Comparative analysis of the efficiency of SAMPL and AFLP in assessing genetic relationships among *Withania somnifera* genotypes

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***Withania somnifera* is a subtropical shrub with important medicinal properties. In the present study, the efficiency of Selectively Amplified Microsatellite Polymorphic Loci (SAMPL) assay in assessing the levels of genetic diversity among *W. somnifera* genotypes was analysed. Some genotypes of *W. coagulans* were used as outlier. A comparison of the SAMPL assay was made with the standard Amplified Fragment Length Polymorphism (AFLP) technique. The SAMPL assay revealed higher levels of polymorphism among the tested *W. somnifera* genotypes compared to the use of AFLP. Significantly higher level of polymorphism was detected with SAMPL within both Kashmiri and Nagori genotypes. Cluster analysis showed clear groupings within the *W. somnifera* Kashmiri, *W. somnifera* Nagori and *W. coagulans* genotypes. The Nagori genotypes are separated from the Kashmiri genotypes at low similarity value, indicating that Nagori and Kashmiri types are highly divergent. One of the *W. somnifera*-specific bands generated with SAMPL was used to develop a simple PCR-based assay. The diagnostic markers thus generated can be used at the seedling stage to distinguish *W. somnifera* Kashmiri, *W. somnifera* Nagori and *W. coagulans* genotypes.**

Keywords: AFLP, genetic diversity, SAMPL, *Withania somnifera*.

THE genus *Withania* is an important member of the family Solanaceae. Twenty-three species of the genus *Withania* have been reported. Of these, only two *W. somnifera* (Linn.) Dunal and *W. coagulans* (Linn.) Dunal have been reported from India. The dried roots of the shrub, *W. somnifera*, commonly known as 'Ashwagandha', are important ingredients in Ayurvedic medicine. Roots of *Withania* contain several alkaloids of medicinal value. These include 13 Dragendroff-positive compounds, withasomine and visamine. These alkaloids have sedative, anti-inflammatory and antispasmodic properties. Ashwagandha has been found useful in the treatment of mental illness, asthma, ulcer and arthritis. The roots and leaves of this shrub contain another family of alkaloids, the withanolides. The most important withanolide, withaferin A was first isolated from leaves and has been shown to possess antitumour and antibacterial activity[1,2]. A related species, *W. coagulans*, is commercially important because of the ability possessed by its berries to coagulate milk. Plants of *Withania* genus are distributed in the east of the Mediterranean region and South Asia. In India, the medicinally important *W. somnifera* genotypes have been reported from the mountain regions of Jammu and Kashmir, submountain areas of Punjab and Uttaranchal and in the plains of western and central India (Rajasthan, Delhi, Gujarat and Madhya Pradesh). *W. coagulans* occurs mainly in the dry areas of Punjab.

Commercial cultivation of *W. somnifera* is carried out in about 4000 ha, mainly in Manasa, Madhya Pradesh and in some parts of Rajasthan[3]. Despite its commercial value, there is apparent lack of improved varieties of *W. somnifera* to make its cultivation cost-effective. Wild *W. somnifera* genotypes may possess genes important for the development of new varieties of this important species. Cataloguing and characterization of wild genetic resources of this species is therefore desirable. Assessment of genetic diversity is the first step towards designing strategies for the conservation of genetic resources of this species. Studies based on morphometric data have been undertaken to analyse the nature and extent of genetic diversity in this important medicinal plant[4,5]. However, such studies are not highly reliable as these are influenced by environmental effects. DNA-based markers are more efficient tools for the assessment of genetic diversity as they are developmentally stable, detectable in all tissues, remain uninfluenced by environmental factors and provide a choice of codominant or dominant markers.

In a previous study, AFLP markers were employed to assess genetic variation among 35 genotypes of *W. somnifera* (Kashmiri and Nagori genotypes) and five genotypes of *W. coagulans*[6]. High percentage of polymorphism was revealed among *W. somnifera* genotypes. However, the study revealed low levels of variation within both the Kashmiri and Nagori genotypes. Therefore, the present study was undertaken to detect higher levels of genetic variation

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within these genotypes. The Selectively Amplified Microsatellite Polymorphic Loci (SAMPL) marker technique was employed for this purpose. This assay can detect high levels of polymorphism between closely related genotypes due to its association with the hypervariable microsatellite region. Another species, *W. coagulans* was included as an outlier in the analysis. The Amplified Fragment Length Polymorphism (AFLP) fingerprints were also generated for these genotypes in order to compare the efficiency of the SAMPL markers with the AFLP marker technology. Another objective of the study was to design technically simple and high throughput microsatellite-based diagnostic SCAR markers for discriminating the two *Withania* species and also the *W. somnifera* Kashmiri and Nagori genotypes.

Material and methods

Plant material and DNA extraction

Twenty-five *Withania* genotypes were analysed with the AFLP and SAMPL assay. A comprehensive list of the genotypes used in this study is presented in Table 1. Leaf material of *W. somnifera* genotypes was collected from Tropical Forest Research Institute (TFRI), Jabalpur (Madhya Pradesh); Central Arid Zone Research Institute (CAZRI), Jodhpur; Jai Narayan Vyas University (JNVU), Jodhpur, and Banki Nursery, Udaipur (Rajasthan), India, while that of *W. coagulans* was obtained from JNVU.

Table 1. *Withania* genotypes analysed, codes given to them in this study and their place of collection

Code	Source	Species/morphotype
A	TFRI, Jabalpur	*W. somnifera*, Kashmiri
B	TFRI, Jabalpur	*W. somnifera*, Kashmiri
C	TFRI, Jabalpur	*W. somnifera*, Kashmiri
D	Jaipur University, Jaipur	*W. somnifera*, Kashmiri
E	Jaipur University, Jaipur	*W. somnifera*, Kashmiri
F	RRL, Jammu	*W. somnifera*, Kashmiri
G	Banki Nursery, Udaipur	*W. somnifera*, Kashmiri
H	Banki Nursery, Udaipur	*W. somnifera*, Kashmiri
I	Banki Nursery, Udaipur	*W. somnifera*, Kashmiri
J	Banki Nursery, Udaipur	*W. somnifera*, Kashmiri
K	CAZRI, Jodhpur	*W. somnifera*, Kashmiri
L	CAZRI, Jodhpur	*W. somnifera*, Kashmiri
M	CAZRI, Jodhpur	*W. somnifera*, Kashmiri
N	CAZRI, Jodhpur	*W. somnifera*, Kashmiri
O	TFRI, Jabalpur	*W. somnifera*, Kashmiri
P	Banki Nursery, Udaipur	*W. somnifera*, Nagori
Q	Banki Nursery, Udaipur	*W. somnifera*, Nagori
R	Banki Nursery, Udaipur	*W. somnifera*, Nagori
S	Banki Nursery, Udaipur	*W. somnifera*, Nagori
T	Banki Nursery, Udaipur	*W. somnifera*, Nagori
U	JNVU, Jodhpur	*W. coagulans*
V	JNVU, Jodhpur	*W. coagulans*
W	JNVU, Jodhpur	*W. coagulans*
X	JNVU, Jodhpur	*W. coagulans*
Y	JNVU, Jodhpur	*W. coagulans*

The *W. coagulans* plants were originally obtained from Balochistan and subsequently planted in the Barmer district, Rajasthan. Young, unexpanded leaves were collected and lyophilized before DNA extraction. All DNA extractions were done using the modified CTAB procedure[7].

AFLP and SAMPL analysis

The AFLP procedure was performed following the protocol developed by Vos *et al.*[8] with minor modifications[9]. All reagents required for AFLP analysis were obtained from Life Technologies Inc, USA. SAMPL analysis was performed using the procedure described by Singh *et al.*[10]. Pre-amplified AFLP library was used as template for selective amplification using radiolabelled SAMPL and *Mse*I + 3 primers. Sequences of the SAMPL primers are as follows:

Primer S2: 5′C$(TC)_4(AC)_4$A 3′
Primer S3: 5′G$(TG)_4(AG)_4$A 3′

The parameters for PCR reaction were the same as those for AFLP by Vos *et al.*[8]. The amplified fragments were size fractionated on 6% polyacrylamide gel and detected by autoradiography.

Data analysis

The multiplex ratio (MR) was calculated as the total number of loci detected per assay, while the effective multiplex ratio (EMR) was the number of polymorphic loci detected per assay[11]. The heterozygosity (H) or PIC for each marker was calculated using the formula PIC = $2f_i\,(1 - f_i)$, where f_i is the frequency of the genotypes showing the presence of band i[12]. The average heterozygosity (H_{av}) was calculated for both total number of bands (H_{av}) and polymorphic bands only ($H_{av\text{-}p}$). The marker index (MI) was calculated[11] as the product of EMR and $H_{av\text{-}p}$.

The amplified fragments in each of the 25 genotypes were scored manually for their presence (denoted as '1') or absence (denoted as '0') for each primer combination. The binary matrix was used to estimate Jacquard's genetic similarity coefficients, $GS_{ij} = a/(a + b + c)$[13], where GS is the measure of genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The similarity matrices were subjected to UPGMA (Unweighted Pair Group Method of Arithmetic averages)[14] method of clustering in order to generate the dendrograms. The Mantel matrix correspondence test[15] was used to test the significance of the correlation coefficients between the similarity matrices for AFLP and SAMPL, to calculate the goodness-of-fit of the clustering to the data matrix and to compare the dendrograms generated by the two markers systems. The COPH and MAXCOMP programs

were employed for the purpose. All the above-mentioned statistical analyses were performed using NTSYS-pc software (version 2.02)[16]. The reliability and robustness of the phenograms were tested by bootstrap analysis[17] to compute probabilities in terms of percentage for each node of the tree. Bootstrapping was done using the WINBOOT software[18].

Conversion of SAMPL markers to diagnostic markers

Three bands specific for *W. coagulans*, *W. somnifera* Kashmiri and Nagori genotypes were excised from dried polyacrylamide gel, re-hydrated in TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for 1 h at room temperature and transferred to 500 µl elution buffer (0.5 M NH_4Ac, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, 0.1% SDS) at 37°C. From this, 1.0 µl of supernatant was used as template for PCR amplification using primers and reaction conditions similar to those used for SAMPL reaction. The amplified products were purified through PCR prep columns (Promega), ligated into pGEM-T plasmid vector (Promega) and transformed into *E. coli* JM109 competent cells. Recombinant plasmids were isolated and sequenced with an ABI prism automatic sequencer (Perkin Elmer) using a fluorescent dye terminator. Based on the sequences of the cloned fragments, the three primers were designed and synthesized. Sequences of these primers are as follows:

Primer WSK3: 5′AGCAGAGCCTCGACATTTTAG 3′
Primer WSN2: 5′GTCAACATTTATCTGAAATTACC 3′
Primer WC2: 5′GGCTCTAAAAGAGGAAATTTG 3′.

PCR amplification of *Withania* genotypes was carried out with these primers in conjunction with the respective SAMPL primers. Primers WSK2, WSN2 and WC2 were labelled with γP^{32}-ATP and the standard AFLP–PCR parameters were employed for amplification. The fragments were size-fractionated on 6% polyacrylamide gel and detected by autoradiography.

Results and discussion

Identification of diverse germplasm that has high chances of conserving potentially useful genes for plant improvement is an essential prerequisite towards formulating conservation strategies for plant genetic resources. Molecular markers represent powerful and rapid tools for characterizing diversity within the target species. A wide array of molecular markers is now available. Two of these marker systems, AFLP and SAMPL, were employed in the present study for detecting genetic relationships within the *Withania* genotypes. These included 20 *W. somnifera* (15 Kashmiri and 5 Nagori) and five *W. coagulans* genotypes (Table 1).

AFLP analysis

The technique of AFLP has high MR and does not require prior sequence information. Though dominant in nature and technically demanding, this assay has become a popular tool for genetic diversity studies in plants, due to its robust and highly reproducible nature. Some of the recent studies using this marker include assessment of genetic diversity in *Brassica nigra*[19], *Vitis vinifera*[20], *Cucurbita pepo*[21] and maize[22]. In the present study also, AFLP analysis of the *Withania* species was performed. Seven primer combinations were employed and these are listed in Table 2. All the primer combinations generated amplification products in the size range of 50 to 400 bp. A typical AFLP profile generated by employing the primer combination $E_{ACC} \times M_{CAG}$ is shown in Figure 1 *a*. Both monomorphic and polymorphic bands were amplified with this primer combination. Only unambiguous bands were analysed. A total of 79 bands were scored with this primer combination, of which 80% was polymorphic. The amplification product marked M indicates a monomorphic band. Polymorphic bands such as R were of rare occurrence, while the band marked F was frequent in occurrence. Several species-specific bands were also identified. S and C exemplify bands specific for *W. somnifera* and *W. coagulans* respectively. Furthermore, distinct fingerprint profiles were obtained for the Kashmiri and the Nagori genotypes of species *W. somnifera*. The band K was amplified only in Kashmiri genotypes, while band N was detected only in Nagori genotypes. Results obtained using the seven AFLP primer combinations are summarized in Table 2. A total of 520 AFLP bands were generated with an average of 74 bands per assay. The number of amplification products varied from 53 to 101 with primer combinations $E_{ACG} \times M_{CAT}$ and $E_{AAC} \times M_{CAA}$ respectively. The percentage polymorphism ranged from 79 with the primer combinations $E_{ACC} \times M_{CTC}$ and $E_{AAC} \times M_{CAA}$ to 89 with $E_{ACG} \times M_{CAT}$ with an average of 82% bands being polymorphic.

SAMPL analysis

In our previous study, the AFLP marker was not able to discriminate within the *W. somnifera* Kashmiri and Nagori genotypes[6]. Another marker system, SAMPL, was therefore employed in the present study. SAMPL is a microsatellite-based modification of the AFLP assay and has all the advantages of the latter. Moreover, it can detect higher levels of polymorphism per locus compared to AFLP due to its ability to survey the hypervariable microsatellite region in the genome. The SAMPL assay has been employed for analysis of genetic diversity in lettuce[23], neem[10], wheat[24], cowpea[25] and sweet potato[26] and for linkage mapping in Kentucky bluegrass[27] (*Poa pratensis* L.). SAMPL assay was standardized for the *Withania* species by testing six SAMPL primers in conjunction with several *Mse*I primers.

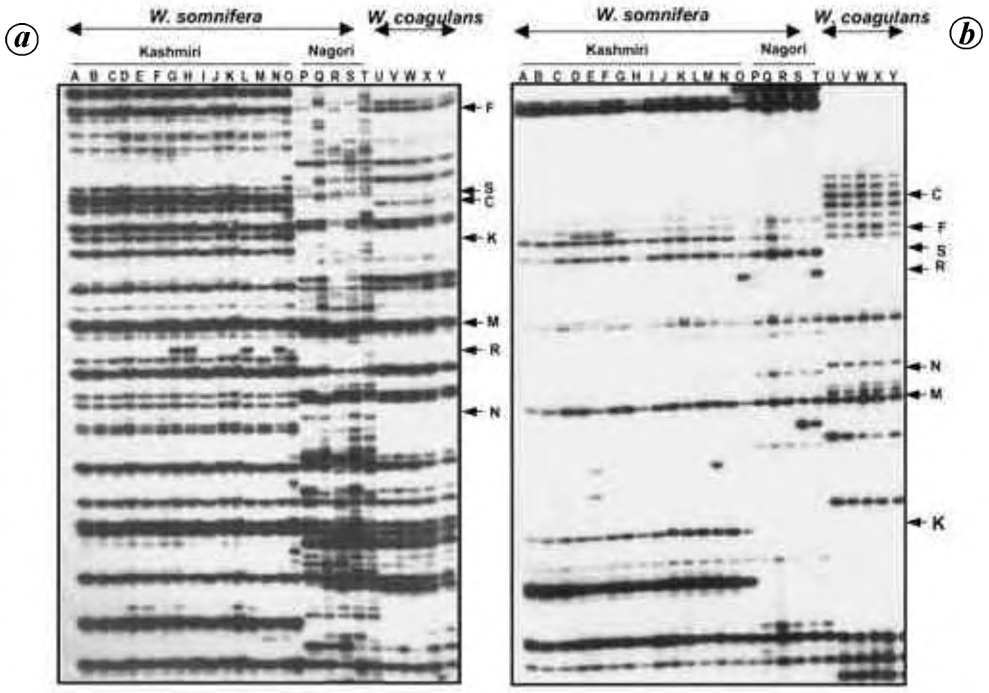

Figure 1. Comparison of AFLP (*a*) and SAMPL (*b*) profiles. The AFLP profile was generated with the primer combination $E_{ACC} \times M_{CAG}$. The SAMPL profile was generated with the primer combination $S3 \times M_{CTG}$. Fingerprint profiles for fifteen *W. somnifera* Kashmiri (A–O), five *W. somnifera* Nagori (P–T) and five *W. coagulans* (U–Y) genotypes are also shown. Arrow M indicate monomorphic bands, while arrows R and F represent rare and frequent polymorphic bands respectively. The *W. coagulans* and *W. somnifera*-specific bands are marked by arrows C and S respectively. Arrow K indicates Kashmiri, while arrow N represents Nagori genotype-specific bands.

Table 2. Information conveyed by seven AFLP and seven SAMPL primer combinations in 20 *W. somnifera* and five *W. coagulans* genotypes in terms of total number of bands detected (*n*), total number of polymorphic bands detected (*np*) and percentage polymorphism (*%P*) per assay

AFLP				SAMPL			
Primer combination	*n*	*np*	*% P*	Primer combination	*n*	*np*	*% P*
$E_{ACC} \times M_{CAT}$	90	69	71	$S2 \times M_{CTT}$	69	69	100
$E_{ACC} \times M_{CAG}$	79	63	80	$S2 \times M_{CTC}$	47	41	87
$E_{AAC} \times M_{CAA}$	101	80	79	$S2 \times M_{CAG}$	58	58	100
$E_{ACC} \times M_{CTC}$	67	53	79	$S3 \times M_{CAA}$	43	36	86
$E_{ACG} \times M_{CAT}$	53	47	89	$S3 \times M_{CTT}$	53	50	94
$E_{ACG} \times M_{CTG}$	65	56	86	$S3 \times M_{CTA}$	102	93	91
$E_{ACG} \times M_{CTT}$	65	53	82	$S3 \times M_{CTG}$	52	41	79
Total	520	423			423	390	
Average	MR = 74	EMR = 60	82		MR = 60	EMR = 56	92

MR, Multiplex ratio; EMR, Effective multiplex ratio.

Stuttering of bands was obtained with many primer combinations (results not shown). This may be attributed to inherent slippage problems during PCR amplification of microsatellites that result in stuttering of bands in the fingerprint profiles. However, use of two SAMPL primers resulted in reliable fingerprint profiles.

Seven *Mse*I primers were utilized in combination with two SAMPL primers for analysis of genetic diversity within the 25 *Withania* genotypes. These primer combinations are listed in Table 2. A representative SAMPL profile generated by employing the primer combination $S3 \times M_{CTG}$ is shown in Figure 1 *b*. This primer combination yielded a total of 52 bands, of which 79% was polymorphic. A monomorphic band is marked M in the fingerprint. The band R is rare, while band F is frequent polymorphic. Distinct fingerprint profiles, including several species-specific bands were generated for both the species. The band S exemplifies a *W. somnifera*-specific band, while

band C is specific for *W. coagulans*. Among the *W. somnifera*-genotypes, different fingerprint profiles were generated for both Kashmiri and Nagori genotypes. Several bands specific for Kashmiri (such as band K) and Nagori genotypes (such as band N) were generated. Results of the analysis carried out by employing the seven SAMPL primer combinations are summarized in Table 2. A total of 423 bands were amplified, with a mean of 60 bands per assay. The number of bands ranged from 102 with primer combination $S3 \times M_{CTA}$ to 43 with $S3 \times M_{CAA}$. The percentage polymorphism across the 25 *Withania* genotypes varied from 100 with primer combinations $S2 \times M_{CTT}$ and $S2 \times M_{CAG}$ to 79 with $S3 \times M_{CTG}$. The average percentage polymorphism was 92 across the *Withania* genotypes. Bands specific for the two species as well as for the *W. somnifera* Kashmiri and Nagori genotypes were obtained with all seven SAMPL primer combinations.

Comparison of AFLP and SAMPL markers

A comparative analysis of AFLP and SAMPL in assessing the genetic diversity among the 25 *Withania* genotypes was carried out and the results are shown in Table 3. Three main aspects of the performance of these marker systems were considered. These included the average number of polymorphic bands detected per assay (EMR)[11], overall efficiency of detecting polymorphism in the germplasm pool and between any two lines taken at random from that pool (per cent polymorphism, heterozygosity)[11] and overall utility of marker for detecting genetic variation (MI)[11]. The results of these analyses are shown in Table 3.

Higher MR was detected within *Withania* genotypes with the AFLP assay (MR = 74), compared to the SAMPL assay (MR = 60; Table 2). However, SAMPL assay detected higher polymorphism ($\%P = 92$, $H_{av\text{-}p} = 0.32$) compared to AFLP ($\%P = 81$, $H_{av\text{-}p} = 0.34$). Similar observations have been made in neem, where SAMPL detected higher levels of per cent polymorphism (85) compared to AFLP (57)[10]. In cowpea (*Vigna unguiculata*) also[25], per cent polymorphism detected for SAMPL was 21.5, while that for AFLP was 15.4. Furthermore, SAMPL assay detected higher levels of polymorphism within *W. somnifera* Kashmiri genotypes ($\%P = 55$, $H_{avp} = 0.22$) compared to the AFLP assay ($\%P = 24$, $H_{av\text{-}p} = 0.16$). Similarly, SAMPL assay also detected higher levels of polymorphism ($\%P = 30$, $H_{av\text{-}p} = 0.42$) than the AFLP assay ($\%P = 2$, $H_{av\text{-}p} = 0.35$) in the Nagori genotypes. Due to the high information content, the SAMPL assay has previously been shown to be more suitable for studies where low genetic variation is expected[23]. For instances in neem the per cent polymorphism within the Kanpur accessions detected by SAMPL was higher (69.2) compared to AFLP (35)[10]. Similarly, the average heterozygosity value obtained using the SAMPL assay[24] among wheat cultivars was 0.22, while that for AFLP was 0.07. This can be explained by the high levels of polymorphism associated with the microsatellite region, expected due to the unique mechanism by which this variation is generated. Replication slippage responsible for SSR diversity occurs more frequently than single-nucleotide mutations and deletions/insertion events that generate polymorphism detectable by AFLP analysis.

A convenient estimate for marker utility is MI, which is calculated as the product of EMR and $H_{av\text{-}p}$. MI was slightly higher for AFLP than SAMPL (20.5 for AFLP and 18 for SAMPL) when calculated for all the *Withania* genotypes. However, SAMPL detected higher MI than AFLP within *W. somnifera* genotypes (8.7 for AFLP and 10 for SAMPL), within Kashmiri genotypes (1.9 for AFLP and 4.8 for SAMPL) and within Nagori genotypes (0.25 for AFLP and 4.2 for SAMPL). This was due to the effective MR component, which was higher for SAMPL assay. This is in corroboration with the studies in wheat[24] and *Vigna*[25], where the MI value for the SAMPL markers was higher than that for the AFLP markers. This was attributed to low proportion of polymorphic bands obtained using the AFLP markers, even though the AFLP system generated higher number of loci per assay.

Genetic similarity matrices were constructed for AFLP and SAMPL marker systems. The Mantel matrix correspondence test[15] was used to compare the similarity matrices for the two assays. Correlation between the similarity matrices was high ($r = 0.89$, $P = 1$); however, poor correlation (0.65) was obtained when only *W. somnifera* genotypes were considered for analysis. Comparison of the average GS

Table 3. Comparison of total number of bands (N), total number of polymorphic bands (N_p), percentage polymorphism ($\%P$), average heterozygosity for polymorphic markers ($H_{av\text{-}p}$), average number of polymorphic markers detected per assay (EMR) and marker index (MI) for the AFLP and SAMPL dataset

	AFLP						SAMPL					
Comparison within	N	N_p	$\%P$	$H_{av\text{-}p}$	EMR	MI	N	N_p	$\%P$	$H_{av\text{-}p}$	EMR	MI
Withania somnifera and *W. coagulans*	520	423	81	0.34	60	20.5	423	390	92	0.32	56	18
W. somnifera (Kashmiri and Nagori)	402	211	53	0.29	30	8.7	333	252	76	0.28	36	10
W. somnifera Kashmiri	354	85	24	0.16	12	1.92	284	157	55	0.22	22	4.8
W. somnifera Nagori	306	5	2	0.35	0.71	0.25	254	71	30	0.42	10	4.2

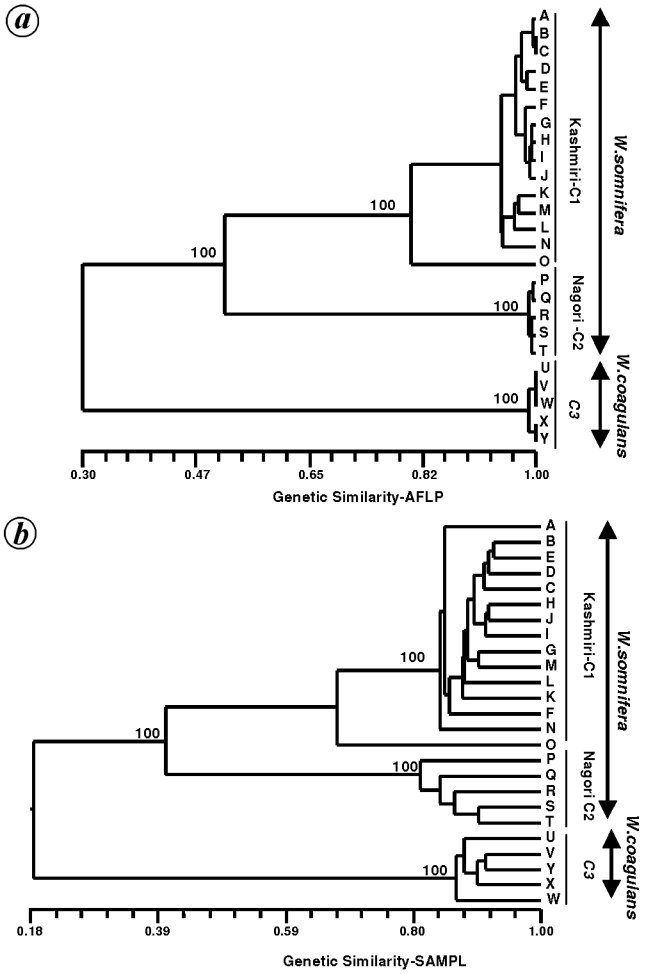

Figure 2. UPGMA dendrogram of *Withania* genotypes generated using AFLP (***a***) and SAMPL (***b***) marker data.

values revealed that SAMPL was more efficient than AFLP in detecting polymorphism. Lower genetic similarity value was obtained for SAMPL (0.46) compared to AFLP (0.61) within all the *Withania* genotypes as well as within the *W. somnifera* genotypes (SAMPL, GS = 0.76 and AFLP, GS = 0.84) respectively. Similar observations were made in neem where Kanpur accessions grouped at a GS value of 0.85, while in SAMPL analysis[10] these grouped at a GS of 0.72. The GS values within *V. unguiculata* landraces were lower for SAMPL (0.51) compared to AFLP (0.61)[25]. Low similarity values (0.65) were also obtained among the wheat accessions using SAMPL assay[24].

The genetic similarity matrices for AFLP and SAMPL data were used to cluster the genotypes by the UPGMA method. The dendrograms thus constructed are shown in Figure 2 *a* and *b* respectively. *Withania* genotypes were grouped into three major clusters in both the dendrograms. Each of these clusters was supported by high bootstrap values. The *W. somnifera* Kashmiri genotypes formed the cluster 1 (C1), the *W. somnifera* Nagori genotypes grouped together as cluster 2 (C2), while the *W. coagulans* genotypes comprised cluster 3 (C3). As expected, the genotypes of the two species were linked to each other at low similarity coefficients. GS values at which the three clusters joined, were different for the two marker systems. The *W. coagulans* genotypes were linked to the *W. somnifera* genotypes at a low GS value of 0.3 in the AFLP dendrogram, while in the dendrogram generated with the SAMPL markers, a much lower GS value of 0.18 was obtained. An interesting observation in the study was the separation of the Nagori genotypes from the Kashmiri genotypes at extremely low similarity values. The GS value at which the cluster for the Kashmiri and the Nagori genotypes joined was 0.52 and 0.38 for AFLP and SAMPL respectively. The Nagori genotype may be the wild form from Nagaur (Rajasthan), whose roots were distributed in India in the ancient times. However, now except for a limited collection of roots from the wild plants growing in Rajasthan, most of the roots available in the markets in the country are obtained from the cultivated plants grown in Madhya Pradesh[3]. The cultivated plants are reported to greatly differ from the wild forms of *W. somnifera* in morphology, especially that of the root and their therapeutic action, though both have the same alkaloids[3,28]. Previous studies also suggest that there is an extreme degree of variability in *W. somnifera* with respect to growth habit and morphological characteristics in different parts of India, especially in the genotypes grown in Rajasthan[3,28]. These studies indicate that either the species is highly polymorphic or the name *W. somnifera* has been indiscriminately applied to a wide variety of dissimilar forms, some of which deserve an independent rank as subspecies[3,28]. These reports also suggest that the Nagori wild form may be a species different from *W. somnifera*[3,28]. Furthermore, in both the dendrograms *W. somnifera* genotype 'O' did not fall in any cluster, and was positioned between the clusters for the Kashmiri and Nagori types.

In both the dendrograms the variant 'O', an intermediate to Kashmiri and Nagori genotypes was observed. This variant was indistinguishable morphologically from the remaining Kashmiri genotypes. However, within the two *W. somnifera* clusters, differences were observed in the grouping of genotypes. This was also indicated in the low correlation value obtained between the cophenetic matrices of two marker systems (0.72, $P = 1$) and can be ascribed to the fact that various markers target different regions of the genome and thus explore genetic variation differently. Though SAMPL analysis revealed higher levels of polymorphism within the Kashmiri and the Nagori genotypes, clustering patterns generated by AFLP corresponded more with the eco-geographic data. Within the *W. somnifera* cluster, groupings were observed for the Kashmiri genotypes from Jabalpur (A, B, C), Jaipur (D, E), Udaipur (G, H, I, J) and Jodhpur (K, L, N) and also for the Nagori genotypes procured from Udaipur (P, Q, R, S, T) with the AFLP markers. However, no such groupings based on geographic

origin were detected in the SAMPL dendrogram. Studies based on morphology have also shown that the genotypes of *W. somnifera* form distinct groups that correlate well with their geographic origin[28]. Previous studies have also indicated the tendency of microsatellite-based markers to overemphasize differences between closely related species and to attribute less variation to differences over larger geographical distances[29].

Conversion of SAMPL markers to diagnostic markers

The AFLP markers and their modifications such as SAMPL are generally expensive to generate, technically tedious and dominant in nature. This limits their large-scale application as diagnostic markers for species or varietal identification and in marker-assisted plant breeding. For practical applications these markers need to be converted to rapid, technically simple assays that can be used on crude DNA preparation. For this, three bands specific for *Withania* species as well as for *W. somnifera* Kashmiri and Nagori genotypes detected in the SAMPL profile were eluted from the gel, cloned and sequenced. The microsatellite regions associated with the SAMPL primer were present at one end of the sequence, while the other end corresponded to the *Mse*I primers. Microsatellites were also identified within the sequences. A Kashmiri genotype-specific band obtained with the primer combination $S3 \times M_{CTA}$ was cloned. This clone, designated as $WSK3_{CTA}$ contained an insert of 203 bp. On sequencing, the insert showed the presence of microsatellite repeat $(GA)_5$ at nucleotide position 118 and a 'GAAA' motif at 45, 137, 143 and 154 nucleotide positions. A band specific for the Nagori genotypes generated with the primer combination $S2 \times M_{CTT}$ was also cloned. This clone named $WSN2_{CTT}$, had a 246-bp insert and showed the presence of microsatellite repeats $(GT)_4$ $(GA)_6$ at 92 and 116, 'TTTGAAA' repeat at 73 and 218 as well as a 'GAAAAT' repeat at 157 and 180 nucleotide positions. A *W. coagulans*-specific band was amplified with the primer combination $S2 \times M_{CTT}$, cloned and sequenced. This clone designated as $WC2_{CTT}$, contained a 177-bp sequence and showed a stretch of $(TG)_5$ $(AG)_5$ repeat at the SAMPL primer end of the sequence. Based on the internal sequence, primers WSK3, WSN2 and WC2 were designed for the three clones $WSK3_{CTA}$, $WSN2_{CTT}$ and $WC2_{CTT}$ respectively.

Primers WSK3, WSN2 and WC2, in conjunction with respective SAMPL primers, were used for amplification in *Withania* species. The primer combination $WSK3 \times S3$ amplified one band in *W. somnifera* genotypes, but no amplification was obtained in the *W. coagulans* genotypes. The primer combination can therefore be used to differentiate *W. somnifera* and *W. coagulans* genotypes. The primer combination $WSN2 \times S2$ failed to generate amplification products in *Withania* genotypes. However, the primer combination $WC2 \times S2$ was able to distinguish between the two *Withania* species as well as between *W. somnifera* Kashmiri and Nagori genotypes (Figure 3). The band S was amplified only in *W. somnifera*, while the band C was detected only in *W. coagulans*. Furthermore, a band N specific for the *W. somnifera* Nagori genotypes was also amplified. These diagnostic markers generated with the primer combination $WC2 \times S2$ can be employed at the seedling stage to discriminate between the two *Withania* species and the *W. somnifera* Kashmiri and Nagori genotypes.

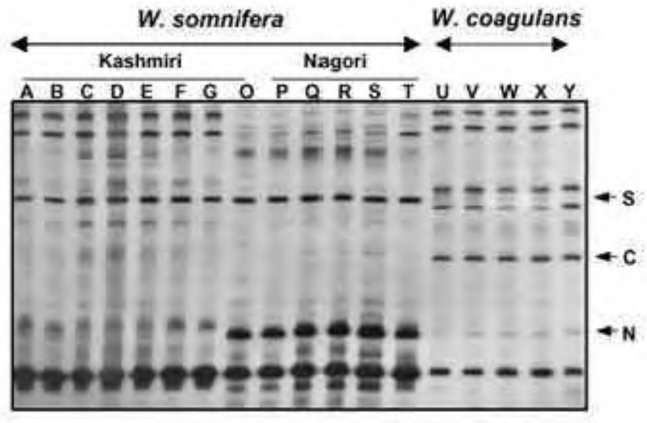

Figure 3. Fingerprint profile generated using SAMPL primer S2 in combination with locus-specific primer WC2. Band S is *W. somnifera*-specific, band C is *W. coagulans*-specific and band N is *W. somnifera* Nagori-specific genotype.

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