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GENETIC DIVERSITY ASSESSMENT OF TWELVE ACCESSIONS OF FIVE *SESBANIA* SCOP SPECIES USING INTER SIMPLE SEQUENCE REPEAT (ISSR) MARKERS

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Abstract

The genus *Sesbania* Scop is one of the important genera of the family Fabaceae and is well-known for being highly promising green manure crops. Crop improvement for increased utilization of the members of this genus can be done with the help of conventional and molecular breeding programs for which the assessment of genetic variability is a pre-requisite. In this study, twelve accessions representing five species of *Sesbania* Scop are studied for their genetic diversity. The DNA was isolated from fresh leaf samples and analyzed by using eight Inter Simple Sequence Repeat primers using Polymerase Chain Reaction and Electrophoresis techniques. Analysis of the band statistics and efficiency parameters of the primers is done after obtaining the DNA banding patterns. The total number of bands amplified are 79 of which 76 are polymorphic indicating a very high degree of genetic variability. The overall percentage of polymorphism observed was 96.2%.Jaccard's similarity coefficients are calculated and the highest similarity value of 0.895 is seen between the two accessions of S2 (*S. sericea* (Willd.) Link IC0631284) and S3 (*S. sericea* (Willd.) Link IC0635936).Principal Component Analysis and Dendrogram analyses grouped all the twelve accessions into 2 major clusters and 5 sub-clusters.Based on the genetic variability, the most useful germplasms for breeding programs are found to be S2 (*S. sericea* (Willd.) Link IC0631284) , S5 (*S. grandiflora* (L.) Pers. IC0564742) and S6 (*S. aculeata* (Willd.) Pers. IC0445741). The exclusive clustering of *S.grandiflora* accessions was observed in addition to the grouping of accessions based on their biological statuses.

Keywords – Dendrogram; moleculargenetic variability;ISSR markers; *Sesbania*Scop.

Introduction

Consisting of nearly 60 species which are widely distributed in tropical and subtropical areas of the world, the genus *Sesbania* Scop. belongs to the family Fabaceae and subfamily Papilionoideae (1). The members of this genus are mainly grouped as annuals or perennial herbs, shrubs or trees and based on their pod morphology are classified into four sub-genera – *Agati, Daubentonia*, *Pterosesbania* and *Sesbania* (2). Many members of this genus are well known for their usage as a green manure crop, especially in rice and wheat cropping systems (3), in addition to their usage in animal feed and fodder (4) and traditional agroforestry systems (5).The assessment of genetic variability in plants is done by studying and comparing by using various types of markers like morphological, biochemical (protein and isozyme) and molecular

(DNA). But in the recent decades, the DNA-based markers are being used predominantly due to their ability to reveal more polymorphism (6) with less sample and in a short amount of time. The usage of Inter Simple Sequence Repeat (ISSR) markers for the studying the polymorphism is based on the presence of microsatellites throughout genomes (7). These ISSR markers are simple and cost effective when compared to Amplified Fragment Length Polymorphism (AFLP) markers and are highly reproducible and reliable than Random Amplified Polymorphic DNA (RAPD) markers (8).Various researches have attempted to evaluate the genetic diversity and variability of the members of genus *Sesbania* Scop. by using DNS based molecular markers like RAPD and ISSR markers.Among these, ISSR markers have been utilized by Bisoyi *et al* (2010)for studying interspecific diversity in 6 species of *Sesbania* (9).Joshi Saha and Gopalakrishna (2007) have also used ISSR markers for evaluating genetic variability in 5 shrubby species of *Sesbania* collected from India, Philippines and Australia (10).In the present study, a total of twelve accessions representing five species of *Sesbania* Scop. with different biological statuses have been used to study their genetic diversity to establish the phylogenetic relationship.

Materials and methods

Plant Materials

A total of twelve accessions were studied out of which elevenaccessions representing five species of the genus *Sesbania* Scop.wereobtained from the Indian Council of Agricultural Research – National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi, India and one was collected from Vizianagaram District, Andhra Pradesh are used in the present study. The source, biological status and accession numbers are mentioned in Table 1. Fresh leaf samples were collected after 3 weeks of germination and for genomic DNA isolation.

Table 1. Species and accessions of *Sesbania* Scop. used in the study

S.No	Accessions (Label used)[a]	Source / Place of collection	Biological Status
1	*S. aculeata* (Willd.) Pers.IC0445741 (S6)	Andhra Pradesh	Traditional Cultivar/ Folk Variety
2	*S. aculeata* (Willd.) Pers. IC0445742 (S7)	Andhra Pradesh	Traditional Cultivar/ Folk Variety
3	*S. aculeata* (Willd.) Pers.IC0430859 (S8)	Uttar Pradesh	Landrace
4	*S. aculeata* (Willd.) Pers.IC0427550 (S11)	Haryana	Wild
5	*S.bispinosa* (Jacq.) W.Wight IC0638686 (S10)	Assam	Wild
6	*S. grandiflora* (L.) Pers. IC0524635 (S4)	Andhra Pradesh	Landrace
7	*S. grandiflora* (L.) Pers. IC0564742 (S5)	Andhra Pradesh	Wild
8	*S. grandiflora* (L.) Pers. L1 (S12)	Andhra Pradesh	Wild
9	*S. rostrata* Bremek. & Oberm. IC0637338 (S1)	Uttar Pradesh	Wild
10	*S. rostrata* Bremek. & Oberm. IC0637340 (S9)	Haryana	Wild
11	*S. sericea* (Willd.) Link IC0631284 (S2)	Andaman & Nicobar Islands	Wild
12	*S. sericea* (Willd.) Link IC0635936 (S3)	Kerala	Wild

[a]All samples are obtained from NBPGR, New Delhi, India, except S.No. 8 which is a local sample obtained from Vizianagaram district, Andhra Pradesh.

Isolation of genomic DNA

The genomic DNA is isolated from leaf materials by the Cetyl Trimethyl Ammonium Bromidemethod (CTAB)(11).The leaves were homogenized in a mortar and pestle along with CTABbuffer and β-mercaptoethanol. It was followed by two washings with Chloroform and Isoamyl alcohol (24:1 V/V). Then, the supernatant was collected and DNA (Deoxyribo Nucleic Acid)precipitated with pre-chilled Isopropanol. The extracted DNA was washed twice with ethanol, dried and then dissolved in $T_{10}E_1$(Tris(hydroxy methyl) amino methane-HCl and EDTA (EthyleneDiamine Tetra aceticAcid)) buffer with pH 8. The DNA was electrophoresed on 0.8% agarose gel to check itsintegrity.

Inter Simple Sequence Repeats (ISSR) analyses

A total of 8 oligonucleotide ISSR primers from the UBC series (University of British Columbia) were obtained from Ira Biotech Pvt Ltd., Hyderabad(Table 2) and used for Polymerase Chain Reaction (PCR) for analysis.Each amplification reaction mixture of 25 µl consisted of 8 µl of DNA (30ng/µl), 4 µl of primer, 0.2 µl of Taq DNA polymerase (5U/ µl), 2.5

µl of 10X Taq buffer, 0.5 µl of 10mM dNTPs and 9.8 µl of sterile double-distilled water. The amplification was carried out in a Thermal Cycler (Bio-Rad T100 Thermal Cycler) and was programmed for 40 cycles with initial denaturation at 95℃ for 4 minutes, followed by cycling conditions of denaturation at 95℃ for 1 minute, annealing at 50℃ for 45 seconds and extension at 72℃ for 1 min. After 40 cycles, there was a final extension step of 5 minutes at 72℃. The PCR products obtained were subjected to submerged gel electrophoresis in a 1.4% agarose gel containing ethidium bromide solution (0.05 µg/ml of gel solution). The PCR products were loaded with the help of micropipette and electrophoresis was carried out at 70 V for 1.5 hours in 1X Tis-Borate-EDTA (TBE) buffer. The gel was then viewed under UV (Ultra-Violet) transilluminator and photographed (Fig.1) with the help of gel documentation system (Bio-Rad Gel Doc Imaging System).

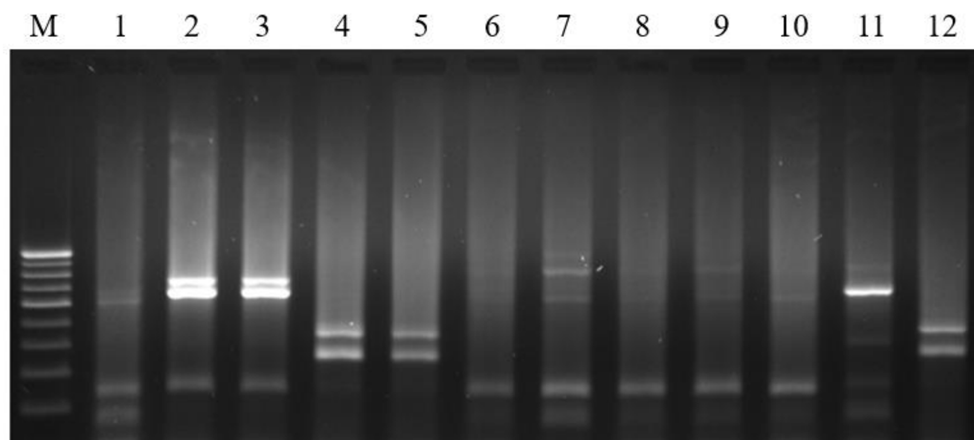

Fig.1 ISSR banding pattern generated by the primer UBC 822. Lane M is the 100 bp molecular ladder and the lanes 1-12 represents the accessions and species S1 to S12 respectively (details in Table.1)

Data Analysis

The amplified PCR products were scored for the presence (1) or absence (0) of bands. A rectangular data matrix was thus obtained for all the primers. The efficiency parameters like Polymorphic Information Content (PIC), Effective Multiplex Ratio (EMR), Marker Index (MI) and Resolving Power (Rp) of each of the primers was calculated by utilizing this data matrix (Table.2). The PIC values were calculated by using the formula $PIC = \sum 2(fi)(1\text{-}fi)$, where fi stands for the proportion of accessions containing the band (12, 13). The values of EMR were calculated by using the formula $EMR = \eta \times \beta$, which is defined as the product of the total number of loci/ bands per primer (η) and the proportion of polymorphic loci/ bands (β) (14). The MI was calculated according to the formula MI = PIC x EMR (15). The Rp value was calculated according to the formula $Rp = \sum I_b$, where I_b represents the bands' informativeness(16). The I_b can be represented on a 0–1 scale using the following formula: $I_b = 1\text{-} (2 \times (0.5 – p))$, where p is the proportion of the accessions containing the band.It wasfurther used to generate Jaccard's coefficients of similarity (17) by using the statistical package NTSYS pc (version 2.10) (18) (Table 3). Subsequently, a Dendrogram was generated by the Unweighted Pair Group Method with Average (UPGMA) method (19) and SAHN(Sequential Agglomerative Hierarchical Non-overlapping) clustering method (9) by using NTSYS program (18)(Fig.2).The Principal Component Analysis (PCA) was also done and a 3D plot was generated for the visualization of the clustering pattern of the analyzed population using the NTSYS program (18) (Fig.3).

Results and Discussion

Banding patterns and characteristics

In order to assess the measure of genetic diversity and heterogeneity in a given population, a variety of molecular markers are used out of which the PCR based molecular markers are now being mostly used in addition to the morphological and agronomic evaluation as they complement the amount of information obtained. ISSR markers are mostly preferred for genetic diversity assessments as they are more reproducible and reliable the RAPD markers and are also simple and cost effective when compared toAmplified Fragment Length Polymorphism (AFLP) markers (10).In the present study, the polymorphism in genomic DNA is detected by ISSR profiles through the PCR reactions and the data obtained from electrophoresis is used to evaluate the genetic diversity among the accessions. The bands' statistics have been presented in Table.2. All of the eight primers that were used showed good amplification profiles.The total number of bands scored from all the primers are 79. The number of bands scored per primer ranged from 6 to 12 with the average being 9.875 bands per primer. The highest number of bands (12) is generated by the primers UBC 807 and UBC 811 and the lowest (6) is by the primer UBC 822.Out of the total 79 bands, 76 are polymorphic and 3 bands aremonomorphic. This indicates that the overall polymorphism is 96.2%. These monomorphic bands are amplified one each by the primers UBC 808, UBC 811 and UBC 817. The total number of unique bands amplified is 6, out of which the highest number (2) is generated by the primer UBC 814. The primers UBC 807, UBC 811, UBC 817 and UBC 825 amplified 1 unique band each and the remaining

primers did not amplify any unique band. The high level of polymorphism percentage (96.2%) seen in this study has also been observedpreviously where the observed polymorphism percentage was 93.5% (10)

Efficiency parameters of the primers

Various efficiency parameters like PIC, EMR, MI and Rp have been calculated in order to compare the efficiency of the primers(2).ISSR markers are considered as dominant markers as only two alleles are assumed in its analysis. Therefore, the maximum value for PIC in case of ISSR analysis is 0.5, as PIC is the measure that is influenced by the number and frequency of alleles (20). The highest PIC value of 0.4153 is shown by the primer UBC 808 whereas the lowest value of 0.3347 is shown by UBC 817 with the overall mean value of 0.3793. Hence, the mean PIC value of 0.3793 in this study indicates a high degree of genetic diversity in the accessions and species studied. The highest value of EMR of 12 is shown by the primer UBC 807 while the lowest value of 6 is being shown by the primer UBC 822. The mean value of the EMR values is 9.1604. In the case of MI, the highest value (4.0694) is shown by UBC 807 and the lowest (2.2361) is by the primer UBC 822 and the mean value is 3.4657.In the case of Rp, the highest value (10.8333) is shown by UBC 807 and the lowest (4.1667) is by UBC 822, with the mean value being 8.5833. According to Prevost and Wilkinson (16) the primer with the highest Rp value should be the most informative primer for distinguishing the samples. Therefore, the primer UBC 807 showing the highest Rp value of 10.8333 is the most informative one among the primers studied.

Table 2. Details of ISSR amplification banding patterns

Primer Name[a]	Nucleotide Sequence	TNB[b]	TPB[b]	TMB[b]	NUB[b]	PIC[b]	EMR[b]	MI[b]	RP[b]
UBC 807	$(AG)_8T$	12	12	0	1	0.3391	12	4.0694	10.8333
UBC 808	$(AG)_8C$	10	9	1	0	0.4153	8.1	3.3638	9.8333
UBC 811	$(GA)_8C$	12	11	1	1	0.3854	10.08	3.8863	9.8333
UBC 814	$(CT)_8A$	10	10	0	2	0.3736	10	3.7361	6.1667
UBC 817	$(CA)_8A$	10	9	1	1	0.3347	8.1	2.7113	9.8333
UBC 822	$(TC)_8A$	6	6	0	0	0.3727	6	2.2361	4.1667
UBC 825	$(AC)_8T$	9	9	0	1	0.4105	9	3.6944	7.3333
UBC 881	$(GGGTG)_3$	10	10	0	0	0.4028	10	4.0278	10.6667
	TOTAL	79	76	3	6	3.0341	73.283	27.7252	68.6666
	MEAN	9.875	9.5	0.375	0.75	0.3793	9.160	3.4657	8.5833

[a] Primers selected from Joshi-Saha and Gopalakrishna (2007) and obtained from Ira Biotech Pvt Ltd., Hyderabad, India.
[b]TNB: Total Number of Bands; TPB: Total Polymorphic Bands; TMB: Total Monomorphic Bands,NUB: Number of Unique Bands; RP: Resolving Power; PIC:Polymorphic Information Content; EMR: Effective Multiplex Ratio; MI: Marker Index

Similarity and cluster analysis

With the help of the above data, Jaccard's (1908) similarity coefficients were calculated which showed the highest similarity value of 0.895 between the accessions S2 (S. sericea (Willd.) Link IC0631284) and S3 (S. sericea (Willd.) Link IC0635936),followed by the accession pairs of S9 (S. rostrata Bremek. & Oberm. IC0637340) and S10 (S.bispinosa (Jacq.) W.Wight IC0638686)and of S8 (S. aculeata (Willd.) Pers. IC0430859) and S10 (S.bispinosa (Jacq.) W.Wight IC0638686) showing the similarity values of 0.809 and 0.775 respectively.The accessions showing the maximum value of Jaccard's similarity (0.895) between them, i.e., S2 (S. sericea (Willd.) Link IC0631284) and S3 (S. sericea (Willd.) Link IC0635936),belong to the same species and biological status type of Wild. Similarly, the accessions of S9 (S. rostrata Bremek. & Oberm. IC0637340) and S10 (S.bispinosa (Jacq.) W.Wight IC0638686) showing the second highest similarity value of 0.809 belong to the same biological status type of Wild. The lowest similarity value of 0.175 was shown between the accessions of S2 (S. sericea (Willd.) Link IC0631284) and S5 (S. grandiflora (L.) Pers. IC0564742).

Table 3. Jaccard's similarity matrix among the twelve accessions of five *Sesbania* Scop. species using the ISSR banding patterns

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
S1	1.000											
S2	0.365	1.000										
S3	0.365	0.895	1.000									
S4	0.190	0.207	0.228	1.000								
S5	0.200	0.175	0.218	0.757	1.000							
S6	0.455	0.300	0.275	0.189	0.176	1.000						
S7	0.451	0.339	0.339	0.216	0.228	0.581	1.000					
S8	0.380	0.273	0.273	0.236	0.250	0.658	0.697	1.000				
S9	0.370	0.316	0.316	0.281	0.273	0.511	0.773	0.738	1.000			
S10	0.412	0.327	0.327	0.224	0.236	0.500	0.727	0.775	0.809	1.000		
S11	0.444	0.375	0.435	0.231	0.245	0.372	0.380	0.333	0.327	0.396	1.000	
S12	0.196	0.307	0.307	0.500	0.432	0.245	0.224	0.245	0.268	0.277	0.265	1.000

The dendrogram constructed based on UPGMA analysis of ISSR matrix data is one of the most effective methods in numerical computation showing the phylogenetic relationships between the accessions and species. The Dendrogram obtained by the UPGMA method and the 3D plot obtained by PCA (Principal Component Analysis) showed 2 distinct clusters I and II. The cluster I consists of 9 accessions which are further sub-clustered into 3 sub-clusters, namely sub-cluster IA consisting of two accessions S1 (S. rostrata Bremek. & Oberm. IC0637338) and S11 (S. aculeata (Willd.) Pers. IC0427550); sub-cluster IB consisting of five accessions S6 (S. aculeata (Willd.) Pers. IC0445741), S7 (S. aculeata (Willd.) Pers. IC0445742), S8 (S. aculeata (Willd.) Pers. IC0430859), S9 (S. rostrata Bremek. & Oberm. IC0637340), S10 (S.bispinosa (Jacq.) W.Wight IC0638686); and sub-cluster IC consisting of two accessions S2 (S. sericea (Willd.) Link IC0631284) and S3 (S. sericea (Willd.) Link IC0635936). The cluster II consists of 3 accessions which are also further divided into 2 sub-clusters, namely sub-cluster IIA consisting of accessions S4 (S. grandiflora (L.) Pers. IC0524635) and S5 (S. grandiflora (L.) Pers. IC0564742) and sub-cluster IIB consisting of only one accession S12 (S. grandiflora (L.) Pers. L1). It can be seen here that all the accessions of *S.grandiflora* have been grouped into a single cluster II and all the remaining accessions have been grouped into cluster I. This was also observed in a study by Bisoyi et al (2010)where *S. grandiflora* was singled out into a separate cluster (9). This observation supports the segregation of *S.grandiflora* which is a tree species into the sub-genus *Agati* Desv. (21). It is also observed that the accessions that have been grouped into the same sub-clusters possess the same type of biological statuses.This explains the similarity between them in relation to their usage and biological status.

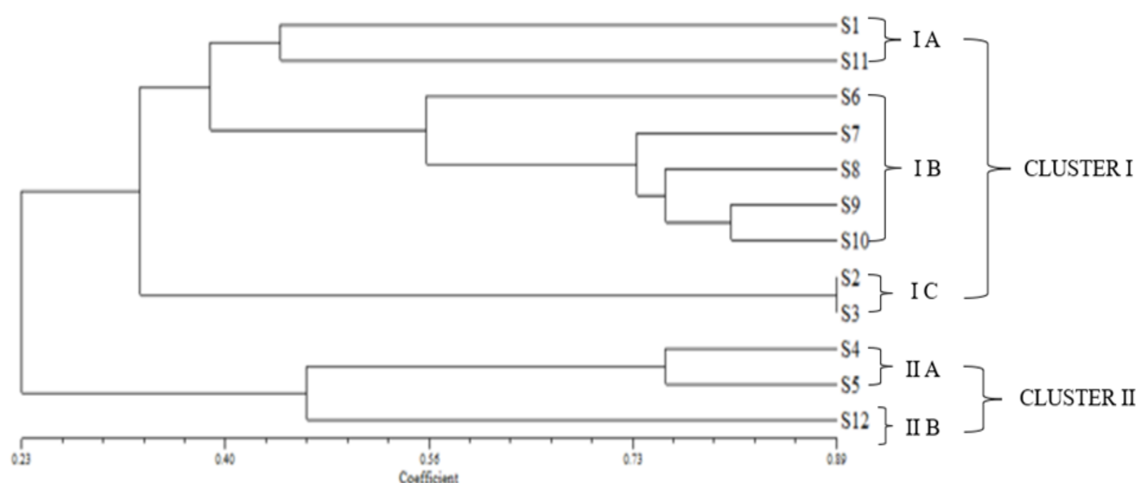

Fig.2. Dendrogram generated for the twelve accessions of five species of *Sesbania* Scop. using UPGMA cluster analysis based on Jaccard's similarity coefficient

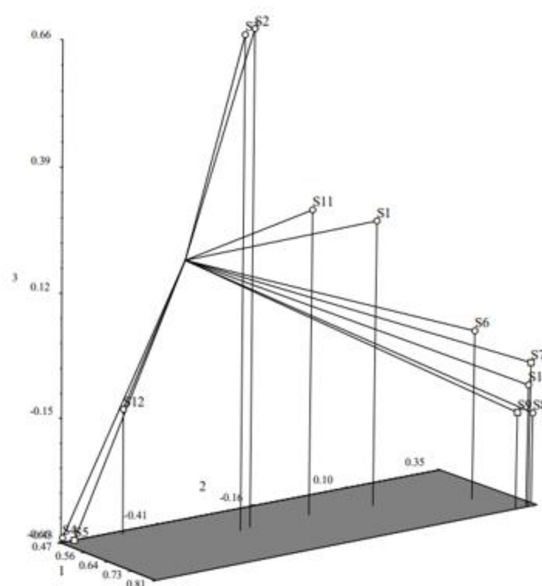

Fig.3. Principal Component Analysis showing relationship among the twelve accessions of the five species of *Sesbania* Scop.

Conclusion

The regaining importance of green manuring in tropical and sub-tropical countries is a result of increasing fertilizer costs and the resulting environmental degradation. Many species of the genus *Sesbania* Scop. are well known as a promising green manure crop especially in rice wheat cropping systems. The extensive assessment of genetic variability is an important factor in breeding programs aiming to improve the quality of germplasm in order to increase their utilization as a green manure crop. In the past, some researchers have studied the genetic diversity in the members of the genus *Sesbania* Scop by using ISSR markers (9, 10). High levels of polymorphism were reported but these studies were either intraspecific or interspecific comparisons. In the present study, both intraspecific and interspecific comparisons of genetic diversity have been taken into account along with the biological status of the accessions and species. The genetically diverse germplasms which are useful for breeding programs as revealed in this study are S2 (S. sericea (Willd.) Link IC0631284), S5 (S. grandiflora (L.) Pers. IC0564742) and S6 (S. aculeata (Willd.) Pers. IC0445741). The exclusion of the accessions of S.grandiflora into a separate cluster was also observed. These germplasms can be used in breeding programs to combine the important characteristics of the studied accessions and increase the usage of *Sesbania* Scop species as biofertilizers.

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