Full Length Research Paper

Study of inter-specific relationship in six species of *Sesbania* Scop. (Leguminosae) through RAPD and ISSR markers

Manoj Kumar Bisoyi[1], Laxmikanta Acharya[2], Arup Kumar Mukherjee[3, 4]* and Pratap Chandra Panda[2]

[1]Department of Botany and Biotechnology, Khallikote Autonomous College, Berhampur, Orissa, India.
[2]Division of Taxonomy and Conservation, Regional Plant Resource Centre, Nayapalli, Bhubaneswar, Orissa, India
[3]DNA Fingerprinting Laboratory, Division of Plant Biotechnology, Regional Plant Resource Centre, Nayapalli, Bhubaneswar, Orissa, India.
[4]Central Institute for Cotton Research, Shankar Nagar PO, Nagpur-440010, Maharashtra, India.

Six species of *Sesbania* were fingerprinted using RAPD and ISSR markers. Both markers yielded a total of 249 bands out of which 243 were polymorphic in nature indicating high degrees of genetic diversity in the genus. Cluster analysis using the combined data revealed segregation of the lone species *Sesbania grandiflora* from the rest of the species. High boot strap values in the dendrograms show the accuracy and authenticity of the result. All other five species got separated to a distinct cluster. This supports the taxonomic division of the genus *Sesbania* into *Agati* Desv. comprising the only species *S. grandiflora* (Linn.) Poir. and sub-genus *Eusesbania* Baker containing all other species.

Key words: *Sesbania*, RAPD, ISSR and phenogram.

INTRODUCTION

The genus *Sesbania* Scop. belongs to the family Leguminosae under the tribe Robinieae and is comprised of about 60 tropical and subtropical species of herbs, shrubs or small trees (Mabberley, 1997; Veasey et al., 1999). Their nitrogen fixing ability enables these plants to grow rapidly on nitrogen deficient soils and allows their utilization for green manuring in paddy fields, for intercropping and ground cover, and for agroforestry and wood production (Ndoye et al., 1990). Baker (1876) divided the genus *Sesbania* occurring in British-India into two sub-genera namely, *Agati* Desv. comprising of the only species *Sesbania grandiflora* (Linn.) Poir. and all other species were included under the sub-genus *Eusesbania* Baker. Others preferred to split the genus in to four subgenera: *Agati*, *Daubentonia*, *Pterosesbania* and *Sesbania* (Monteiro, 1984). Subgenus *Sesbania*, originating from the Old World and later dispersed throughout the New World, has the greatest number of species including the perennial *Sesbania*

sesban, which has been widely utilized in agriculture for livestock forage and green manure (Brewbaker et al., 1990) . *S. grandiflora* of subgenus *Agati*, originating in the Old World (Australia and Asia), together with *S. sesban* have been utilized extensively in traditional agroforestry systems (Bray et al., 1997) and as high protein fodder, fuelwood and construction material.

The infra-generic classification and phylogeny of the genus *Sesbania*, which is based entirely on morpho-logical characters like habit and pod characters, have been a matter of controversy over the years. Though genetic diversity assessment have been made on some African species of *Sesbania* (Jamnadass et al., 2005), very little work has been done in Asia except that of Joshi-Saha and Gopalakrishna (2007), who studied the agro-morphological and genetic variability in five shrubby species of *Sesbania* (under the sub-genus *Sesbania*) collected from India, Philippines and Australia using only ISSR marker. Besides, Saraswati et al. (1993) and Veasey et al. (2002) have used seed proteins and iso-zymes for identification and characterization of *Sesbania* species and germplasm. The present investigation on

*Corresponding author. E-mail: titirtua@gmail.com. Tel: 91-7103-275536 (O), 2556912. Fax: 91-7103 -275529.

molecular profiling of six species of *Sesbania* aims at authenticating the classification and phylogeny of this economically important group of plants through RAPD and ISSR analysis.

During the last two decades, molecular markers have been widely used for molecular taxonomic studies in plants as a whole and flowering plants in particular. This has been due to parallel revolutions in theory, computing and molecular technology, whose synergies have resulted in the focusing of the discipline of systematics on the production of phylogenetic trees.

Different molecular markers used for genetic finger-printing and deriving phylogenetic relationship include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), inter simple sequence repeat (ISSR), etc. Among the molecular markers, RAPD is the most common, less complex and easy technique having application in genetic variability assessment and breeding programmes (Bonnin et al., 2004; Fritsch and Rieseberg, 1996). Besides, ISSR marker is also used for genetic characterization of different plant species as an alternative technique to study polymorphism based on the presence of micro-satellites throughout genomes (Zietkiewicz et al., 1994).

We report here the inter-specific relationship and phylogeny of six species of *Sesbania* as revealed through RAPD and ISSR markers and to authenticate the taxonomic status of the two sub-genera *Agati* Desv. and *Eusesbania* Baker under *Sesbania* Scop.

MATERIALS AND METHODS

Plant materials

Fresh leaf samples from six species of *Sesbania* were collected from Bhubaneswar and its adjoining regions for DNA isolation. After extraction, the DNA samples were preserved for future use at -85°C. The voucher specimens were deposited in the Herbarium of Regional Plant Resource Centre, Bhubaneswar, India.

Isolation of genomic DNA

Genomic DNA isolation

DNA was isolated from freshly collected young leaves by the CTAB method as described by Doyle and Doyle (1990). RNA was extracted with RNaseA (Quiagen) treatment: @ 60 μg for 1 ml of crude DNA solution at 37°C followed by two washings with phenol/ chloroform/ iso- amyl-alcohol (25:24:1v/v/v) and subsequently two washings with chloroform/ iso-amyl-alcohol (24:1 v/v). After centrifugation, the upper aqueous phase was separated, 1/10 volume 3 M-sodium acetate (pH 4.8) added and DNA precipitated with 2.5 volume of pre-chilled absolute ethanol. The extracted DNA was dried and then dissolved in 10 mM Tris-HCl [Tris(hydroxy methyl) amino methane]/1 mM EDTA (ethylene diamine tetra acetic acid, disodium salt)[$T_{10}E_1$ buffer pH 8]. Quantification was made by running the dissolved DNA in 0.8% agarose gel along side uncut λ DNA of known concentration. The DNA was diluted to 25 ng/μl

for RAPD and ISSR analysis.

Random amplified polymorphic DNA (RAPD) analyses

Nineteen random decamer oligonucleotide primers of A, AF, C, D and N series (Operon Technologies, Alameda, USA) as detailed in Table 1 were used for polymerase chain reaction (PCR) for RAPD analysis. The primers were dissolved in double sterilized $T_{10}E_1$ buffer (pH 8.0) to the working concentration of 15 ng/μl. The RAPD analysis was performed as per the methods of Williams et al. (1990). Each amplification reaction mixture of 25 μl contained 20 ng of template DNA, 2.5 μl of 10X assay buffer (100 mM Tris- HCl, pH 8.3, 0.5 M KCl and 0.01% gelatin), 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 20 ng of primer and 0.5U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler (Applied Biosystems, 9700). The first cycle consisted of denaturation of template DNA at 94°C for 5 min, primer annealing at 37°C for 1 min and primer extension at 72°C for 2 min. In the subsequent 42 cycles, the period of denaturation was reduced to 1 min while the primer annealing and primer extension time was maintained as in the case of the first cycle. The last cycle consisted of only primer extension at 72°C for 7 min.

Inter simple sequence repeat (ISSR) analyses

Two selected inter simple sequence repeats (Bangalore Genei Pvt. Ltd., Bangalore, India) were used for PCR amplification. Each amplification reaction mixture of 25 μl contained 20 ng of template DNA, 2.5μl of 10X assay buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl and 0.1% gelatin), 1.5 mM $MgCl_2$, 200 μm each of dNTPs, 15 ng of primer and 0.5U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler (Applied Biosystem, Model 9700). The first step consisted of holding the sample at 94°C for 5 min for complete denaturation of the template DNA. The second step consisted of 42 cycles, each cycles consisted of three temperature steps that is, 92°C 1 min for denaturation of template, 45 - 54°C; 1 min for primer annealing and 72°C for 2 min. for primer extension followed by 72°C for 7 min for complete polymerization.

The PCR products obtained from RAPD and ISSR were separated in 1.5 and 2% agarose gel respectively containing ethidium bromide solution (0.5 μg/ml of gel solution). The size of the amplicons was determined using standards (100 bp ladder plus, MBI Fermentas, Graiciuno, Vilnius, Lithuania). The DNA fragments were observed under UV light and photographed.

Data analysis

Jaccard's coefficient of similarity (Jaccards et al., 1908) was measured and a phylogram was generated by outweighed pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and SAHN clustering. Most informative primers were obtained by comparing all the primers with that of the pooled data using Mantel Z statistics (Mantel, 1967). All analyses were performed using the statistical package NTSYS pc 2.02e (Rohlf, 1997). Resolving power of the RAPD and ISSR primer were calculated as per Prevost and Wilkinson (1999). The RAPD primer index (RPI) was calculated from the polymorphic index (PIC), which was calculated as PIC = 1- P^2_i; P_i being the band frequency of the ith allele (Smith et al., 1997). In the case of RAPDs and ISSRs, the PIC was calculated as $1-p^2-q^2$, where p is band frequency and q is no band frequency (Ghislain et al., 1999). PIC value was then used to calculate the RAPD/ISSR primer index (RPI/SPI). RPI is the sum of the PIC of all the markers amplified by the same primer. Principal

Table 1. Details of RAPD banding pattern in six different species of *Sesbania*.

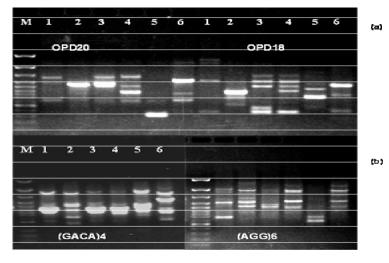| Primer	Nucleotide sequence	Range of amplicons (Bp)	Total no. of Bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Resolving power	Primer index
OPA04	AATCGGGCTG	2800-350	15	15	0	9	8.67	5.11
OPAF03	GAAGGAGGCA	2900-475	17	17	0	9	10.33	6.28
OPAF04	TTGCGGCTGA	2100-475	12	12	0	8	7.67	4.28
OPAF06	CCGCAGTCTG	3200-550	10	10	0	6	5.67	3.5
OPAF11	ACTGGGCCTG	2500-400	12	11	1	7	9.33	3.44
OPAF14	GGTGCGCACT	2400-500	9	8	1	4	6.33	2.94
OPAF15	CACGAACCTC	3000-650	11	11	0	10	4.67	3.22
OPC06	GAACGGACTC	1900-850	8	8	0	7	3	2.39
OPC10	TGTCTGGTGG	2100-250	8	8	0	5	5	2.61
OPC11	AAAGCTGCGG	2600-550	10	10	0	6	5	3.5
OPC12	TGTCATCCCC	2900-500	13	13	0	12	5	3.83
OPD02	GGACCCAACC	2100-900	3	2	1	1	2.67	0.55
OPD03	GTCGCCGTCA	1700-900	2	1	1	1	2.33	0.27
OPD08	GTGTGCCCCA	3000-400	17	17	0	12	8	5.66
OPD18	GAGAGCCAAC	2800-350	14	14	0	7	8	5.11
OPD20	ACCCGGTCAC	2300-350	9	8	1	5	5.67	2.72
OPN03	GGTACTCCCC	1600-575	6	6	0	1	3.33	2.11
OPN06	GAGACGCACA	3500-350	15	15	0	10	8.33	5.05
OPN15	CAGCGACTGT	1800-450	11	11	0	9	4.33	3.39
Total			202	197	5	131		

Figure 1. RAPD (a) and ISSR (b) banding pattern in 6 species of *Sesbania*.

co-ordinate analysis (PCA) was used to retrieve information about the clustering pattern of the analyzed population. PCA was performed based on the RAPD and ISSR data for all the primers (Semagn et al., 2000).

RESULTS AND DISCUSSION

Out of the twenty-five random decamer oligonucleotide primers used for RAPD analysis, 19 primers responded well and gave very good amplification. The RAPD banding pattern is represented in Figure 1a. All the primers gave wide range of fragments ranging from 250 to 3500 bp. The highest number of fragments (17) were amplified by the primer OPAF3 and lowest by OPD03 (2). The primer OPAF3 yielded maximum number of poly-morphic bands (17) and the least number polymorphic

Table 2. Details of ISSR banding pattern in six species of *Sesbania*.

Primer	Range of amplicons (Bp)	Total no. of bands	No. of monomorphic bands	No. of polymorphic bands	No. of unique bands	Resolving power	Primer index
(GA)9T	2000-450	11	11	0	5	6	4.11
T(GA)9	1500-400	9	9	0	5	6	3
(AGG)6	2300-550	13	13	0	8	8.33	4.5
(GACA)4	2100-450	14	13	1	9	8.67	4.44
Total		47	46	1	27		

Table 3. Details of RAPD and ISSR band sharing in six species of *Sesbania*.

Primer name	*S. javanica*	*S. speciosa*	*S. cannabina*	*S. rostrata*	*S. grandiflora*	*S. aculeata*
OPA04	5	4	6	4	4	3
OPAF03	4	5	5	6	5	6
OPAF04	4	5	3	4	2	5
OPAF06	1	4	4	2	2	4
OPAF11	3	6	6	5	3	5
OPAF14	3	3	4	3	3	3
OPAF15	4	2	1	2	3	2
OPC06	3	1	3	1	1	0
OPC10	4	2	4	2	2	1
OPC11	2	3	3	4	2	1
OPC12	4	3	3	2	1	2
OPD02	1	1	1	1	1	3
OPD03	1	1	1	1	2	1
OPD08	4	4	2	6	2	6
OPD18	3	4	5	5	3	4
OPD20	4	2	4	3	2	2
OPN03	4	3	1	1	2	0
OPN06	6	4	4	2	4	5
OPN15	2	3	2	3	2	1
(GA)9T	3	2	3	4	2	4
T(GA)9	3	3	3	3	3	3
(AGG)6	5	5	4	5	2	5
(GACA)4	6	4	4	4	5	4
Total	78	74	74	73	58	68

bands in case of OPD03 (1). Interestingly, each of the primers OPDO2, OPDO3, OPD20, OPAF06, and OPAF11 produced one mono-morphic band and none in case of other primers. The total numbers of unique bands amplified were 131. The primers OPD08 and OPC12 produced the maximum number of 12 unique bands. The resolving power (RP) and RAPD primer index (RPI) was highest (10.33 and 6.277 respectively) in case of OPAF03 and lowest (2.33 and 0.277 respectively) for OPD03.

Four ISSR primers yielded 47 bands out of which 46 were polymorphic and only one band was monomorphic in nature. Of the polymorphic bands, only 27 bands were unique. The amplicons were in the range of 400 to 2300 base pairs. The resolving power for the four primers (GA) 9T, T (GA)$_9$, (AGG)$_6$, (GACA)$_4$ were 6, 6, 8.33 and 8.67 and the primer index of 4.11, 3, 4.5 and 4.44 respectively (Table 2). The ISSR banding pattern is represented in Figure 1b.

The total number of bands amplified by RAPD and ISSR markers was highest in *Shorea javanica* (78) and lowest in *S. grandiflora* (58) (Table 3). The dendrogram constructed on the basis of both RAPD and ISSR data showed two distinct clusters (Figure 2). *S. grandiflora* was singled out from the rest five species, which formed a distinct clade and shared a node with high bootstrap
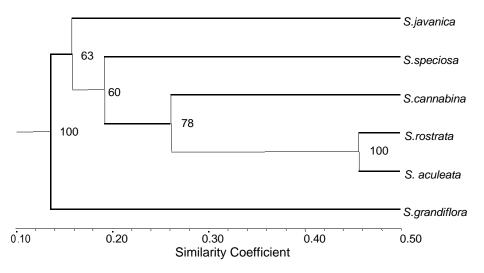
Figure 2. Dendrogram representing genomic relationship among 6 species of *Sesbania* using RAPD and ISSR markers (values written in between nodes indicating 100 bootstrap value).

Table 4. Jaccard's similarity among different members of *Sesbania*.

	S. javanica	*S. speciosa*	*S. canabina*	*S. rostrata*	*S. grandiflora*	*S. aculeata*
S. javanica	1.00					
S. speciosa	0.125	1.00				
S. cannabina	0.22	0.18	1.00			
S. rostrata	0.15	0.21	0.29	1.00		
S. grandiflora	0.11	0.15	0.15	0.17	1.00	
S. aculeata	0.14	0.17	0.24	0.45	0.13	1.00

value (100). *Sesbania rostrata* and *Sesbania aculeata* were closely related to each other with Jaccard's similarity of 0.455 (Table 4) and bootstrap value of 100. *S. javanica* and *S. grandiflora* were remotely placed with a similarity of 0.112. Co-phenetic correlation was found to be maximum between *S. aculeata* and *S. rostrata* (0.455) and minimum between *S. javanica* and *S. grandiflora* (0.112). This was further supported by the high bootstrap value (60 - 100) of different nodes of the dendrogram.

When the pairing by chance was calculated from the combined data it was found to be negligible (1.226×10^{-51}). Principal co-ordinate analysis (PCA) showed that all the species were distinctly placed (Figure 3) and follow a pattern similar to that obtained in the dendrogram. *S. grandiflora* was clearly separated out from all others and *Sesbania cannabina*, *S. aculeata* and *S. rostrata* formed a distinct cluster. Correlation between combined markers with RAPDs and ISSRs showed a high degree of correlation with 'r' values of 0.991 and 0.922 respectively. The correlation between RAPD and ISSR marker was also equally high (r = 0.922).

In the present investigation, six species of *Sesbania* were fingerprinted using RAPD and ISSR markers. Of the 25 RAPD primers used, six primers did not give

satisfactory amplification. Similar observation was earlier made in the legume sub-tribe Millettieae (Acharya et al., 2004) and in ginger cultivars (Nayak et al., 2005). RAPD and ISSR markers yielded a total number of 249 bands out of which 243 were polymorphic ones and only 6 bands were found to be mono- morphic. This is indicative of the fact that high degree of polymorphism do exist in the genus *Sesbania*. Jamnadass et al. (2005) observed high level of polymorphism in populations of *S. sesban* from Africa. While making an evaluation of the agro-morphological and genetic variability in five species of *Sesbania* collected from India, Philippines and Australia using, Joshi-Saha and Gopalakrishna (2007) analyzed the relationships among and with in populations of different species of the genus and found very high level of polymorphism (93.5%). However, they used only ISSR marker for molecular profiling and did not include the tree species *S. grandiflora*, which belong to a different sub-genus *Agati*. In the present study, both RAPD and ISSR markers have been used in combination and species wild or naturalized in the locality have been studied for genetic diversity.

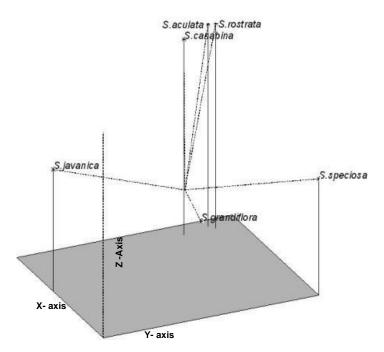The phylogenetic trees and similarity values among different species (Figure 2 and Table 4) show clear

Figure 3. Principal Co-ordinate analysis showing relationship among six species of *Sesbania.*

segregation of *S. grandiflora*, a tree species, from rest of the shrubby or herbaceous taxa. This justifies inclusion of the lone species under the sub-genus *Agati* Desv. (Baker, 1876). Of the other species, *S. javanica* got separated from the other four species. *S. rostrata and S. aculeata* were the closest in terms of similarity between them. High boot strap values in the dendrograms shows the accuracy and authenticity of the result, which is sup-ported by the findings of the previous workers like Baker (1876), Bairiganjan et al. (1984) and Veasey et al. (1999). Clustering of all the five species of *Sesbania* other than *S. grandiflora* also justify their classification under the sub-genus *Eusesbania (*Baker, 1876) or sub-genus *Sesbania* as described by Monteiro (1984) and Veasey et al. (1999). The segregation of *S. grandiflora* is supported by the cytotaxonomic study on *Sesbania* by Bairiganjan et al. (1984), who reported the species as a tetraploid with 2n = 24. From morphological, cytotaxonomical and molecular study, it is treating *S. grandiflora* as a sub-genus of *Sesbania* seems justified.

The high degrees of correlation between ISSR and RAPD (0.922) suggested that any of the markers could be used for the present study. Garcia-Mars et al. (2000) made similar observations using RAPD and AFLP markers in watermelon. Both ISSR and RAPD showed exactly same pattern of clustering of species. The principal coordinate analysis showed placement of all the species in a similar coordinate except *S. grandiflora* and the result was supported by morphological and cytotaxonomical studies described earlier.

This is the first report on genetic diversity assessment in the genus *Sesbania* using both RAPD and ISSR markers and taking into consideration species from two sub-genera *Agati* and *Eusesbania/ Sesbania.* Both the markers were found suitable for characterization of *Sesbania* species and germplasm.

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