African Journal of Plant Science Vol. 3 (8), pp. 168-173, August, 2009
Available online at http://www.academicjournals.org/AJPS
ISSN 1996-0824 © 2009 Academic Journals

Full Length Research Paper

Genetic differentiation of *Senna tora* (L.) Roxb. and *Senna obtusifolia* (L.) Irwin & Barneby by using RAPD markers

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Accepted 5 of June, 2009

Genetic relationships were examined among 19 accessions belonging to two *Senna* species by using RAPD markers. Within 60 tested primers, 9 primers only produced clear banding patterns that have been expected. An initial test of 60 primers, gave only 9 with consistently clear banding patterns. These 9 primers generated 108 scorable amplified products, of which 72 were polymorphic (66.6%). This degree of polymorphism is relatively low. An average of 12 bands was obtained per primer, ranging in size from 150 to 3530 bp. A UPGMA cluster analysis of genetic similarity indices grouped all the accessions into two major clusters corresponding to the pre-existing, species-level classification. Our result showed that RAPD technique is a sensitive, precise and efficient tool for genomic analysis in genetical discrimination of *Senna* species that may be useful in future studies by assigning new, unclassified germplasm accessions to specific taxonomic groups and reclassifying incorrectly classified accessions of other *Senna* species.

Key words: Molecular taxonomy, *Senna tora* L. and *Senna obtusifolia* L. RAPD.

INTRODUCTION

Senna tora (L.) Roxb. and *Senna obtusifolia* (L.) H.S. Irwin & Barneby belong to subfamily Caesalpinioideae of the Leguminosae. Both species are non-nodulating legumes (Parson and Cuthbertson, 1992; Randell, 1988) and thus do not have nitrogen-fixing bacteria asso-ciated with their roots (Waterhouse and Norris, 1987). *S. tora* and *S. obtusifolia* are important medicinal plants. Their roots have purgative and antihelminthic properties, and their leaves are used to treat skin diseases, dysentery and opthalmia (Quisumbing, 1951).

Singh (1965) observed that both species usually grow in association in each other. In India, *S. tora* rarely grows to more than two Feet. whereas *S. obtusifolia* grows to 2.5 m (James and Fossett, 1982), but, in both species, plant height depends on environmental condition. *S. tora* and *S. obtusifolia* have pantropical distributions (Flient et al., 1984), originally restricted to the Old World, princi-pally from the Indian subcontinent eastwards, and it is is likely that the species evolved in the Asia-pacific region (Randell, 1988). There are two morphological types of *S. obtusifolia* found in the USA (Irwin and Barneby, 1982). The fiirst type has narrow needle-like pods outwardly and downwardly curved, whereas the second has broader, less curved pods with compressed seeds that are obliquely tilted.

Many workers, including Brenan (1958), Retzinger (1984), and Singh (1978), separated these two species on the basis of anther shape and characteristics of the seed areole, extrafoliar nectaries, and seed testa. But the identification of both species are difficult due to their overlapping morphological characteristics. Upadhaya and Singh (1968) noted that there was no natural interspecific hybridization when both species were grown in adjacent plots. *S. tora* may have evolved from *S. obtusifolia* (Cock and Evans, 1984). Tandon and Bhatt (1971) have shown both species are caryologically distinct. *S. obtusifolia* has n= 12, 13, 14, whereas in *S. tora* n= 13, 14 and Indian forms of *S. tora* L. have n= 13, 14 so *S. tora* L.

DNA markers are considered the best tool for deter-mining genetic relationships, as they are nearly unlimited in number, can show high polymorphism and typically

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Table 1. Collected *Senna tora* and *Senna obtusifolia* L accessions.

S/No	Accession No.	Accession code	Taxon	Location
1.	200456	St1	*Senna tora*	Madhya Pradesh, India
2.	200457	St2	*Senna tora*	Himachal Pradesh, India
3.	200458	St3	*Senna tora*	Uttar Pradesh, India
4.	200459	St4	*Senna tora*	Karnataka, India
5.	200460	St5	*Senna tora*	Uttrakhand, India
6.	200461`	St6	*Senna tora*	Uttar Pradesh, India
7.	200462	St7	*Senna tora*	Uttar Pradesh, India
8.	200463	St8	*Senna tora*	Uttrakhand, India
9.	200464	St9	*Senna tora*	Uttrakhand, India
10.	200465	St10	*Senna tora*	Karnataka, India
11.	200466	So1	*Senna obtusifolia*	Madhya Pradesh, India
12.	200467	So2	*Senna obtusifolia*	Himachal Pradesh, India
13.	200468	So3	*Senna obtusifolia*	Karnataka, India
14.	200469	So4	*Senna obtusifolia*	Uttar Pradesh, India
15.	200470	So4	*Senna obtusifolia*	Uttrakhand, India
16.	200471	So5	*Senna obtusifolia*	Uttar Pradesh, India
17.	200472	So6	*Senna obtusifolia*	Himachal Pradesh, India
18.	200473	So7	*Senna obtusifolia*	Uttar Pradesh, India
19.	200474	So8	*Senna obtusifolia*	Karnataka, India

typically are independent of environmental interactions, that is, highly heritable. Various types of DNA markers are now available, but Random amplified polymorphic DNA technique has gained importance due to its simplicity, efficiency and the absence of a need for DNA-sequence information. RAPD technique has been very useful in studies of genetic relationships, phylogeny, systematics, genetic linkage mapping and gene tagging (Chalmer et al., 1994; Millan et al., 1996; Sun et al., 1998; Cheung et al., 1997; Tiwari et al., 1998).

In spite of the economic and medicinal value of *S. tora* and *S. obtusifolia*, no serious attempt has been paid to their diversity and taxonomy. We carried out RAPD analysis to assess its ability to differentiation between these two taxa and verify existing identifications.

MATERIALS AND METHODS

Plant materials

Ten accessions of *S. tora* and nine accessions of *S. obtusifolia* were collected from various locations in the Indian states of Uttrakhand, Uttar Pradesh, Madhya Pradesh, Karnataka, Rajasthan and Himachal Pradesh (Table 1).

Genomic DNA isolation

DNA was isolated from the leaves following the CTAB method described by Doyle and Doyle (1987) with few modifications. All DNA samples were diluted to a concentration of 5 ng/µl for use in Polymerase Chain Reactions (PCR).

PCR analysis

Sixty decamer random oligonucleotide primers of series B, C and AP from Operon Technologies (Alameda, California, USA) were used in PCR analysis. A standard 20 µl reaction containing 50 ng template DNA, 0.5 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 1X PCR reaction buffer, 10 picomoles primer and 100 µM of each dNTPs (MBI Fermentas, California, USA).

PCR conditions

DNA amplification was performed in a Gene Amp. 9700 thermal cycler (Applied Biosystem, location?). The thermal cycling program was performed according to Williams et al. (1990) with some modifications: incubation at 94^{o}C for 2 min., 44 cycles at 94^{o}C for 1 min., 36^{o}C for 1.30 min., and 72^{o}C for 1.30 min. Amplified products were separated on 1.4% agarose gels visualized by ethidium bromide and photographs were taken with the Gel Documentation system AlphaImager^{TM} 3400 (System & Control). λ DNA double digest (Hind III / EcoRI) was included as a molecular-weight marker.

Reproducible of RAPD data

Amplifications were repeated at least three times to ensure reproducibility. RAPD bands were considered reproducible only when they were observed in three separate amplifications that tested different DNA samples. Smeared or faint bands were not scored. Reaction components, template (DNA), dNTPS, primer and *Taq* DNA polymerase were standardized.

Data analysis: For each accession, the presence of band (1) or its absence (0) was recorded in a binary matrix. Pair-wise similarity coefficients were generated by using the Simqual subprogram of NTSYS-pc Version 2.02 (Rohlf, 2000) and used for cluster analysis with the SHAN subprogram of NTSYS-pc. A dendrogram was created based on the Unweighted Pair-Group Method with Arithmetic

Table 2. Results of nine polymorphic RAPD primers when evaluated on *Senna tora* and *Senna obtusifolia*

Primer No.	Total No. of amplicons	Total No. of bands	Polymprphic bands	Monomorphic bands	PIC Values	Average	Average No of bands	Size range of amplified product (bp)
B-2	130	9	5	4	0.2	0-0.4	14.4	300-2000
B-5	150	11	6	5	0.4	0.0.45	13.6	200-2500
B-10	245	12	9	3	0.1	0-.51	20.4	250-3500
B-12	180	15	13	2	0.5	0-0.32	12	300-2500
B-15	280	16	12	4	0.3	0-0.4	17.5	150-2700
AP-7	149	11	5	6	0.16	0-0.5	7.8	250-3530
AP-8	344	15	10	5	0.10	0-0.3	18.1	300-2700
AP-12	160	10	6	4	0.4	0-0.51	16	400-2500
AP-15	130	9	6	3	0.3	0-0.4	14.4	200-2400
	1768	108	72	36	0.273		Max= 20 Min=7	

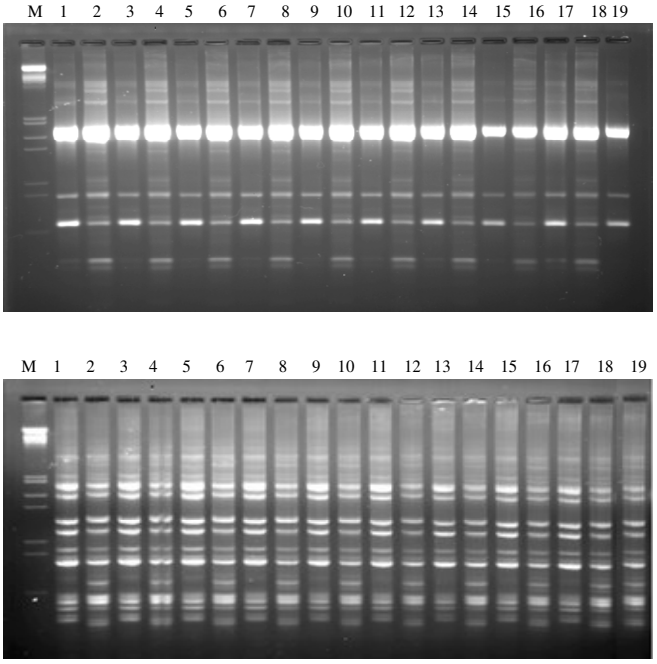

Figure-1. Lane M- Eco RI and Hind III digested λ DNA Lanes 1-19 Different accessions of *Senna tora* and *Senna obtusifolia.*

Average (UPGMA).

Polymorphic Information Content (PIC) was calculated by applying the formula given by Powell et al. (1996):

$$PIC = 1-\sum_{i=1}^{n} f_i^2$$

Where f i is the frequency of the i^{th} allele, and the summation extends over n alleles.

RESULTS

Of the 60 primers used to differentiate *S. tora* and *S. obtusifolia,* 10 gave no amplification among all accessions, 5 generated smeared banding patterns, 35 amplified only one or two bands, while 9 primers amplified polymorphic products. These 9 primers were then used for RAPD analysis of 10 accessions of *S. tora* and 9 accessions of S. *obtusifolia*. Amplification of 19 accessions yielded a total of 108 scorable bands, of which 72 were polymorphic (Table 2). An average of 12 bands was obtained per primer, and the amplification products ranged in size from 150 to 3530 bp. The highest number of bands was obtained with primer B-15, while the lowest number was obtained with primer AP-15. Figure 1 shows a representative amplification pattern obtained from primers B-6 and AP-17.

The polymorphism obtained in these 19 accessions showed a distinct variation. The highest degree of similarity (1) was observed among *S. obtusifolia* accessions while lowest (0.36) among *S. tora* accessions. Different primers varied (Table 3) in their ability to detect polymerphism. For example, primers B-12 and B-15 revealed the highest levels of polymorphism (100%) with all their amplification products being polymorphic, while primers C-2 and AP-15 revealed the lowest polymorphism (66%).

Cluster analysis of the RAPD data led to a clear distinction between *S. tora* and *S. obtusifolia*. The dendrogram based on the UPGMA clustering algorithm (Figure 2) arranged all the accessions into two major clusters; cluster I consist of the 9 accession of *S. obtusifolia* and cluster II with the 10 accessions of *S. tora*. Cluster I divided into 4 subcluster; subcluster I included 3 accessions (So1, So3, So2) subcluster II consists of 3 accessions (So6, So4 and So7), subcluster III included one accessions So5 and subcluster IV consisted two accessions (So8 and So9). Cluster II divided into three subclusters; subcluster I consisted one accessions of *S*

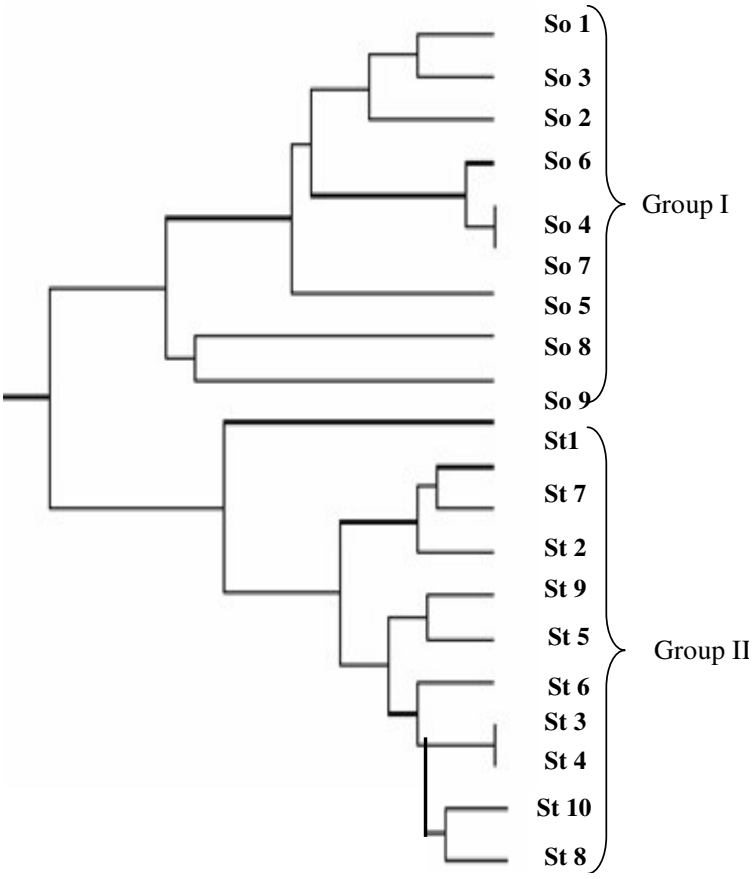

Figure 2. Dendrogram showing genetic relationships among *Senna*.

. *tora* L. (St1), subcluster II consisted three accessions (St7, St2, St9), subcluster III included seven accessions (St5, St6, St3, St4, St10 and St8).

DISCUSSION

Several doubts have been raised regarding the suitability of RAPD for genetic-relationship studies, the most important one being that comigrating bands may not be allelic. However, the homology of comigrating RAPD bands has been demonstrated in some species of *Glycine* and *Allium* (Williams et al., 1993; Wilkie et al., 1993). In addition, the conformity of phenetic groupings based on RAPD data to those based on conventional approaches, such as morphology, cytology and isozyme analysis, is in itself indirect, but significant evidence in support of a reasonable degree of allelism in comigrating RAPD bands (Virk et al., 2005). Some specific examples of such conformity include studies of ocimum, Gliricidia, *Musa* and *Brassica* (Howell et al., 1994; Demeke et al., 1992, Singh et al., 2004 and Chalmers et al., 1992)) and between species of *Stylosanthes* (Kazan et al., 1993). The use of large number of polymorphic markers would minimize the skewing of results due to non-allelism.

Another problem often encountered in RAPD analysis is that of the reproducibility of banding patterns between different PCR runs. This aspect can be overcome by using a thoroughly optimized PCR protocol and scoring only reproducible bands, as we have attempted to do in this study.

Cassiinae usually do not secrete nectar as the pollen is shed through short slits or pores in the anthers and the pollen is released through the vibration of the flowers by bees during 'buzz pollination' (Gottsberger and Silberbauer-Gottsberger 1988). Many species of *Senna* are well adapted to buzz pollination but in *S. obtusifolia* the gynoecium is curved over another pore. Pollination in this way would be unusual and it appears *S. obtusifolia* is self-fertile (Retzinger 1984). Indeed, self-fertilization is probably normal in *S. obtusifolia* as the flower is commonly fertilized in late bud, before the flower is open, when the style is curved inward to present the stigmatic cavity directly to the face of the precociously dehiscent anthers and have been shown to detect high polymerphism than RFLP and AFLP markers (Thormann et al., 1994; Das et al., 1999). RAPD markers revealed low degree of polymorphism among the 19 acces-sions belonging to two species of *Senna*. Virk et al. (1995) have analyzed the germplasm collection of rice acces-sions by RAPD marker and classified the unclassified rice accessions as *indica* or *japonica* types. Similarly, Howell

Table 3. Pair-wise genetic distances among different accessions of *Senna tora* and *Senna obtusifoli*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		1	2	3
1	1.00																		
2	0.363																		
3	1.00	0.363																	
4	0.363	1.00	0.363																
5	1.00	0.363	1.00	0.363															
6	0.363	1.00	0.363	1.00	0.363														
7	1.00	0.363	1.00	0.363	1.00	0.363													
8	0.363	1.00	0.363	1.00	0.363	1.00	0.363												
9	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363											
10	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363										
11	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363									
12	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363								
13	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363							
14	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363						
15	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363					
16	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363				
17	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363			
18	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	0.363	
19	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00	0.363	1.00

and Newburg (1994) have used RAPD for identifying and classifying *Musa* germplasm. Pipe et al. (1995) supported the separation of two groups of *Opiostoma* piceac into two species based on the clear cut divergence revealed by RAPD. In another case the genus *Scaevola*, which was initially misclassified by Linnaeus in 1753, and further rearranged several times by other scientists (Bentham, 1868; Krauze, 1912; Carolin, 1992), has now been classified, resolving the previous confusions through RAPD analysis (Swoboda and Bhalla, 1997), Kumar et al. (2007) solve the taxonomic problem of *Cassia glauca*. And separated two species Senna sulfurea and Senna surattensis.

Our study on *S. tora* L. and *S. obtusifolia* accessions has shown that RAPD is a robust and reliable method to detect genetic differentiation and genetic relationships assessment.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Department of Biotechnology, Government of India for financial support.

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