Random amplified polymorphic DNA as marker for genetic variation and identification of *Senna surattensis* Burm, f. and *Senna sulfurea* DC. ex Collad.

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The taxonomy and nomenclature of various species of the genus *Cassia* L. are quite complex due to overlapping morphological characters. They are not easily differentiated from closely related species due to a great range of similarities. *Senna surattensis* Burm. f. is one of the important species of the *Cassia* group. Besides its medicinal importance, this species also has taxonomic importance. The taxonomy of *S. surattensis* is based primarily on morphology, which often makes identification difficult, because this species is separated on the basis of fine differences in morphological traits of closely related species, *Senna sulfurea* and others. These two species are so closely related that their status has been variously interpreted. RAPD analysis was carried out to assess the authentic identification as well as to solve the taxonomic problems between *S. surattensis* and *S. sulfurea*. Amplification with 10-mer primers was performed under pre-standardized condition of 38 accessions along with *Senna occidentalis*, *Cassia fistula*, *Senna tora* and *Senna siamea* collected from different mixed populations. Out of sixty primers utilized, fifty-four were successful in amplification and among them one was species-specific. The results demonstrate the ability of RAPD markers to reliably differentiate between *S. surattensis* and *S. sulfurea*.

Keywords: Genetic variation, markers, RAPD, *Senna sulfurea*, *Senna surattensis*.

CASSIA L. Sensu lato is one of the twenty-five largest genera of dicotyledonous plants in the world and originally treated as a large heterogeneous genus. Irwin and Barneby[1] proposed an improved classification and raised the genus *Cassia* L. to the level of subtribe *Cassiinae*; comprising of three genera, viz. *Cassia* L. Sens. str., *Senna* P. Mill. and *Chamaecrista* Moench. There has been considerable divergence of opinion concerning the delimitations and taxonomic status of its three constituent subgenera. This genus has about 600 species occurring mostly in the tropics and subtropics[2], and it is an economically as well as medicinal important genus[3]. Within its bounds may be seen great diversity in habit, ranging from tall trees to delicate, prostrate, annual herbs[4], and 2C DNA amounts range from 1.30 to 2.54 pg/cell in ten *Cassia* species at the same ploidy level belonging to herb, tree and shrub. These form a natural grouping of herbs, trees and shrubs with respect to mean 2C DNA amount, except *Cassia excelsa* which increases stepwise, approximately 0.05 pg among the successive groups[5]. Also, there is a wide variation in foliar characters and mode of pod dehiscence[4]. The taxonomy and nomenclature of various species and intraspecific taxa have been quite complex and intriguing. They are not easily differentiated from closely related species due to a great range of similarities. This usually leads to mis-identification and mis-interpretation of components. With a taxonomical point of view, *Cassia glauca* has been selected for molecular studies.

According to Chatterjee[6], 'The plant commonly known in India as *C. glauca*, is a shrub or a small tree with attractive yellow flowers'. Rheede[7] described this plant under the name *wellia-tagera*. Burman[8] described the plant and named it *Cassia surattensis*. Later, Lamarck[9] described the plant as *C. glauca* and Vahl[10] named it *Cassia arborescens*. Both Lamarck and Vahl referred to Rheede's description. Bentham[11] in his revision of the genus *Cassia*, agreed that all these names refer to the same species, but he accepted the name *C. glauca*. Merrill[12], however, indicated that the name *C. surattensis* should receive priority over *C. glauca*. More recently, Raizada and Hingorani[13] accepted the name *C. surattensis*.

The species has two well-marked varieties: (i) var. *surattensis* and (ii) var. *suffruticosa*. Bentham[11] found it difficult to separate the Indian collection into two distinct varieties. He has, however, stated the existence of two 'types'. *S. surattensis* (Burm. f.) Irwin & Barneby and *S. sulfurea* (DC. ex Collad.) Irwin & Barneby are so closely related that their status has been variously interpreted earlier. The most confusing names are *C. surattensis* Burm. f., *C. glauca* Lam. and *C. suffruticosa* Koen. ex Roth. The last two species have been considered synonymous under *C. surattensis* Burm. f. by some workers[11,14]. As such the types could not be analysed and the specific epithet *surattensis* was referred to species with few larger leaflets, and epithet *glauca* and *suffruticosa* with more but smaller leaflets. Earlier workers also overlooked the protologe which describes just the reverse of the prevailing concept. Irwin and Barneby[1] recognized this mistake in their work and placed those specimens under *S. surattensis* (Burm. f.) Irwin & Barneby, which have more but smaller leaflets, keeping *C. suffruticosa* Koen. ex Roth under its synonymy, and specimens with fewer and larger leaflets under *S. sulfurea* (DC. ex Collad.) Irwin & Barneby, keeping *C. glauca* Lam. under its synonymy. Randell[15], however, considered *C. sulfurea* DC. ex Collad. as a variety under *S. surattensis* (Burm. f.)[16].

In spite of the economic and medicinal value of *C. glauca*, no serious attention has been paid to diversity, characterization and taxonomical identification at the molecular level. This is a prerequisite to the exploitation of the vast genetic variability available for the improvement of the

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quality and quantity of their drug contents. Morphological characters and behavioural patterns, which have until recently provided the major guide to classification, can sometimes evolve independently in unrelated species in response to common environmental challenges. Thus the central problem of phylogenetics has been to distinguish the component of biological similarity due to descent from common ancestry (homology) from that due to convergence from different ancestors[17]. For this interpretation, research on DNA markers was carried out between mixed populations of *S. surattensis* and *S. sulfurea* to solve the taxonomical confusion.

A number of molecular marker systems are available for such studies and RAPD (Random Amplification of Polymorphic DNA) method[18] was used for the analysis of genetic variation and identification in both the species and to screen primer(s) to test the purity of species. RAPD markers provide an efficient assay for polymorphisms, which allow rapid identification and isolation of chromosome-specific DNA fragments. Genetic polymorphism detected with RAPD reveals one allele per locus, which corresponds to the amplification product. RAPD is not expected to identify heterozygous loci. In this context, RAPD is limited. It may be used to identify heterozygous individuals when a single primer generates at least one complementary polymorphic amplification product from each parent[19]. Species- and population-specific loci were also identified by this marker. In particular, RAPD is a useful predictive tool to identify areas of maximum diversity and may be used to estimate levels of genetic variability in natural population.

Thirty-eight selections of mixed population of *S. surattensis* and *S. sulfurea* along with *S. occidentalis*, *C. fistula*, *S. tora* and *S. siamea* located in different parts of India from Uttar Pradesh to Uttarakhand (about 2000 km) were collected as two selections per place (Table 1 and Figure 1). These thirty-eight selections were either maintained by local people or found in the wild. After morphological studies, two groups of plants on the basis of leaflets were distinguished in different mixed populations (Figure 2).

Table 1. Place of collection and accessions number of selections

Place of collection	Location on map (Figure 1)	Accession no.
Aligarh (UP)	1	A and B
Raebareilly (UP)	2	C and D
Unnao (UP)	3	E and F
Kanpur (Urban) (UP)	4	G and H
Kanpur (Rural) (UP)	5	I and J
Kanauj (UP)	6	K and L
Mainpuri (UP)	7	M and N
Etah (UP)	8	O and P
Lucknow (UP)	9	Q and R
Bulandsahar (UP)	10	S and T
Meerut (UP)	11	U and V
Muzaffarnagar (UP)	12	W and X
Haridwar (U)	13	Y and Z
Saharanpur (UP)	14	AA and AB
Pauri (U)	15	AC and AD
Uttarkashi (U)	16	AE and AF
Teharigarhwal (U)	17	AG and AH
Rudraprayag (U)	18	AI and AJ
Dehradun (U)	19	AK and AL

UP, Uttar Pradesh; U; Uttarakhand.

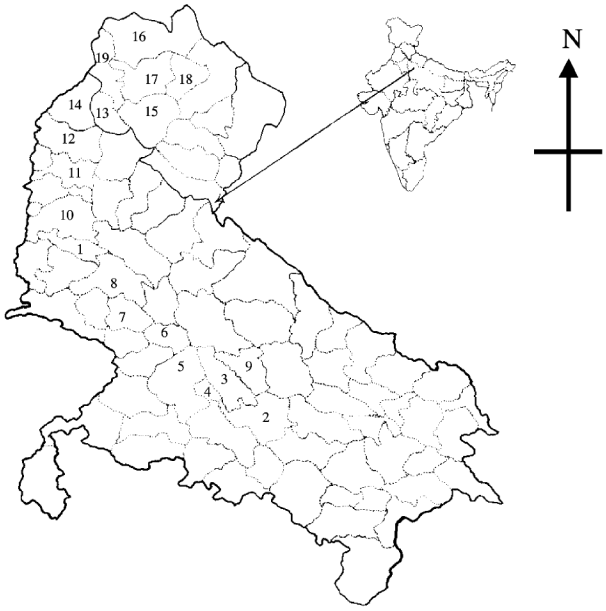

Figure 1. Place of collection in Uttarakhand and Uttar Pradesh.

Figure 2. Leaf of *Senna sulfurea* leaflets (***a***) and *Senna surattensis* (***b***) showing leaflets.

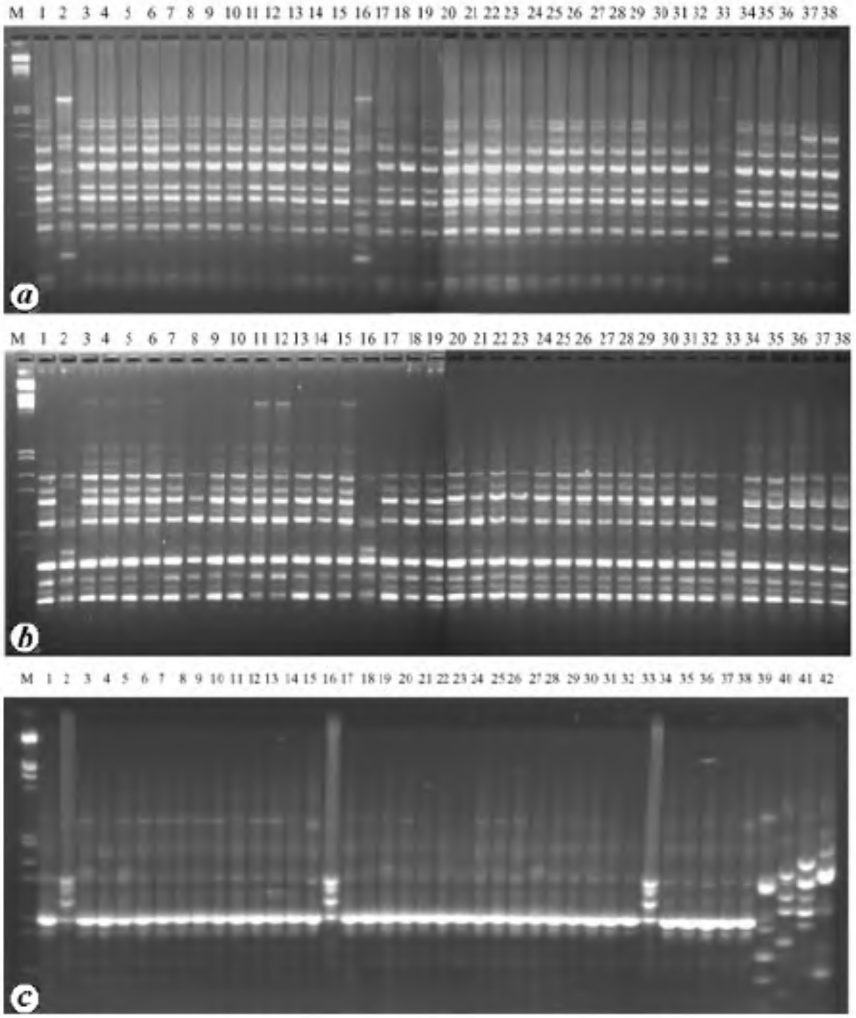

Figure 3. ***a***, ***b***, RAPD profile of accessions of *S. sulfurea* and *S. surattensis* listed in Table 1, after amplification with primer OP-C-8. (***a***) and digested λ-DNA marker; lanes 1, 3–15, 17–32 and 34–38, *S. sulfurea*, and lanes 2, 16 and 33, *S. surattensis*. Primer OP-C-20 (***b***) Lane M, *Hind*III and *Eco*RI digested λ-DNA marker, lanes 1, 3–15, 17–32 and 34–38, *S. sulfurea* and lanes 2, 16 and 33, *S. surattensis*. (***c***) RAPD profile of accessions of *S. sulfurea* and *S. surattensis* listed in Table 1, after amplification with primer OP-AP-6. Lane M, *Hind*III and *Eco*RI digested λ-DNA marker; lanes 1, 3–15, 17–32 and 34–38, *S. sulfurea*; lanes 2, 16 and 33, *S. surattensis* and lanes 39–42, *Senna occidentalis*, *Cassia fistula*, *Senna tora* and *Senna siamea* respectively.

Young and healthy leaves from a single tree of each accession were used for DNA isolation by CTAB method, with minor modifications[20]. The extraction buffer composition was 2% w/v CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.2% 2-mercepto-ethanol v/v. DNA was treated with bovine pancreatic RNAase and extracted once with phenol, followed by phenol : chloroform (1 : 1) and twice with chloroform : isoamyl alcohol (24 : 1). After precipitation with iso-propanol, a 70% ethanol wash was given. DNA was dissolved in TE buffer and quantified in a fluorometer (DyNA Quant[200]).

DNA prepared from all the selections was used for the PCR reactions. RAPD variations within and among populations were assessed using DNA from individual seedlings. Conditions have been optimized to allow reproducible RAPD amplification. PCR reactions were individually primed with different primers. These oligonucleotide primers were supplied by Operon, USA.

PCR reaction was preceded by a hot start at 94°C for 5 min. The PCR reaction was performed using a Gene Amp PCR System 9700 with 44 cycles of 94°C for 60 s, 36°C for 90 s and 72°C for 90 s, followed by 5 min of final polymerization step, the reaction was held at 4°C until further processing. Each reaction contained 10× thermostable PCR buffer, 25 mM $MgCl_2$, 0.2 mM dNTPs in equimolar ratio, 1 unit Taq polymerase (Genei, Banglore), 0.5 mM primer (Operon, USA) and 50 ng template DNA to a final volume of 20 µl.

The reactions were then electrophoresed through 1.5% agarose gels under constant voltage (11 V) overnight.

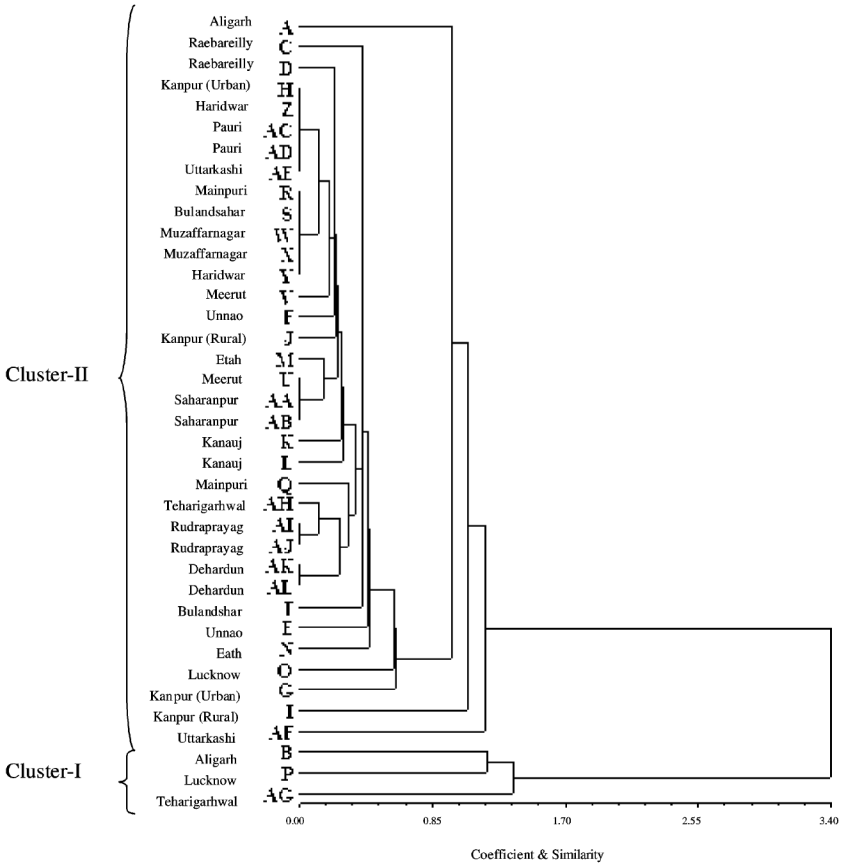

Figure 4. Dendrogram showing the relationship among 38 accessions of *S. sulfurea* and *S. surattensis* from mixed populations based on UPGMA and sequential agglomerative hierarchical nested clustering.

*Eco*RI and *Hind*III digested λ-DNA standards were used to assign molecular weight to individual RAPD bands. After completion of electrophoresis, the gels were stained in a dilute solution of ethidium bromide. RAPD bands were scored as present or absent on gels with PCR reaction being done in duplicate to confirm reproducibility of banding patterns, and data were entered in a binary data matrix as discrete variables. Analysis of RAPD markers was based on the assumption that each marker represented a single locus comprising two alleles, a marker allele (amplified product present) and a non-marker allele (amplified product absent). Only bands that were between 100 and 2500 bp in size and that appeared consistently between runs were rated as present in composite scores of entries. Bands that were classified as faint were not included in the dataset, as these were assumed to be unreliable markers. A dendrogram was prepared using UPGMA. The computer package NTSYS-pc, ver. 2.1 was used for cluster analysis[21].

For the isolation of good-quality DNA, a CTAB-based procedure was optimized in the present study that yielded high-quality DNA free of polyphenols, polysaccharides and other secondary compounds, which may inhibit the activity of *Taq* polymerase. To minimize polymerization errors in the present study, many factors were standardized. Two accessions were used for a preliminary amplification test to standardize the DNA amplification conditions on *S. surattensis* and *S. sulfurea*.

In this study, 60 Operon, random, ten-base long, single-stranded primers (twenty primers from each kit of OP-B, OP-C and OP-AP) were screened with 35 accessions of *S. sulfurea* and three accessions of *S. surattensis* collected from different mixed populations. Among these, 54 primers gave on an average 10.37 and 11.74 bands per primer per sample in *S. sulfurea* and *S. surattensis* respectively, with 10.48 bands on an average in all 38 accessions of *S. surattensis* and *S. sulfurea*.

Four primers yielded no amplification products, and two primers yielded no clear or scorable bands. Seven and five primers yielded few polymorphic bands in *S. sulfurea* and *S. surattensis* respectively, and nine primers gave few polymorphic bands in either case (Figure 3 *a* and *b*). Most of the primers gave monomorphic bands among *S. surattensis* and *S. sulfurea*, though they belong to different populations and different places. No primer gave banding pattern for the intermediate between *S. surattensis* and *S. sulfurea*.

A single band was amplified for *S. surattensis* with primer OP-AP-6 (GTCACGTCTC) along with the genomic

DNA of *S. sulfurea*, *S. occidentalis*, *C. fistula*, *S. tora* and *S. siamea* (Figure 3 *c*) and this primer can be treated as species-specific.

The dendrogram (Figure 4) demonstrates the ability of RAPD markers to reliably differentiate between *S. surattensis* and *S. sulfurea*, since their phenotypic differences are not clear. The 38 accessions analysed clustered mainly into two groups, which support the classification of Irwin and Barneby[1]. Cluster I (B, P and AG) of three accessions of *S. surattensis* with range up to 1.36 and cluster II consisting of the remaining 35 accessions with range 0.00–1.19 and accession 'A' were more distinct than 'I' and 'AF'. A single marker, OP-AP-6 (GTCACGTCTC) has potentially informative value for distinguishing both the species. DNA sequences related to particular phenotypic characters like leaf size have not yet been identified. Cloning and characterization of such markers, possibly genes, could provide valuable tools for breeding programmes. The presence of few polymorphic bands with less number of primers and more monomorphic bands with most primers among *S. surattensis* and *S. sulfurea*, and the presence of more number of polymorphic bands between the two species strongly support the classification of Irwin and Barneby[1], and justify that both the species have separate status in *C. glauca*.

The present investigation of DNA profiling in *Senna* species clearly demonstrates that it is possible to analyse the RAPD patterns for correlating their similarity and distance between species and accessions, by which one can predict the origin of the species to a great extent. Further, the species-specific band can be utilized to define the uniqueness, which will be helpful in the identification of species and taxa. On the basis of clustering data for the RAPD profile, accessions of the first and second group exhibit greater similarity amongst each other, and genetic variation has been found in accessions of the same population because samples have been collected from the wild as well as selections maintained by local people.

In *Cassia*, where floral structure is adapted for outbreeding, the isolating mechanism appears to be weak, and hybridization is undoubtedly of frequent occurrence, sexual reproduction would be expected to produce rapid and wide segregation, which has been indicated by population surveys[15]. However, in the study there was no finding of intermediate between *S. surattensis* and *S. sulfurea*. Apart from this, no fruit formation was found after several reciprocal crosses had been done between the species. This may support the results of Dulberger[22] and Owens[23] that interspecific pollen transfer may be particularly critical in Cassiinae, where the species examined proved to be self-compatible or pollination between species flowering concurrently in a given locality, and visited by the same bee species, is prevented by structural differences between their flowers, i.e. by mechanical–morphological isolation[24] or by different location of pollen on the bees.

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