Genetic diversity of 28 wild species of fodder legume *Cassia* using RAPD, ISSR and SSR markers: a novel breeding strategy

Suprava Mohanty[1], A. B. Das[2,*], Nabarun Ghosh[3,*], B. B. Panda[4], Don W. Smith[5]

[1]Cytogenetics Laboratory, Regional Plant Resource Centre, Nayapalli, Bhubaneswar 751015, Orissa, India. [2]Department of Agricultural Biotechnology, College of Agriculture, Orissa University of Agriculture & Technology, Bhubaneswar 751003, India. [3]Department of Life, Earth and Environmental Sciences, West Texas A&M University, Canyon, TX 79016, USA. [4]Departemnt of Botany, Berhampur University, Orissa, India. [5] Department of Biological Sciences, University of North Texas, Denton, Texas 76203

RAPD, ISSR and SSR primers were used to assess genetic diversity and phylogenetic relationships among 28 species of *Cassia* (2n = 16, 26, 28). RAPD, ISSR and SSR primers revealed 36.12, 42.7 and 54.4% polymorphism, respectively. The Dendograms based on RAPD, ISSR, and SSR data precisely organized 28 species of *Cassia* into different clusters. SSR primer could distinguish all species analyzed within the genus. Polymorphic index varied from 0.1 to 0.5 for both SSR and RAPD markers; primer index values were substantially higher for RAPD primers (0.35 – 4.65) than for SSR primers (0.35 – 1.73). It was possible to identify accessions with the help of RAPD, ISSR and SSR markers. Dendograms constructed from RAPD, ISSR and SSR data revealed DNA marker-based genetic identification in *Cassia*. Four groups of *Cassia* that were resolved corresponded to species grouped earlier taxonomically. *Cassia mimosoides* with a different genomic set up showed close relation to *C. javanica*. *Cassia siamea* and *C. spectabilis*; *C. grandis* and *C. nodosa* in a core group with close morphological similarities. *Cassia artemisioides* and *C. covesii*, both showing morphologically drought tolerance characters are closely placed, indicating that they are the wild progenitors of these species. The broad adaptation of some species of *Cassia* implies a large probability that nuclear DNA mutations occurred in the past and that resulted in diversification and genetic polymorphism in growth habit, vigor, and stem, leaf, seed, and pod characteristics and a physiological polymorphism, the water-use efficiency.

* Corresponding authors: (1) Dr. Nabarun Ghosh, Department of Life, Earth and Environmental Sciences, West Texas A&M University, Canyon, TX 79016, USA. E-mail: nghosh@mail.wtamu.edu. (2) Dr. A. B. Das, Department of Agricultural Biotechnology, College of Agriculture, Orissa University of Agriculture & Technology, Bhubaneswar 751003, Orissa, India. E-mail: a_b_das@hotmail.com or drabdas@yahoo.com.

Abbreviations: ISSR= Inter-Simple Sequence Repeats, RAPD= Random Amplified Polymorphic DNA, SSR= Simple Sequence Repeat.

Introduction

Cassia L. is a large genus of the family Fabaceae, subfamily Caesalpinioideae with over 300 species distributed worldwide that commonly grows in all types of ecological sites in arid regions of India over a large area in all types of ecological sites. Species of *Cassia* are valued for their medicinal properties and industrial value. In *Cassia* the biomass production rate is very high and it is commonly used for firewood. The extensive variability in its growth habit ranging from tall trees to delicate annual herbs, numbers and size of the leaflets, form and foliar characteristics has added difficulties to taxonomists in identification of species or the intraspecific taxa for influence of habitat conditions (1). Some exotic species like *C. grandis, C. laevigata, C. multijuga, C. nodasa, C. renigera and C. speciosa* are grown as garden

plants for their graceful appearance and beautiful flowers. All of them exhibit excellent drought resistance. This immense natural variation in *Cassia* may be harnessed to enhance the yield products like gums, tannins, proteins, carbohydrates and oils, biological nitrogen fixation and resistance to biotic and abiotic stresses (2). Hence, cataloging and characterizing the genomes in terms of DNA markers is of immense importance and can serve as a DNA database for future improvement programs.

During the last decade numerous novel DNA-markers (RAPD, RFLP, SSR, ISSR etc.) have been rapidly integrated into the tools available for genome analysis (3). Salimah et al. (4) have used DNA fingerprinting for assessing genetic diversity. Presence or absence of DNA bands in the gel may be used as RAPD markers to study closeness genetic relationship (5), inter- and intra-specific genetic variations (6), identification of specific genes (7) and patterns of gene expression (8). In cultivated legumes, genetic variation for resistance to different abiotic and biotic stress is low. This has motivated breeders to extend their search into the secondary gene pool, involving wild relatives, some of which are known to possess agronomically useful characters (9). The utility of PCR-based RAPD or inter-simple sequence repeat (ISSR) variations as phylogenetic markers for investigating evolutionary relationships among plants has been clearly established (10-12) Kass and Wink (13) reported phylogenetic studies of three sub families of Leguminosae using *rbcL* sequences. Genetic diversity of 30 accessions of *Cassia brewsteri* using randomly amplified DNA were reported by Cunningham et al. (14). Isozyme profiles of *Cassia auriculata* were reported earlier to enumerate the genetic differences (15). Moreover, cDNA encoding isoforms of chalcone synthase were isolated from a cDNA library of RNA from root tissue of an important medicinal plant *Cassia alata* (16). Recently interspecific variations of 4C DNA content and somatic chromosome number in 15 species of Cassia were reported by our group to establish the genetic relationships among the species (17). However, these parameters are not sufficient to interpret the phylogenetic relationships of *Cassia.* Not much has been reported on phylogenetic studies of *Cassia* using molecular markers like RAPD, ISSR and SSR. Thus, in the present study, we have used RAPD, ISSR- and SSR fingerprinting data to assess first, the level of genetic diversity within the genus; second, to highlight any observed trends in the genetic diversification and differentiation of *Cassia* at the species level; and third, to estimate to what extent the results of molecular analysis will be helpful in reconstructing phylogenetic relationships among the species in the genus *Cassia*.

Materials and Methods

Plant Source

Table 1 shows the 28 species of *Cassia* we investigated. The seed samples were collected from the Regional Plant Resource Campus (RPRC), Bhubaneswar and nurseries in Dehradun, Uttar Pradesh, India and from the Royal Botanical Gardens, Kew, UK. The seeds were germinated *in vitro* and were planted on the experimental gardens of RPRC, Bhubaneswar. Voucher specimens are kept in the herbarium of RPRC, Bhubaneswar.

Isolation of genomic DNA

Isolation of genomic DNA is difficult in *Cassia* species due to heavy cytoplasmic contents like polyphenol, gum and polysaccharides. Nuclear DNA was isolated from leaf tissues of *Cassia*

Table 1. Collection of seeds: 28 species and their accessions

SPECIES	COLLECTED FROM
1. Cassia tora (L) Roxb.	Kew Botanical Garden, Kew and Local collections
2. Cassia obtusifolia (L). Irwin & Barneby	Kew Botanical Garden, Kew and Local collections
3. Cassia spectabilis (DC) Irwin & Barneby	Kew Botanical Garden, Kew and Local collections
4. Cassia roxburghii DC.	Local collections
5. Cassia siamea (Lam) Irwin & Barneby	Dehradun nursery and Local collections
6. Cassia occidentalis (L) Link	8 Accessions
7. Cassia absus (L.) Irwin & Barneby	Local collections
8. Cassia mimosoides (L) Link	Local collections
9. Cassia fistula L.	Dehraun Nursery and Local collections
10. Cassia senna	Dehradun Nursery
11. Cassia javanica Linn.	Local collections
12. Cassia renigera Wall.	Local collections
13. Cassia grandis	Dehraun Nursery and Local collections
14. Cassia biflora	Local collections
15. Cassia glauca Lam.	Dehraun Nursery and Local collections
16. Cassia alata Linn.	Dehraun Nursery and Local collections
17. Cassia nodosa Ham.	Dehraun Nursery and Local collections
18. Cassia italica Mill.	Kew Botanical Garden, Kew
19. Cassia wislizenii	Kew Botanical Garden, Kew
20. Cassia polyantha	Kew Botanical Garden, Kew
21. Cassia versicolor	Kew Botanical Garden, Kew
22. Cassia petersiana	Kew Botanical Garden, Kew
23. Cassia floribunda (Cav.) I. & B.	Kew Botanical Garden, Kew
24. Cassia lindheimeriana	Kew Botanical Garden, Kew
25. Cassia martiana	Kew Botanical Garden, Kew
26. Cassia atomaria (L) Irwin & Barneby	Kew Botanical Garden, Kew
27. Cassia artemisioides (Gaud. ex DC)	Kew Botanical Garden, Kew
28. Cassia hirsuta (L) Irwin & Barneby.	Kew Botanical Garden, Kew

following the modified CTAB method of Saghai-Maroof et al (18). The DNA was extracted with 3 volumes of CTAB extraction buffer (2% CTAB, 100mM Tris HCl, pH 8.0, 20 mM EDTA, 1% 2-mercaptoethanol). The concentration was measured using a Versafluor TM Fluorometer (Bio-Rad, USA) with Hoechst 33258 fluorimetric dye and gel electrophoresis. A known quantity of Lambda DNA (Bangalore Genei) was used as the standard. The DNA was diluted to a final concentration of 25 ng/µl with $T_{10}E_1$ buffer and was used as the template for Polymerase Chain Reaction (PCR).

RAPD fingerprinting

A total of 21 selected 10-mer primers from Operon kits A, C, N and AF (Operon Technologies) were used for PCR amplification. Each 25 ng/µl reaction volume contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM each dNTP (Promega), 200 nM primer, 25 ng DNA template, and 1 U Taq DNA

polymerase (Bagalore Genei). DNA amplification was performed in a Perkin Elmer Cetus Thermal Cycler programmed for 45 cycles, as described by Williams et al., (3). Initial denaturation was 7 min at 94^{o}C, followed by 45 cycles of 30 sec at 94^{o}C, 45 sec at 52^{o}C and 2 min at 72^{o}C, with a final extension at 72^{o}C.

ISSR fingerprinting

Inter-simple sequence repeats microsatelite markers were synthesized and PCR were performed using different ISSR sequences- $(GATA)_4$, $(ACTG)_4$, $(GACA)_4$, $(GACAGATA)_2$, $(ACAG)_4$, AGGGCTGGAGGAGGGC, AGAGGTGGGCAGGTGG, $(CAG)_5$, CAGCGACAAG, $(CT)_8$, $(CAG)_5$, GGGC-$(GA)_8$. These primers were mostly 14 to 16-mers. The amplification was carried out in a 25 µl reaction volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM each of dNTP, 200 nM primer, 1 U Taq DNA polymerase and 20 ng of genomic DNA. For denaturation the same protocol was repeated from RAPD.

SSR fingerprinting

To develop SSR markers for *Cassia* species, we conducted a Gene Bank database search to identify SSR sequences present in *Cassia* DNA. We designed the primers both forward and backward (20- and 20-mer primers) using the flanking sequences of the SSR repeat (Table 3) , PCR reaction and size separation of the PCR amplified fragments in 3% agarose gel were carried out to examine the existing level of genetic diversity using microsatellite sequences in the *Cassia* species. The amplification was carried out in a 25 ng/µl reaction volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM each of dNTP, 200 nM primer, 0.6 U Taq DNA polymerase and 40 ng of genomic DNA. Initial denaturation was for 3 min at 94°C; followed by 42 cycles of 1min at 94°C, 1min at 45-58°C, and 2 min at 72°C; with a final 15 min extension at 72°C.

The amplification products in all the cases were size-separated by standard horizontal electrophoresis in 3.0 % agarose gels and stained with ethidium bromide. The reproducibility of the DNA profiles was tested by repeating the PCR amplifications twice with each of the primers analyzed.

Results and Discussion

Data analysis

The molecular size of each fragment was estimated using Gene Ruler 100bp DNA ladder plus (MBI Fermantas, Lithuania) as standard. Since RAPD markers are dominant, a particular DNA band which is generated from the genome of one individual, but absent in a second individual represents a polymorphism. In an attempt to identify species-specific diagnostic markers, samples from each species were amplified simultaneously and amplified products were run side by side on the same gel. RAPD, ISSR and SSR markers were scored as presence (1) or absence (0) of a band, and the data obtained were placed in a rectangular matrix. The data matrix was then used to generate a genetic similarity index (19), using NTSYS software. Cluster analysis was carried out based on genetic distance (GD = 1– GS), using UPGMA (unweighted pair-group method using arithmetic averages) (20) and the Neighbour join program of the software package Phylip 3.5 (21). Bootstrap analysis was carried out with 100 replicate data sets (22) and the trees thus generated were used to make a consensus tree viewed in Tree View 1.5 software.

Polymorphism information content (PIC) was calculated from the frequency of a RAPD bands

Table 2. Details of RAPD, ISSR and SSR analysis in different species of *Cassia*.

SPECIES	RAPD				ISSR				SSR			
	Total Bands	Polym bands	Unique bands	% of polym	Total bands	Polym bands	Unique bands	% of polym	Total Bands	Polym Bands	Unique Bands	% of polym
Cassia javanica	218	190	04	83.33	121	115	6	95.04	78	75	1	96.15
Cassia mimosoides	211	170	03	82.40	127	121	6	95.27	64	58	4	90.62
Cassia occidentalis	***230***	***208***	***08***	***96.38***	***121***	***118***	***3***	***97.52***	***55***	***52***	***1***	***94.54***
Cassia renigera	172	160	1	87.09	133	124	9	93.23	72	65	3	90.27
Cassia grandis	188	182	4	85.52	114	100	9	87.71	80	75	2	93.75
Cassia absus	***215***	***200***	***12***	***83.33***	***124***	***118***	***6***	***95.16***	***72***	***65***	***1***	***90.27***
Cassia biflora	204	192	10	93.24	124	115	6	92.74	71	69	3	97.18
Cassia spectabilis	207	195	2	82.51	127	124	3	97.63	68	61	3	89.70
Cassia siamea	192	170	02	80.09	108	107	9	99.07	59	52	1	88.13
Cassia obtusifolia	176	154	02	89.23	136	130	6	95.58	67	62	2	92.53
Cassia tora	***158***	***140***	***08***	***90.00***	***109***	***107***	***2***	***98.16***	***78***	***70***	***5***	***89.74***
Cassia glauca	209	178	10	96.19	112	102	9	91.07	72	70	1	97.22
Cassia alata	200	192	9	97.10	126	123	3	97.61	68	57	1	83.82
Cassia fistula	145	144	0	99.71	131	135	0	98.54	75	73	1	97.33
Cassia roxburghii	221	211	5	98.28	112	110	2	98.21	64	61	1	95.31
C. artemisioides	216	210	1	98.10	126	109	5	86.50	72	68	2	94.44
C. atomaria	201	192	9	97.00	115	111	4	96.52	62	51	2	82.25
C. covesii	211	190	8	97.31	131	121	3	92.36	74	70	1	94.59
C. italica	205	199	2	99.28	123	120	2	97.56	52	50	0	96.15
C. lindheimariana	206	189	7	97.26	108	105	1	97.22	58	50	1	86.20
C. martiana	221	212	6	97.19	129	120	7	93.02	61	56	2	91.80
C. petersiana	225	221	4	98.76	111	102	6	91.89	68	61	1	89.70
C. polyantha	***212***	***200***	***9***	***96.15***	***116***	***110***	***4***	***94.82***	***73***	***65***	***2***	***89.04***
C. floribunda	194	183	1	97.61	132	126	5	95.45	56	53	4	94.64
C. spectabilis	164	156	5	96.96	133	131	2	98.49	63	57	1	90.47
C. versicolor	202	194	5	97.35	125	117	6	93.60	51	41	2	80.39
C. wislizenii	218	210	4	96.89	111	106	4	95.49	72	66	2	91.66
C. hirsuta	213	208	2	98.23	102	95	3	93.13	65	62	1	95.38

Table 3. RAPD primer index (RPI), ISSR primer index (IPI) and SSR primer index (SPI) for the polymorphic primers in 28 Cassia species

Primer	Total no. of bands	No. of polymorphic bands	RPI/IPI/SPI value
RAPD			
OPA02	18	10	3.65
OPA 04	21	13	4.7
OPA 08	15	9	3.15
OPA 11	19	12	4.14
OPA 14	16	10	2.21
OPC 02	9	7	1.99
OPC 08	11	8	0.91
OPN 04	10	6	0.82
OPN 05	14	9	0.83
OPN 15	18	15	0.95
OPN 18	12	8	0.72
OPD 01	11	8	0.77
OPAF 05	17	14	0.48
OPAF 06	6	4	0.41
OPAF 12	7	2	0.45
OPAF 19	13	11	0.55
OPAF 09	13	9	0.35
Total	**230**	**178**	
ISSR			
(GATA)4	9	8	1.73
(CAG)5	10	7	1.7
(CAA)5	12	9	1.35
(ACTG)4	18	16	1.32
(GACA)4	16	15	2.25
(ACAG)4	12	8	1.95
(CAA)5	13	12	2.10
(CT)8	9	7	1.0
(CA)6	8	5	0.81
(GAG)4	14	12	0.45
(GA)7	15	12	0.59
Total	**136**	**111**	
SSR			
(CA)14	11	10	1.25
(TG)15	10	8	1.95
(AC)8	13	12	2.10
(GA)12	9	7	1.0
(CT)10	8	5	0.81
(CA)15	14	12	0.45
(TG)14	15	12	0.59
Total	**80**	**66**	

using the formula, PIC= 1 $^{羊}\sum(Pi)^2$, where Pi is the allele frequency of the i^{th} allele (23). In the case of RAPDs, ISSRs and SSRs, the PIC was considered to be 1 – $p2$ – $q2$, where p is band frequency and q is no band frequency. Then PIC values were used to calculate RAPD primer

index (RPI) and SSR primer index (SPI), which were generated by adding up the PIC values of all the markers amplified by the same primer.

RAPD analysis

A set of ten-mer oligonucleotide primers applied to 28 species of *Cassia* produced a total of 230 marker bands of which 178 markers were polymorphic (Tables 2 and 3). The size range of the amplified DNA was 250 bp to 3.5 kb (Figs. 1a & b). Maximum 21 and 19 RAPD markers were produced in OPA-4 and OPA-11 primers. A total of 33 unique genetic markers were produced in different species of *Cassia. C. absus,* produced 12 unique amplified DNA markers in different primers. Two unique markers were obtained in *C. siamea, C. spectabilis, C. obtusifolia* and *C. italica*. In *C. artemisioides*, *C. floribunda* and *C. renigera* unique marker bands of 400bp, 500bp, 2.5 kb respectively were found from OPA-4 and OPA-11 primers. *C. mimosoides* showed 2 unique markers of 300bp and 2.5kb size in OPA-08 and OPC-02 primers. The highest RAPD primer index (4.7) was found in primer OPA-04 and the lowest (0.35) in OPAF-09 (Table 3). The similarity index obtained from RAPD patterns of *Cassias* showed relative genetic similarities among the species that ranged from 0.01 to 1.0. The Dendogram drawn on the basis of these genetic distances radically separated 28 species into two major groups of 14 species each which further divided into minor groups (Fig. 1c). Interestingly, the plants collected from RPRC, Orissa, Dehradun, UP, India and Kew seed germinated plants (Kew plants) were uniquely separated and placed in two groups. Only one species *C. uniflora* collected from Kew could be grouped with the local plants. *C. nodosa, C. grandis, C. obtusifolia, C. alata* were grouped with Kew seed germinated plants. *C. mimosoides* with a different genomic set up (2n=16) showed close relation with *C. javanica*. *C. siamea* and *C. spectabilis; C. grandis* and *C. nodosa* were in a core group with close morphological similarities. *C. artemisioides* and *C. covesii*, both having more drought tolerance characters were closely placed.

SSR analysis

80 SSR marker bands and 66 polymorphic bands were amplified from 7 primers designed from the flanking sequences of the microsatellites (Table 3). The size range of the amplified DNA was 100 bp to 3.2 kb (Fig. 2a). Maximum 15 and 8 SSR markers were produced in (TG)14 and (CT)10 respectively. All 15 unique genetic markers were produced in different species of *Cassia.* Five unique marker bands were produced in *C. tora* and no such bands were found in *C. italica*. The highest RAPD primer index (2.10) was found in primer $(AC)_8$ and the lowest (0.45) in $(CA)_{15}$ (Table 3). The similarity index obtained from RAPD patterns of *Cassias* shows relative genetic similarities among the species ranging from 0.02 to 0.47 (Table 3). The Dendogram from SSR analysis forms two major groups (Fig. 2b). *C. floribunda* remains isolated forming an out-group from the rest of the species. The second major group contains rest of the species and is further divided into two groups. In this case also, there is similarity with RAPD and ISSR primers in the local plants and Kew plants are sorted into two groups. Only Kew plants *C. uniflora and C. atomaria* can be grouped with local plants. Here also *C. siamea* and *C. spectabilis* with close morphological similarities are closely placed.

ISSR analysis

A total of 136 marker bands and 111 polymorphic bands were amplified from 11 ISSR primers applied to 28 species of *Cassia* (Tables 2 and 3). The size range of the amplified DNA was

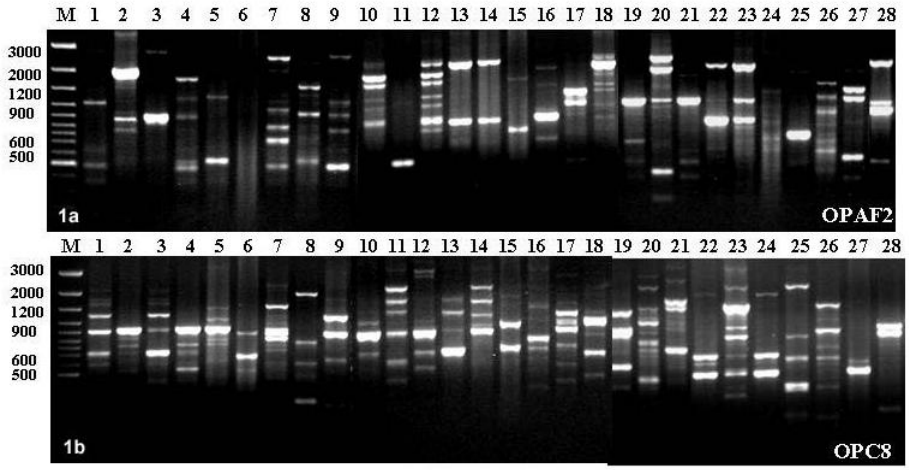

Figure 1a and 1b. RAPD profiles in different species of *Cassia* species using random primers OPAF-2 (Fig. 1a) and OPC-8 (Fig. 1b). M=DNA marker 100bp Ladar plus (MBI Fermentas, Luthmania), 1-*C. javanica,* 2-*C. mimosoides,* 3-*C. Occidentalis,* 4-*C. renigera,* 5-*C. grandis,* 6-*C. absus,* 7-*C. biflora,* 8-*C. spectabilis,* 9-*C. siamea,* 10-*C. obtusifolia,* 11-*C. tora,* 12-*C. glauca,* 13-*C. alata,* 14-*C. fistula,* 15-*C. roxburghii,* 16- *C. nodosa,* 17-*C. hirsuta,* 18- *C. wislizenii,* 19-*C. polyantha,* 20- *C. italica,* 21-*C. versicolor,* 22- *C. petersiana,* 23-*C. floribunda,* 24- *C. lindheimariana,* 25-*C. martiana,* 26- *C. artemisioides,* 27- *C. atomaria* & 28-*C. covesii.*

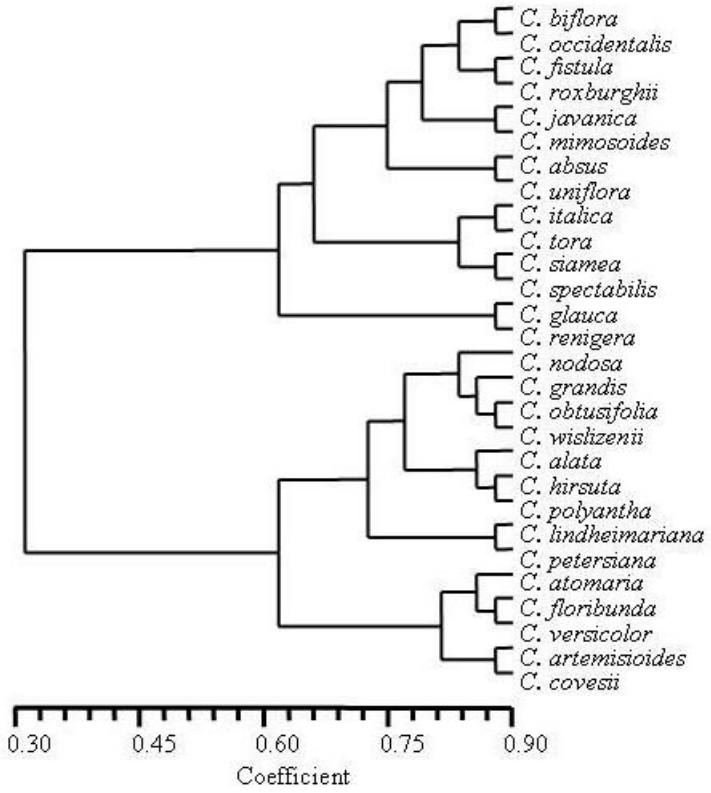

Figure 1c. Dendogram from RAPD profiles of different species of *Cassia.*

100 bp to 3.0 kb (Figs. 3a and b). Maximum 18 and 8 ISSR markers were produced in $(ACTG)_4$ and $(CA)_6$ primers. Total 12 unique genetic markers were produced in different species of

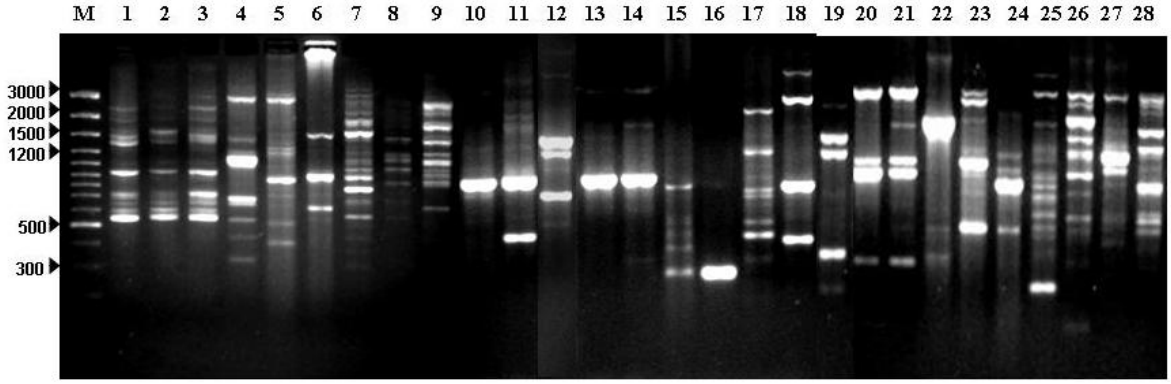

Figure 2a. DNA profile in different species of *Cassia* species using SSR primers. 1-*C. javanica,* 2-*C. mimosoides,* 3-*C. Occidentalis,* 4-*C. renigera,* 5-*C. grandis,* 6-*C. absus,* 7-*C. biflora,* 8-*C. spectabilis,* 9-*C. siamea,* 10-*C. obtusifolia,* 11-*C. tora,* 12-*C. glauca,* 13-*C. alata,* 14-*C. fistula,* 15-*C. roxburghii,* 16- *C. nodosa,* 17- *C. hirsuta,* 18- *C. wislizenii,* 19-*C. polyantha,* 20- *C. italica,* 21-*C. versicolor,* 22- *C. petersiana,* 23-*C. floribunda,* 24-*C. lindheimariana,* 25-*C. martiana,* 26- *C. artemisioides,* 27- *C. atomaria* & 28-*C. covesii.*

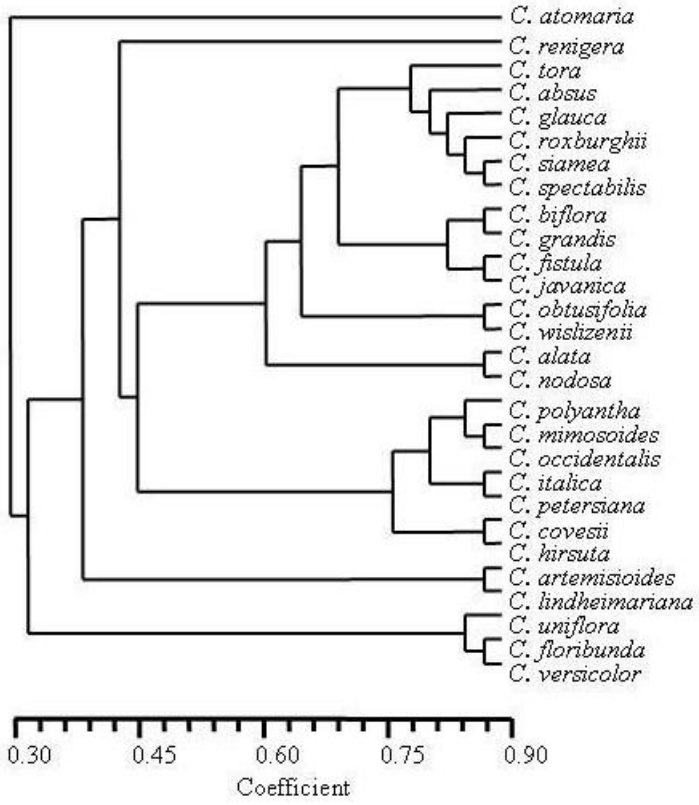

Figure 2b. Dendogram obtained from SSR profiles from different species of *Cassia.*

Cassia. C. renigera and *C. grandis,* produced 9 unique amplified DNA markers in different primers. A unique marker was obtained from *C. lindhemariana*. The highest RAPD primer index (2.25) was found in primer $(GACA)_4$ and the lowest (0.45) in $(GAG)_4$ (Table 3). The similarity index obtained from RAPD patterns of *Cassias* showed relative genetic similarities that ranging from 0.05 to 0.92 among the species (Table 3). The Dendogram from ISSR analysis forms two major groups (Fig. 3c). *C. atomaria* remains single forming an out group from the rest of the

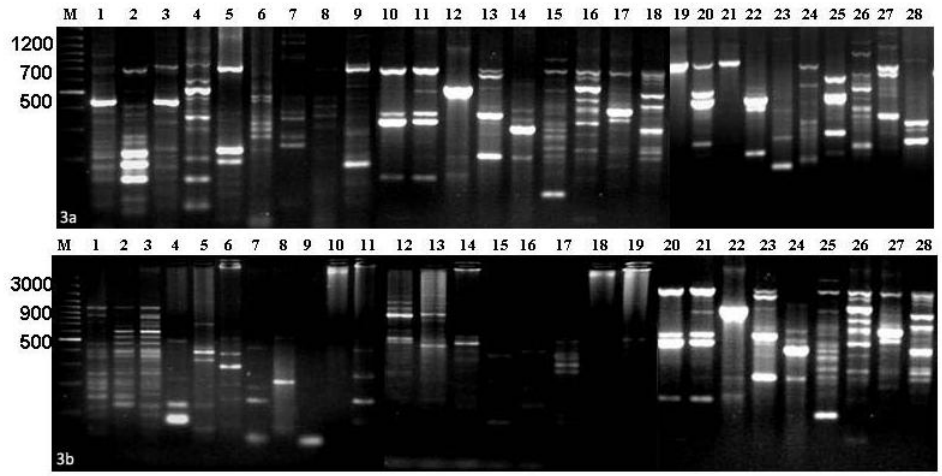

Figure 3a and 3b. DNA profile in *Cassia* species using ISSR primers. 1-*C. javanica,* 2-*C. mimosoides,* 3-*C. Occidentalis,* 4-*C. renigera,* 5-*C. grandis,* 6-*C. absus,* 7-*C. biflora,* 8-*C. spectabilis,* 9-*C. siamea,* 10-*C. obtusifolia,* 11-*C. tora,* 12-*C. glauca,* 13-*C. alata,* 14-*C. fistula,* 15-*C. roxburghii,* 16- *C. nodosa,* 17- *C. hirsuta,* 18- *C. wislizenii,* 19-*C. polyantha,* 20- *C. italica,* 21-*C. versicolor,* 22- *C. petersiana,* 23-*C. floribunda,* 24- *C. lindheimariana,* 25-*C. martiana,* 26- *C. artemisioides,* 27- *C. atomaria* & 28-*C. covesii.*

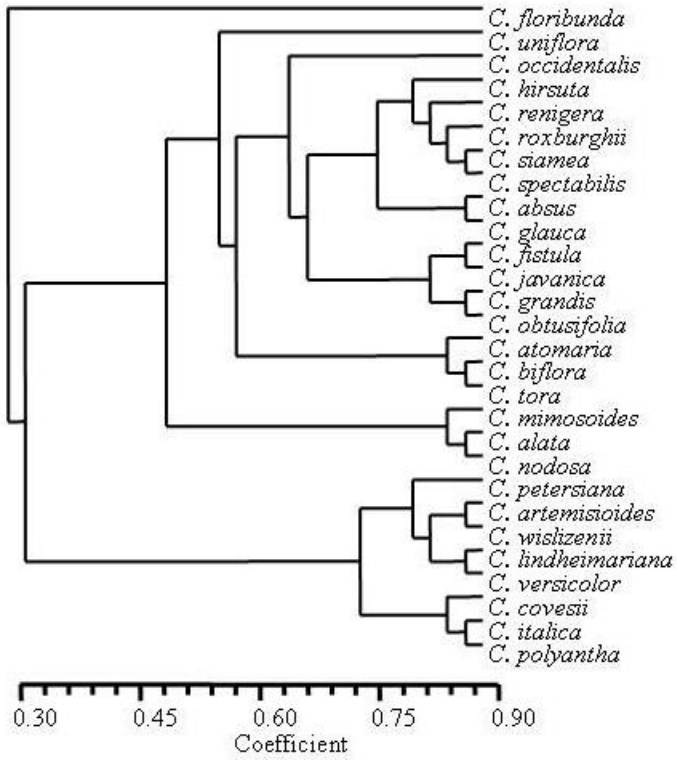

Figure 3c. Dendogram obtained from ISSR profiles from different species of *Cassia*.

species. The second major group containing the rest of the species is further divided into two groups with *C. uniflora*, *C. versicolor* and *C. floribunda* remaining distant from the rest of the group. In this case also, there is some similarity with RAPD primers in that the local plants and Kew plants are placed separately. Only Kew plant, *C. wislizenii* grouped with local

plants and *C. mimosoides* and *C. occidentalis* grouped with Kew plants. Here also *C. siamea* and *C. spectabilis,* with close morphological similarities, are closely placed.

None of the amplification products generated by either RAPD or ISSR or SSR primers was found to be common to all the species. Most of the RAPD as well as the SSR primers produced fingerprints that were diagnostic for each species. On the basis of this analysis, a few RAPD, ISSR and SSR primers were recognized (Table 3) that could preferably be used to fingerprint *Cassia* germplasm resources. To characterize the capacity of each RAPD, ISSR and SSR marker to reveal polymorphic loci in *Cassia*, we analyzed the PIC content. In calculating the PIC, the markers with band frequencies below 0.05 or above 0.95 were not considered, because this level is estimated to be too close to the empirical threshold for the detection of differences by RAPD and SSR analyses (24). The calculated PIC values for the RAPD, ISSR and SSR markers ranged from 0.08 to 0.45. RPI, IPI and SPI values ranged from 0.35 to 4.14, 0.45 to 2.25 and from 0.45 to 2.10 respectively (Table 3).

Three Dendograms based on RAPD, ISSR, and SSR data clearly indicated that there is correlation between the marker-based grouping pattern and geographical origin. The pattern of clustering of the species remained more or less the same in all three Dendograms. The matrices for RAPD and ISSR markers were also compared using Mantel's test. The correlation between the matrices based on RAPD, ISSR and SSR data was very high (r = 0.90). The bootstrap probability values ranged from 75.8 to 99%.

Our investigation revealed significant variation in terms of RAPD, ISSR and SSR fingerprinting among the closely related species thought to be devoid of molecular variation (25) and thereby successfully drawing the interspecific phylogenetic relationships. The broad adaptation of some species of *Cassia* implies a large probability that nuclear DNA mutations occurred in the past. These resulted in diversification and genetic polymorphism in growth habit, vigor, and stem, leaf, seed, and pod characteristics and a physiological polymorphism, the water-use efficiency.

Acknowledgments

The authors are thankful to The Department of Science and Technology, Government of India for providing financial support (Sanction No. SR/FT/L-14/2003) and The Director, Regional Plant Resource Centre, Bhubaneswar for providing Laboratory facilities for this study.

REFERENCES

1. Singh V. 2000. Monograph on the suntribe Cassinae. BSI, Dehra Dun.
2. Singh M**,** Chaudhuri PK and Sharma RP. 1995. Constituents of the leaves of *Cassia angustifolia*. Fitoterapia 66(3): 284.
3. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531-6535.
4. Salimah SS, De Oliveira AC and Godwin ID. 1995. Assessment of genomic origin and genetic diversity in the genus Eleusine with DNA markers. Genomic. 38: 757–763.
5. Lee S and Rasmussen SK. 2000. Molecular markers in some medicinal plants of the Apiaceae family. Euphytica 114: 87–91.
6. M'Ribu HK, and Hilu KW. 1994. Detection of interspecific and intraspecific variation in *Panicum* millets through random amplified polymorphic DNA. Theor. Appl. Genet. 88: 412–416.
7. Paran I, Kesseli R and Michelmore R. 1991. Identification of RFLP and RAPD markers linked to Downey mildew resistance gene in lettuce using near-isogenic lines. Genome, 34: 1021–1027.
8. Valle PR, Souza MB, Pires EM, Silva EF and Gomes MA. 2000. Arbitrarily primed PCR fingerprinting of RNA and DNA in *Entamoeba histolytica*. Rev. Inst. Med. Trop. Sao Paulo, 42(5): 249–253.

9. Lal RK, Sharma JR and Mishra OH. 1997. Genetic diversity of senna (*Cassia angustifolia* Vahl.). Journal of herbs, spices & medicinal plants 5(22): 3-10.
10. Welsh J and McClelland M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213–7218.
11. Morgante M and Olivieri AM. 1993. PCR-amplified microsatellites as markers in plant genetics. The plant Journal 3:175-182.
12. Maugham PJ, Saghai Maroof MA, Buss GR and Huestis GM. 1996. Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. Theor. Appl. Genet. 93:392-401.
13. Kass E and Wink M. 1996. Molecular evolution of the Leguminosae: phylogeny of the three subfamilies based on *rbcL* – sequences. Biochemical Systematics and Ecology 24(5):365-378.
14. Cunningham DC, Walsh KB, Anderson ER, Harrison DK and Carroll BJ. 2002. Analysis of genetic diversity in Cassia brewsteri with randomly amplified DNA fingerprints (RAFS). Australian Systematic Botany 15: 237-244.
15. Siva R and Krishnamurthy KV. 2005. Isozyme in *Cassia auriculata* L. African Journal Biotechnology 4 (8): 772-775.
16. Samappito S, Page J, Schmidt J, De Eknamkul W and Kutchan TM. 2002. Molecular characterization of root-specific chalcone synthases from *Cassia alata*. Planta. 216(1): 64-71.
17. Mohanty S and Das AB. 2006. Interspecific genetic diversity in 15 species of *Cassia* L. evident by chromosome and 4C nuclear DNA analysis. Journal of Biological Sciences 6(4):664-670.
18. Saghai-Maroof MA, Soliman KM, Jorgenson R and Allard RW. 1984. Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81: 8014-8018.
19. Nei M and Li WH. 1979. *Mathematical model for studying genetic variation in terms of restriction endonucleases.* Proc. Natl. Acad. Sci. 76:5269–5273.
20. Sneath P HA and Sokal RR. 1973. Numerical Taxonomy. Freeman, San Francisco.
21. Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.
22. Felsenstein J. 1989. PHYLIP—Phylogeny inference package (version 3.2). Cladistics 5:164–166.
23. Smith JSC, Chin, ECL, Shu H, Smith OS, Wall SJ, Senior ML, Mitchell SE, Kresovich S, and Ziegle J. 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from RFLPs and pedigree. Theor. Appl. Genet. 95:163–173.
24. Nienhuis J, Tivang J, Skroch PW and dos Santos JB. 1995. Genetic relationships among cultivars and landraces of lima bean (*Phaseolus lunatus* L.) as measured by RAPD markers. J Am Soc. Hort Sci 120:300 306.
25. Singh SP, Sharma JR, Mishra HO, Lal RK and Gupta MM. 1998. Genetic variation and strain selection in Senna (*Cassia angustifolia*) for north Indian plains. Journal of Medicinal and Aromatic Plant Sciences 20(2): 375-378.