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Full Length Research Paper

Validation of generic status of different taxa in the sub-tribe Cassiinae (Leguminosae: Caesalpinoidae) using RAPD, ISSR and AFLP markers

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Random amplified polymorphic DNA (RAPD), Inter simple sequence repeat (ISSR) and Amplified fragment length polymorphism (AFLP) markers were used to verify the segregation of the genus *Cassia* L. (*sens. lat.*) into three distinct genera namely, *Chamaecrista* Moench., *Senna* P. Mill. and *Cassia* L. (*sens.str.*). Eighteen representatives of the three taxa were characterized using the molecular markers. 25 RAPD, six ISSR primers and six AFLP primer combinations resulted in the amplification of 612, 115 and 622 bands (loci), respectively. Most of the loci are found to be polymorphic, showing high degrees of genetic diversity among the different taxa studied. The dendrogram constructed on the basis of the RAPD, ISSR and AFLP data using the SHAN clustering, divided *Cassia* L. (*sens. lat.*) into three different clusters as *Chamaecrista* Moench., *Senna* P. Mill. and *Cassia* L. (*sens.str.)*. High bootstrap value revealed that all the clusters were stable and robust. It was observed from the present investigation that these genera have their identity at molecular level, which supports the elevation of the genus *Cassia* L. *sens. lat.* to the level of sub tribe Cassiinae and segregation into three distinct genera instead of intra-generic categories. In the present study taking the molecular markers into account the trifurcation of the sub tribe Cassiinae could be re-established.

Key words: Cassia, molecular phylogeny, RAPD, ISSR, AFLP.

INTRODUCTION

Cassia L. Sens. Lat. is one of the twenty-five largest genera of dicotyledonous plants in the world. The genus extends in all terrestrial habitats from the equator to the edges of dry and cold deserts, but much of its diversity centred in the areas of varied topography with seasonal climates. The taxa have expanded greatly from the Miocene onwards and the versatility of the taxa enhances their great economic importance, which is increasing as human pressure demands more effective use of marginal lands. In the long term, a much wider and more subtle use of the immense natural variation may be harnessed to fix nitrogen, conserve soil, provide timber, fuel, pesticides and amenity value, as well as more carbohydrate, protein and oils.

The species under this taxon have wide variability in habit ranging from tree to delicate annual herbs. There has been considerable divergence of opinion concerning the delimitation and taxonomic status of its three constituent subgenera. Irwin and Barneby (1981, 1982) proposed an improved classification and raised the genus *Cassia* L. to the level of sub tribe Cassiinae; the later comprising of three genera, *viz*., *Cassia* L. (*sens. str.*), *Senna* P. Mill. and *Chamaecrista* Moench. This concept had found wide recognition in recent years (Randel, 1988; 1989; 1990; Lock, 1988, 1989; Larsen and Hou,

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1996). Marazzi and Endress (2008) studied the floral assymetry in *Senna* and found seven groups in it.

Despite several studies by taxonomists, either on the whole family Caesalpiniaceae in restricted area or of certain genera through out the world, there is still great deal of taxonomic work to be done at the level of genus and tribe. The taxonomic investigation of the genus *Cassia* L. *sens. lat.* has been made in some countries in Asia, namely Malaysia (De Wit, 1955) and Pakistan (Ali and Quraishi, 1967). In recent years, taxonomy has developed as a synthetic discipline and evidences are being drawn from anatomical, biochemical, cytological, serological and molecular studies to derive phylogenetic relationships rather than relying only on morphological characters. This has helped taxonomists in proposing newer system of classification or revising the existing ones. As with other taxa, data obtained from cytology, biochemistry and other aspects have resulted in modification of criteria and evolutionary relationship in the sub tribe *Cassiinae.*

Irwin and Barneby (1981, 1982) raised the genus *Cassia* L. *sens lat* to the level of sub tribe and elevated previous subgenera to generic rank *viz*., *Senna* Mill and *Chamaecrista* Moench under the tribe Cassieae Bronn ex Irwin and Barneby of Caesalpiniaceae. However, very little work has been done on the molecular phylogeny of this genus and validation of the trifurcation of this group. Similarly, only a few reports are available on the biochemical and cytological studies in the taxon. There are contradicting reports on chromosome numbers and cytological data failed to reflect the phylogenetic relationship in the group (Singh, 2001).

Keeping all these facts the present investigation was carried out with the objectives to derive an authentic relationship among the studied genera of the sub tribe Cassiinae and to justify of trifurcation the sub tribe into three distinct genera like, *Cassia*, *Senna* and *Chamaecrista* as suggested by Irwin and Barneby (1982), taking the molecular data from RAPD, ISSR and AFLP analyses.

MATREIALS AND METHODS

Plant materials

All the plants were raised from the seed. Eighteen species of the genus *Cassia* were taken for the present study. The correct name, synonym, locality of collection, reported chromosome numbers etc. of each species are given in Table 1. They were grown in the nursery of Regional Plant Resource Centre, Bhubaneswar, Orissa, India. Very young and tender leaves were taken for genomic DNA isolation.

Molecular analysis

DNA was isolated from young and fresh leaves using the CTAB method as described by Saghai-Maroof et al. (1984). For RAPD analysis, PCR amplification of 25 ng of genomic DNA was carried out using standard 30 decamer oligonucleotide primers, out of which in 25 primers reproducible amplification was found. So they are taken for the present RAPD analysis and those are OPA02, OPA03, OPA04, OPA10, OPA16, OPA18, AF14, OPC02, OPC05, OPD02, OPD03, OPD07, OPD08, OPD18, OPD20, OPN02, OPN04, OPN05, OPN06, OPN08, OPN10, OPN11, OPN12, OPN16 and OPN18 (Operon Tech. Alameda, CA. USA). The RAPD analysis was performed as per the standard methods of Williams et al. (1990). PCR products were separated on a 1.5% agarose gel containing ethidium bromide solution (@ 0.5 μg/ml of gel solution). The size of the amplicons was determined using size standards (100 bp ladder plus or DNA ladder mix, MBI Fermentas, Lithuania). DNA fragments were visualized under UV light, documented in Gel Doc (Bio-Rad, USA) and photographed.

Inter-simple sequence repeats has recently been developed which access the variation in the numerous microsatellite regions distributed throughout different genomes (basically the nuclear genome) and bypass the challenges of characterizing individual loci that other molecular techniques require. The PCR products were separated in polyacrylamide gel. Amplified fragment length polymorphism analysis was done as per the standard protocol of Vos et al. (1995) and the protocol supplied by the manufacturer (Invitrogen, USA). All the reagents and chemicals were procured from Invitrogen (Invitrogen life technology, CA, USA). After the completion of the gel run it was stained with 0.0002% of ethidium bromide and destained in distilled water. Gel was documented in a gel doc (Gel Doc 2000, Bio Rad, USA).

Data analysis

The bands amplified from RAPD, ISSR and AFLP were scored as '1' and '0' for present and absent of band, respectively. All the bands whether monomorphic or polymorphic were used for similarity calculation in order to avoid over estimation of distance (Gherardi et al., 1998). Jaccard's coefficient of similarity (Jaccard, 1908) was calculated and a dendrogram based on similarity coefficient was obtained through un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and SHAN clustering. All the analysis was done by using the computer package NTSYS-PC-2.02e (Rohlf, 1997). Resolving power (Rp) of the RAPD, ISSR and AFLP were calculated as per Prevost and Wilkinson (1999). Resolving power is: Rp = ΣIB (IB (Band informative ness) = 1 - [2 × (0.5-P)], P is the proportion of the 18 species containing the band. Primer index (PI) was calculated from the polymorphic index. A polymorphic index (PIC) was calculated as PIC = 1 - $\sum P^2_i$, P_i is the band frequency of the *ith* allele (Smith et al., 1997). In the case of RAPDs, ISSRs and AFLP, the PIC was considered to be 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 primer index (RPI). RPI is the sum of the PIC of all the markers amplified by the same primer. Principal coordinate analysis (PCA) was used to retrieve information about the clustering pattern of the analyzed populations. PCA was performed based on the RAPD, ISSR and AFLP data, for all the primers.

RESULTS

Molecular markers

Three different PCR based molecular markers like random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) were used in the present investigation.

Table 1. Details of species with locality of collection, field number, habit and chromosome number in Cassiinae.

Name of the species	Chromosome number	Habit	Locality with field collection number
Senna tora *(Linn.) Roxb.* (*Cassia tora* Linn.)	2n = 24, 26, 28, 52	Herb or under shrub	R.P.R.C., BBSR. LKA 7643
Senna occidentalis (Linn.) Link {*Cassia occidentalis* Linn. *Senna occidentalis* (Linn.) Roxb.}	2n = 28, n = 13,14	Erect herb or under shrub	R.P.R.C., BBSR. LKA 7650
Chamaecrista absus (Linn.) Irwin and Barneby {*Senna absus* (Linn.) Roxb.}	2n = 26, 28, 56	Erect, viscid-hairy herb	R.P.R.C., BBSR. LKA 7652
Senna alexandrina Gars. ex Miller (*Cassia angustifolia* Vahl)	2n = 26	Herbs or shrubs	Keonjhar, Orissa.
Senna siamea (Lam.) Irwin and Barneby (*Cassia siamea* Lam.)	2n = 28	Moderate-sized tree	R.P.R.C., BBSR. LKA 7625
Cassia fistula Linn.	2n = 28,	Small or medium-sized tree	R.P.R.C., BBSR. LKA 7644
Cassia javanica Linn. var. *javanica* (*Cassia nodosa* Buch-Ham. Ex Roxb.)	2n = 28	Deciduous trees	R.P.R.C., BBSR. LKA 7630
Senna pallida (Vahl) Irwin and Barneby (*Cassia biflora* Linn.)	2n = 28	Shrubs	R.P.R.C., BBSR. LKA 7631
Chamaecrista mimosoides (Linn.) Greene (*Cassia mimosoides* Linn.)	2n = 16, 32	Prostrate or decumbent herbs or under shrub	R.P.R.C., BBSR. LKA 7626
Senna sulfurea (DC. ex Collad.) Irwin and Barneby (*Cassia glauca* Lam. *Senna glauca* Roxb)	2n = 28, 56	Shrub or small tree	OUAT Campus, BBSR. LKA 7648
Cassia grandis Linn.	2n = 28	Trees	R.P.R.C., BBSR. LKA 7642
Cassia javanica Linn.var. *indochinensis* Gagne	2n = 28, n = 12, 14	Trees	Governer's House, BBSR. LKA 7633
Senna alata (Linn.) Roxb. (*Cassia alata* Linn.)	2n = 12, 24, n = 12, 14	Shrubs or small trees	Dhauli, BBSR. LKA-7632
Senna spectabilis (DC.) Irwin and Barneby (*Cassia spectabilis* DC.)	2n = 28	Evergreen trees	I.G. Park, BBSR. LKA 7649
Senna auriculata (Linn.) Roxb. (*Cassia aruiculaia* Linn.)	2n =14,16,28. n = 14	Large shrub	Uppal, Hyderabad A.P. LKA 7641
Cassia roxburghii DC.	2n = 24, 28	Small tree	Rajmahal Square, BBSR. LKA 7651

Table 1. cont.

Chamaecrista pumila (Lam.) Singh (*Cassia pumila* Lam.)	2n = 28	Diffuse or prostrate herb	R.P.R.C., BBSR. LKA 7647
Senna septemtrionalis (Viv.) Irwin and Barneby (*Cassia laevigata* Willd.)	2n = 26, 28	Shrubs	Dhauli, BBSR. LKA 7627

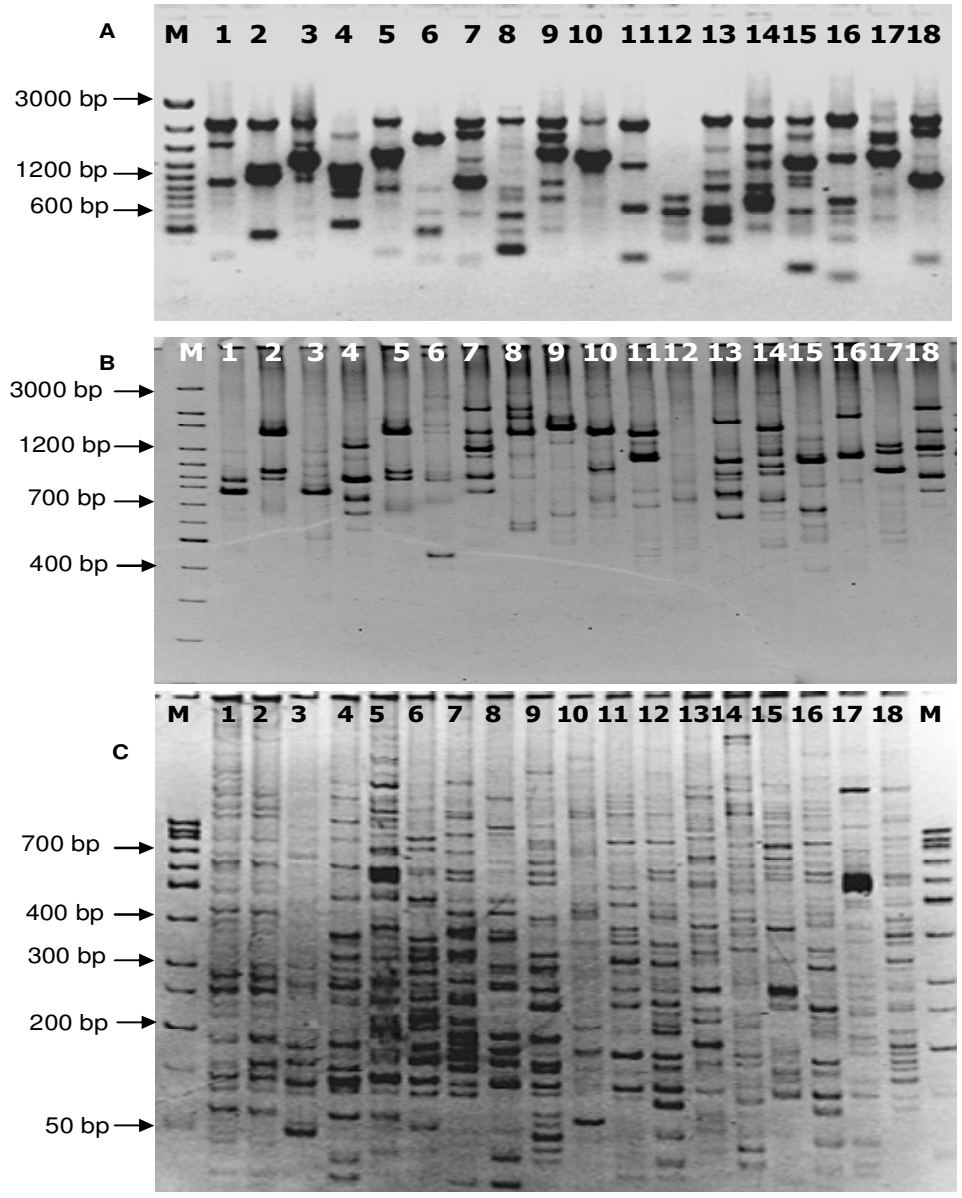

Figure 1. (A,B,C) Banding pattern in different species of Cassiinae as seen in RAPD, ISSR and AFLP technique.

Random amplified polymorphic DNA

All the 18 species produced distinct reproducible amplifications. The DNA profiles as observed in RAPD are represented in (Figure 1a). A total of 612 numbers of bands bands were amplified (Table 2). All the bands were found to be polymorphic. Maximum and minimum amplification was observed in case of the species *Chamaecrista absus* L.

Table 2. Details of RAPD analysis in Cassiinae.

Primer	Sequence of oligonucleotide	Approx. fragment size (bp)	Total bands	Unique bands	Resolving power	RAPD primer index
OPA02	5'TGCCGAGCTG3'	> 3000 - 300	36	8	10.667	8.642
OPAO3	5'AGTCAGCCAC3'	> 3000 - 230	36	7	13.222	9.8704
OPA04	5'AATCGGGCTG3'	> 3000 - 350	26	3	8.222	6.6543
OPA10	5'GTGATCGCAG3'	> 3000 - 300	28	4	10	7.8025
OPA16	5'AGCCAGCGAA3'	> 3000 - 200	24	4	8.556	6.6358
OPA18	5'AGGTGACCGT3'	> 3000 - 200	19	4	7.333	5.5802
OPAF14	5'GGTGCGCACT3'	> 3000 - 200	23	7	5.889	4.821
OPC02	5'GTGAGGCGTC3'	> 3000 - 300	17	4	5.667	4.4012
OPC05	5'GATGACCGCC3'	> 3000 - 200	23	5	6.878	6.6111
OPD02	5'GGACCCAACC3'	> 3000 - 300	25	2	9.778	7.1481
OPD03	5'GTCGCCGTCA3'	> 3000 - 350	22	3	8.111	6.142
OPD07	5'TTGGCACGGG3'	> 3000 - 350	19	4	6.111	4.7222
OPD08	5'GTGTGCCCCA3'	3000 - 300	24	4	8.222	6.3086
OPD18	5'GAGAGCCAAC3'	> 3000 - 200	30	7	11.222	7.8457
OPD20	5'ACCCGGTCAC3'	2000 - 300	19	2	8.111	5.4877
OPNO2	5'ACCAGGGGCA3'	3000 - 200	27	7	8.667	6.7654
OPN04	5'GACCGACCCA3'	3000 - 200	31	4	12.444	9.1235
OPN05	5'ACTGAACGCC3'	> 3000 - 250	22	4	6.778	5.4753
OPN06	5'GAGACGCACA3'	> 3000 - 100	28	10	8	6.321
OPN08	5'ACCTCAGCTC3'	> 3000 - 400	20	9	5.778	3.8025
OPN10	5'ACAACTGGGG3'	3000 - 200	23	6	7.556	5.9259
OPN11	5'TCGCCGCAAA3'	> 3000 - 400	17	3	6.333	4.7593
OPN12	5'CACAGACACC3'	> 3000 - 200	20	5	6.444	4.5556
OPN16	5'AAGCGACCTG3'	> 3000 - 100	31	4	9.556	7.6914
OPN18	5'GGTGAGGTCA3'	> 3000 - 400	22	4	7.222	5.7716
Total			612	124		

(139) and *Cassia spectabilis* L. (57) respectively. The highest numbers of bands were amplified with primer OPA02 and OPAO3 (36 each) and lowest numbers of amplification were observed with the primers OPC02 and OPN11 (17 loci each). Among the polymorphic bands; 124 were exclusive bands, specific for a single species. Highest number of exclusive bands were observed for the primer OP N10 (10) while in case of OP D02 and OP D20 only two bands were found to be exclusive. In *Chamaecrista* there were highest unique bands (20), only two bands were found to be unique in case of *Chamaecrista pumila* as well as for *Cassia grandes*. The dendrogram constructed using the SHAN clustering shows three major groups among the 18 studied taxa.

Inter simple sequence analysis

Six ISSR primers resulted in the amplification of 115 fragments. The ISSR banding pattern in Cassiinae is represented in Figure 1b. The primer (CAA)5 and (GACA)4 produced maximum number of bands (21), while with the primer (GA)9T only 14 loci could be amplified. Of these, only 8 were found to be unique bands.

The bands were amplified in the range of 200 - 3000 base pairs. All the loci amplified with the ISSR primers were polymorphic and prominent. Maximum number of unique bands were observed with primer T (GA)9 (4), while there were no unique band with the use of primer (AGG)6, (GTG)5 and (CAA)5. Among these ISSR primers, maximum resolving power was obtained for (AGG)6 (12) and the minimum Rp was for (CAA) (5.78). Maximum primer index was calculated for (GACA)4 and minimum for (CAA)5. Highest numbers of 36 bands were resolved for the species *Cassia javanica* and the least number of amplicons were amplified for the *species Chamaecrista mimosoides.* Details of ISSR banding pattern and bands amplified in different species with different primers have been represented in Table 3.

Table 3. Details of ISSR analysis in Cassiinae.

Primer	Sequence of oligonucleotide	Approximate fragment size	Total bands	Unique bands	Resolving power	ISSR primer index
(AGG)6	5'AGG AGG AGG AGG AGG AGG3'	2700 - 300	19	0	13.33	7.2098
(GA)9T	5'GAG AGA GAG AGA GAG AGA T3'	2500 - 300	14	3	7.8888	5.6419
(GACA)4	5'GAC AGA CAG ACA GAC A3'	> 3000 - 400	21	1	10.8889	8.1667
T(GA)9	5'TGAG AGA GAG AGA GAG AGA3'	2300 - 400	20	4	8.33333	6.006
$(GTG)_5$	5'GTG GTG GTG GTG GTG3'	> 3000 - 400	20	0	12	7.272
$(CAA)_5$	5'CAA CAA CAA CAA CAA3'	> 3000 - 100	21	0	5.78	4.969
Total			115	8		

Table 4. Details of AFLP analysis in Cassiinae.

Primer	Sequence of primer	Total bands	Monomorphic bands	Unique bands	Resolving power	AFLP primer index
1	EACC/MCTC	104	0	12	42.778	31.3148
2	EACT/MCAG	98	0	2	60.111	37.10494
3	EAAG/MCAG	116	2	1	78.889	43.333
4	EACC/MCAT	62	3	1	45.889	22.89506
5	EACA/MCAG	139	0	10	71.222	46.9444
6	EAAG/MCAC	103	0	2	63.111	39.28395
Total		622		28		

*A*FLP analysis

Six AFLP primer combinations were used for the present work. This primer combination had amplified 622 loci among which five loci were found to be monomorphic in nature and the rest were polymorphic. Banding pattern in AFLP for 18 species of Cassiinae is shown in Figure 1C. From the 594 polymorphic bands amplified, only 28 were found to be unique that to in a single species. Maximum numbers of 139 bands were resolved for the primer combination EACA/ MCAG and the minimum for EACCA/MCAT (62). The average numbers of bands amplified per primer combination was calculated to be as high as 103.6.

The highest numbers of bands (65) were noticed for *C. javanica* var. *indochinensis* for primer combination EACT/ MCAG and the lowest for the primer combination EACC/ MCTC (19) in case of the species *C. javanica.* Bands resolving between 1500 to 20 bp were taken into consideration for the present investigation. Maximum numbers of 3 monomorphic bands were scored for the primer combination EACC/ MCAT, where as no band was found to be common to all the species with the primer combinations EACC/ MCTC, EACT/ MCAG, EACA/ MCAG and EAAG/ MCAC.

The resolving power was maximum for the primer combination EAAG/ MCAG (78.889) and minimum for EACC/ MCTC (42.778). However, the primer index was found to be highest for EACA/ MCAG (46.944) and lowest for EACC/ MCAT (22.895). The details of AFLP analysis are presented in Table 4.

Similarity for RAPD, ISSR and AFLP

The Jaccard's similarity was calculated from the data generated from RAPD, ISSR and AFLP analysis. It was observed that *Cassia grandis* and *C. javanica* var. *indochinensis* were most closely related with Jaccrad's similarity coefficient of 0.473, where as *C. mimosoides* and *Senna sulfurea* were distantly placed with the least Jaccard's similarity coefficient of 0.064. Among all the species, there was an average similarity of 0.1834. The Jaccard's similarity among the taxa is represented in Table 5.

Tree generated from the combined markers

On the basis of the data obtained from all the molecular marker systems, a dendrogram was constructed using UPGMA and SHAN clustering in NTSYS-pc 2.02e (Figure 2). All the 18 taxa of Cassiinae studied were separated into three different clusters each containing members of a particular genus.

The species of *Senna* were further divided into two subclusters each containg 5 species. While the first subcluster contained *Senna tora*, *Senna occidentalis*, *Senna*

Table 5. Jaccard's coefficient of similarity between two species of Cassiinae as revealed from RAPD, ISSR and AFLP.

	S. tora	*S. occidentalis*	*C. absus*	*S. alexandrina*	*S. siamea*	*C. fistula*	*C. javanica var. javanica*	*S. pallida*	*Ch. mimosoides*	*S. sulfurea*	*C. grandis*	*C. javanica var. indochinensis*	*S. alata*	*S. spectabilis*	*S. auriculata,*	*C.roxburghii*	*Ch. pumila*	*S. septemtrionalis*
S. tora	1.000																	
S. occidentalis	0.434	1.000																
Ch. absus	0.194	0.176	1.000															
S. alexandrina	0.274	0.262	0.180	1.000														
S. siamea	0.245	0.282	0.145	0.310	1.000													
C. fistula	0.165	0.178	0.132	0.164	0.246	1.000												
C. javanica var. javanica	0.152	0.154	0.151	0.154	0.179	0.401	1.000											
S.pallida	0.236	0.235	0.139	0.242	0.260	0.153	0.164	1.000										
Ch. mimosoides	0.117	0.107	0.351	0.132	0.107	0.079	0.097	0.145	1.000									
S. sulfurea	0.221	0.221	0.113	0.226	0.249	0.146	0.151	0.227	0.107	1.000								
C .grandis	0.160	0.133	0.127	0.122	0.108	0.303	0.311	0.090	0.065	0.153	1.000							
C. javanica var. indochinensis	0.152	0.135	0.118	0.129	0.151	0.357	0.334	0.135	0.079	0.136	0.473	1.000						
S. alata	0.213	0.234	0.123	0.226	0.230	0.140	0.162	0.240	0.087	0.274	0.128	0.157	1.000					
S. spectabilis	0.257	0.222	0.130	0.219	0.276	0.158	0.125	0.256	0.106	0.244	0.106	0.159	0.295	1.000				
S. auriculata,	0.209	0.198	0.092	0.207	0.241	0.140	0.147	0.212	0.097	0.220	0.125	0.123	0.258	0.303	1.000			
C. roxburghii	0.137	0.137	0.088	0.112	0.127	0.295	0.315	0.133	0.067	0.126	0.376	0.424	0.131	0.128	0.125	1.000		
Ch.pumila	0.109	0.096	0.362	0.131	0.126	0.101	0.082	0.118	0.420	0.109	0.113	0.117	0.095	0.098	0.111	0.107	1.000	
S. septemtrionalis	0.242	0.241	0.147	0.215	0.219	0.142	0.188	0.214	0.082	0.236	0.122	0.108	0.227	0.245	0.246	0.139	0.118	1.000

alexandrina, *Senna siamea* and *Senna pallida*, the second had *S. sulfurea*, *Senna alata*, *Senna spectabilis*, *Senna auriculata* and *Senna septemtrionalis* and both the sub-clusters sharing a common node at approximately 23% level of similarity. All the species of *Chamaecrista* had a similarity of nearly 35.5% among themselves and *Cassia* at 32% similarity level. The tree had a common node at 11% similarity level for the three different genera in the subtribe Cassiinae.

Cophenetic correlation

Cophenetic correlation was calculated for different phenograms generated from three different marker systems. It was found that the maximum cophenetic correlation existed between *C. grandis* and *C. javanica* var. *indochinensis* (0.473). The cophenetic correlation for different markers is represented in Table 6. The average cophenetic correlation between any two species was found to be 0.183392.

Bootstrap analysis

Bootstrap analysis revealed that all the clusters

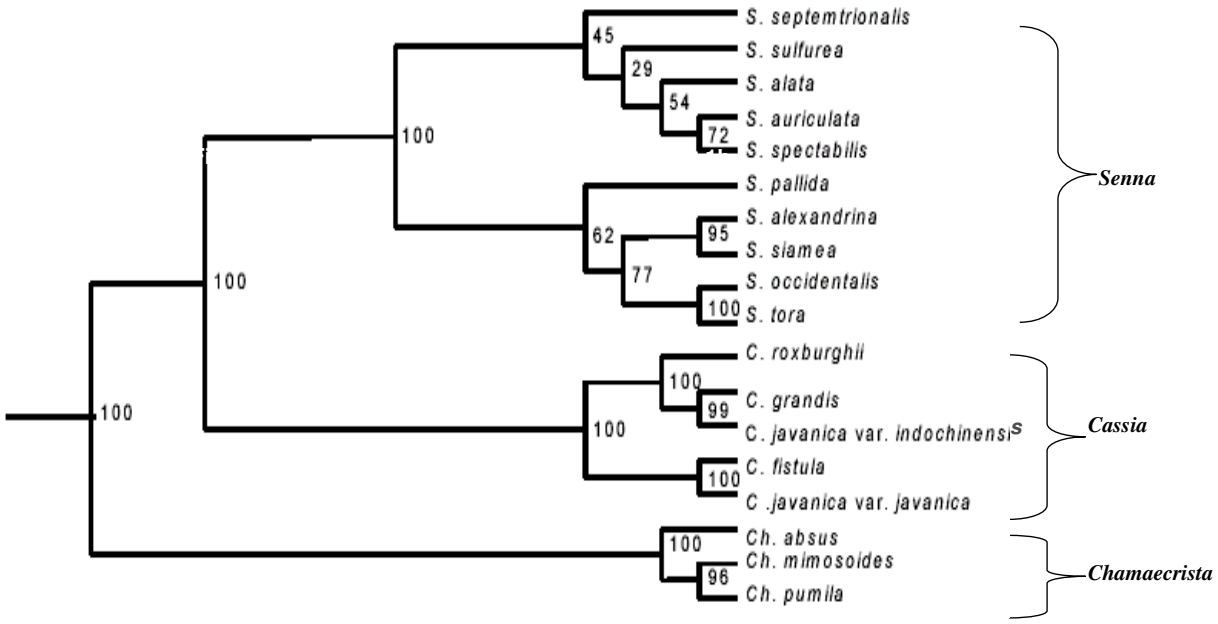

Figure 2. Dendrogram showing genomic relationship and bootstrap value among and within different clusters in Cassiinae as revealed from RAPD, ISSR and AFLP data.

were stable and had bootstrap value of approximately 100. It indicates that the clades containing *C. javanica and Cassia fistula* among the members of *Cassia*, *C. pumila* and *C. mimosoides* in *Chamaecrista* and *S. tora* and *S. occidentalis* in *Senna* were closely linked with bootstrap value of 100 or nearly 100. The bootstrap value among different clusters and sub clusters were presented in fig bootstrap tree (Figure 2).

Principal co-ordinate analysis

In the PRINCORD analysis, all the species were separated in three different plains each containing the representatives of a particular genus. All the species of *Chamaecrista* were grouped together at a corner in the PCA Figure while all the members of *Cassia* were grouped in the opposite corner in the Figure. However, the elements of *Senna* were found in the middle of the diagram maintaining equal distance from *Cassia* and *Chamaechrista* groups. The PCA diagram is represented in Figure 3.

DISCUSSION

The genomic relations among different taxa of Cassiineae were studied on the basis of RAPD, ISSR and AFLP fingerprinting. The relationship obtained among the different taxa of Cassinnae using RAPD was in agreement with the conventional taxonomic classification of the subtribe. The trifurcation of Cassiinae into three distinct genera as suggested by Irwin and Barneby (1981) proved to be justified. Whitty et al. (1994) worked on Cassiinae and justified the grouping of Cassiinae into *Cassia*, *Senna* and *Chamaecrista*. We observed similar type of result. However, the intra-generic relationship among species of *Senna* does not follow the sequence that Irwin and Barneby (1981) worked out. The 3 species represented here of the sect. *Peiranisia* namely, *S. pallida*, *S. auriculata* and *S. spectabilis* formed a cluster. Similarly, *S. tora*, *S. occidentalis*, *S. septemtrionalis* and *S. siamea* all belonging to the sect. *Chamaefistula* came together in a distinct clade. Non-inclusion of *S. alexandrina* in the above cluster could not be reasoned out. Of the three elements of the genus *Chamaecristra,* the lone species of the sect. *Grimaldia* that is, *C. absus* got separated from the other two, which belong to the sect *Chamaecrista*.

The intra-generic arrangement of species in *Chamaecrista* was in agreement with Irwin and Barneby (1981). The deviations with regard to intra-generic relationship may be due to selection of a few numbers of species from such a large taxon for the present investigation and amplification a small portion of the entire genome. Souza and Benko-Iseppon (2004) found significant differences in chromosome size, morphology and condensing behavior among members of the controversial tribe Cassieae (*Cassia*, *Chamaecrista* and *Senna*), revealing the tribe to be a heterogeneous group from the karyological point of view.

The dendrogram obtained from the ISSR data segregated the subtribe into two clusters; the first with the three species of *Chamaecrista* and the second having

Table 6. Cophenetic correlation among different species of Cassiinae as generated from RAPD, ISSR and AFLP phenograms.

	S. tora	*S. occidentalis*	*Ch. absus*	*S. alexandrina*	*S. siamea*	*C. fistula*	*C. javanica var. javanica*	*S. pallida*	*Ch. mimosoides*	*S. sulfurea*	*C. javanica var. indochinensis*	*C. nodosa*	*S. alata*	*S. spectabilis*	*S. auriculata,*	*C.roxburghii*	*Ch. pumila*	*S. septemtrionalis*
S. tora	1.000																	
S. occidentalis	0.430	1.000																
Ch. absus	0.110	0.110	1.000															
S. alexandrina	0.270	0.270	0.110	1.000														
S. siamea	0.270	0.270	0.110	0.310	1.000													
C. fistula	0.140	0.140	0.110	0.140	0.140	1.000												
C. javanica	0.140	0.140	0.110	0.140	0.140	0.400	1.000											
S. palida	0.240	0.240	0.110	0.240	0.240	0.140	0.140	1.000										
Ch .mimosoides	0.110	0.110	0.360	0.110	0.110	0.110	0.110	0.110	1.000									
S. sulfuria	0.230	0.230	0.110	0.230	0.230	0.140	0.140	0.230	0.110	1.000								
C. javanica var indochinensis	0.140	0.140	0.110	0.140	0.140	0.320	0.320	0.140	0.110	0.140	1.000							
C. nodosa	0.140	0.140	0.110	0.140	0.140	0.320	0.320	0.140	0.110	0.140	0.470	1.000						
S. alata	0.230	0.230	0.110	0.230	0.230	0.140	0.140	0.230	0.110	0.250	0.140	0.140	1.000					
S. spectabilis	0.230	0.230	0.110	0.230	0.230	0.140	0.140	0.230	0.110	0.250	0.140	0.140	0.280	1.000				
S. auriculata,	0.230	0.230	0.110	0.230	0.230	0.140	0.140	0.230	0.110	0.250	0.140	0.140	0.280	0.300	1.000			
C .roxburghii	0.140	0.140	0.110	0.140	0.140	0.320	0.320	0.140	0.110	0.140	0.400	0.400	0.140	0.140	0.140	1.000		
Ch. pumila	0.110	0.110	0.360	0.110	0.110	0.110	0.110	0.110	0.420	0.110	0.110	0.110	0.110	0.110	0.110	0.110	1.000	
S. septemtrionalis	0.230	0.230	0.110	0.230	0.230	0.140	0.140	0.230	0.110	0.240	0.140	0.140	0.240	0.240	0.240	0.140	0.110	1.000

all the species of *Cassia* and *Senna*. The second clade was further bifurcated to two sub-clusters; one with all the 10 species of *Senna* and the second sub-cluster had 5 species belonging to the genus *Cassia*. Thus broadly, there was a segregation of the sub-tribe Cassiinae into three genera. This was in conformity with the classification proposed by Irwin and Barneby (1981). Though the division of the subtribe on the basis of ISSR data appear justified, the clustering and arrangement of species under *Senna* and *Cassia* were not at par with the traditional grouping made earlier. As in case of RAPD, in the genus *Chamaecrista*, *Ch. absus* that comes under the sect. *Grimaldia* got separated from the rest two and *C. mimosoides* and *C. pumila* formed a sub-cluster justifying their inclusion under the sect. *Chamaecrista*. In the *Cassia* clade, *C. javanica* var. *indochinensis* and *C. roxburghii*, which are closely related came in a single cluster with distantly related *C. grandis*. As a result, the other variety *C. javanica* var. *javanica* remained isolated and formed a clade with *Chamaefistula*.

The placement of the four species of *Cassia* under the three series *Cassia, Grandes* and *Obolospermae* was not possible. Similarly, from the dendrogram generated from ISSR data,

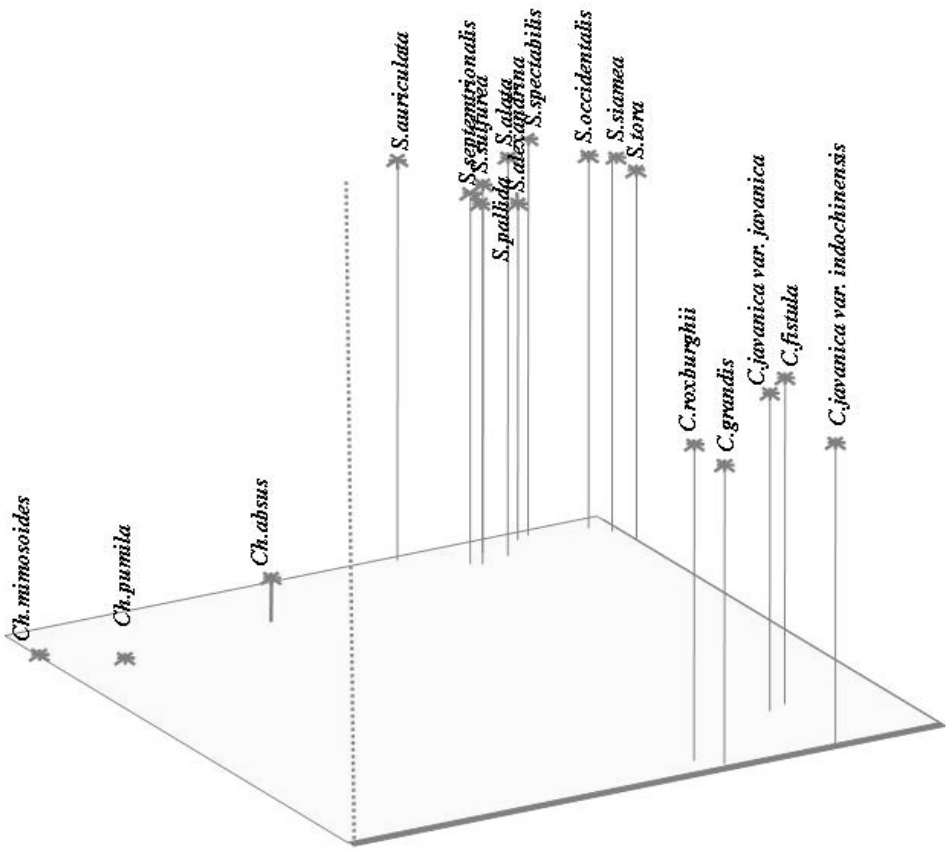

Figure 3. Principal co-ordinate analysis showing cluster arrangement in Cassiinae.

segregation of species of *Senna* into traditionally recognized sections like *Psilorhegma*, *Peiranisia*, *Chamaefistula* and *Senna* could not also be made. Though only six ISSR primers were used for the present investigation, the result was comparable with the RAPD.

As both RAPD and ISSR were dominant markers and arbitrarily amplified the loci, the need for application of better marker system was felt and AFLP was used. Six AFLP primer combinations were used for deciphering the genetic relationship among the 18 species of Cassiinae and a total of 622 bands were obtained, most of which were polymorphic in nature. A high degree of genetic diversity among the species of Cassiinae was noticed. The dendrogram constructed on the basis of the data obtained from the AFLP analysis segregated the subtribe into three distinct groups. As observed in RAPD and ISSR analysis, all members of *Senna*, *Cassia* and *Chamaecrista* formed distinct clusters. The arrangement of species under the genera *Chamaecrista* and *Cassia* was exactly similar as determined from RAPD and ISSR analysis as described earlier. However, the species of *Senna* were clustered in two groups; the first cluster containing *S. tora*, *S. occidentalis*, *S. alexandrina*, *S. siamea* and *S. pallida* and the second cluster with remaining five species. All the five species of the first sub-cluster shared a common node at 34% similarity level; *S. tora* and *S. occidentalis* were the closest. The second sub-cluster was constituted of *S. sulfurea*, *S. alata*, *S. spectabilis*, *S. auriculata* and *S. septemtrionalis* and shared a node with the first sub-cluster at about 27% level of similarity. The species arrangement did not follow any logical pattern and the data obtained was not discernible.

Marazzi et al. (2006) studied phylogetic relationships with *Senna* based on parsimony analyses of three chloroplast regions (*rpS16*, *rpL16* and *matK*) and provided new insights on the evolution of floral symmetry and extrafloral nectaries. Their results supported the monophyly of only one sect. *Psilorhegma* of the six currently recognized sections, while *Chamaefistula*, *Peiranisia*, and *Senna* were paraphyletic and monotypic *Astroites* and *Paradictyon* were nested within two of the seven major clades identified by molecular phylogeny. Their investigation further suggested that flowers in *Senna* were ancestrally monosymmetric with seven fertile stamens and three adaxial staminodes, switched to asymmetry later, and reverted to monosymmetry in most advanced clade. Fertility of all 10 stamens was considered to be a derived state, characterizing the *Psilorhegma* subclade.

The phenogram constructed using combined data

from RAPD, ISSR and AFLP analyses exhibited similar relationships among the genera and species. The bootstrap value obtained for different groups was fairly good which indicated that the branching in the tree was stable. When the correlation among all the markers was calculated it was highly encouraging and all markers showed high degree of correlation with each other and with the combined data.

Data obtained from analysis through molecular markers revealed high degree of genetic diversity among the different taxa of Cassiinae. Similar observations were made by workers, who worked on the subtribe taking different morphological markers (Bhattacharya and Maheshwari, 1971; Lasseigne, 1979; Malik and Krishna, 1978; Shyam et al., 1983; Mathur, 1985; Shyam and Vartak, 1985; Bhattacharya and Saha, 1992; Sahai et al., 1997). In the present study taking the molecular markers into account the trifurcation of the sub tribe Cassiinae could be re-established but the intra-generic classification and phylogeny in different genera of the sub-tribe needs to be worked out in detail taking large number of species and using sophisticated molecular marker systems such as SSR and sequence-based markers like cp-DNA, nr DNA and ITS regions.

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