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Separation of the genera in the subtribe Cassiinae (Leguminosae: Caesalpinioidae) using molecular markers

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RESUMO

(Separação dos gêneros na subtribo Cassiinae (Leguminosae: Caesalpinioidae) utilizando marcadores moleculares). Técnicas de *Random amplified polymorphic DNA* (RAPD), *Inter simple sequence repeat* (ISSR) e *Amplified Fragment Length Polymorphism markers* (AFLP) foram utilizadas para verificar a segregação do gênero *Cassia* L. *senso lato* em três diferentes gêneros, *Chamaecrista* Moench., *Senna* P. Mill. e *Cassia* L. *senso stricto* Dezoito representantes dos três táxons foram caracterizados com o uso de marcadores moleculares: 25 RAPD, seis iniciadores ("primers") ISSR e seis AFLP combinações de iniciadores, resultando na amplificação de 612, 115 e 622 bandas (*loci*), respectivamente. A maioria dos *loci* apresentou-se como polimórfico, mostrando um alto grau de diversidade genética entre os táxons estudados. O dendrograma construído com base nos dados de RAPD, ISSR e AFLP e agrupamento com procedimentos SHAN dividiu *Cassia* L. *senso lato* em três diferentes agrupamentos, chamados de *Chamaecrista* Moench., *Senna* P. Mill. e *Cassia* L. *senso stricto* Valores altos de bootstrap revelaram que todos os agrupamentos foram estáveis e robustos. Foi observado pela presente investigação que estes gêneros possuem identidade ao nível molecular, o que sustenta a elevação do genero *Cassia* L. *senso lato* para o nível de subtribo e a segregação dos três gêneros ao invés de formarem categorias infra-genéricas.

Palavras-chave: AFLP, *Cassia*, filogenia molecular, ISSR, RAPD

ABSTRACT

(Separation of the genera in the subtribe Cassiinae (Leguminosae: Caesalpinioidae) using molecular markers). 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. *senso lato* into three distinct genera namely *Chamaecrista* Moench., *Senna* P. Mill. and *Cassia* L. *sensostricto* 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 SHAN clustering, divided *Cassia* L. *senso lato.* into three different clusters as *Chamaecrista* Moench. *Senna* P. Mill. and *Cassia* L. *senso stricto* 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. *senso lato* to the level of subtribe Cassiinae and segregation into three distinct genera instead of intrageneric categories.

Key words: AFLP, *Cassia*, ISSR, Molecular phylogeny, RAPD

Introduction

Cassia L. senso lato (Leguminosae: Caesalpinioideae) 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 is centred in areas of varied topography with seasonal climates. There has been considerable divergence of opinion concerning the delimitation and taxonomic status of its three constituent subgenera. Irwin & Barneby (1981, 1982) proposed an improved classification and raised the genus *Cassia* L. to the level of subtribe Cassiinae; the latter comprised of three genera, viz *Cassia* L. *senso stricto*,

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Senna P. Mill. and *Chamaecrista* Moench. This concept had found wide recognition in recent years (Randel 1988, 1989,1990; Lock 1988, 89; Larsen & Hou 1996). Despite several studies by taxonomists, either on the whole subfamily Caesalpinioideae in restricted areas or of certain genera throughout the world, there is still a great deal of taxonomic work to be done at the level of genus and tribe. The taxonomic treatment of the genus *Cassia* L. *senso lat*. has been done in some countries in Asia, namely Malaysia (De Wit 1955) and Pakistan (Ali & Quraishi 1967). But nowadays taxonomy is not simply based on floral and vegetative characters but many other types of data, like anatomical, cytological, serological and molecular. These are regarded as very important in determining relationships and affinities of the plants. New trends and new information on various aspects had prompted modern taxonomists to propose a new system of classification. These have also led to some changes in the criteria for determining the evolutionary status of the various taxa in the subtribe Cassinae.

Irwin and Barneby (1981, 1982) raised the genus *Cassia* L. *sensu lato* to the level of subtribe and elevated previous subgenera to generic rank viz. *Senna* Mill and *Chamaecrista* Moench under the tribe *Cassieae* Bronn ex Irwin and Barneby of Caesalpiniaceae (today Leguminosae-Caesapinioideae). However there is little work done on the molecular phylogeny of this genus and conformation of the trifurcation of this group. There are very few reports on other aspects like biochemical and cytological on this taxa. The reports that are present contradict each other in the cytological data (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 subtribe *Cassiinae*. Justification of trifurcation of the subtribe into three distinct categories like *Cassia, Senna* and *Chamaecrista* as suggested by Irwin and Barneby (1982), taking the molecular data obtained from RAPD, ISSR and AFLP analyses.

Material and methods

Plant material

All plants were raised from seed. Eighteen species of the genus *Cassia* were taken for the present study. Their taxonomic rank and changed names are given in Tab.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. Vouchers were deposited at Herbarium of Regional Plant Resource Centre.

Molecular Analysis

DNA was isolated from young, fresh leaves using the CTAB method as described by Saghai-Maroof *et al.* (1984). For RAPD analysis, PCR amplification of 25ng 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 (@0.5µg/ml of gel solution). The size of the amplicons was determined using size standards (100bp 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 have 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 presence and absence 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 unweighted pair group method using arithmetic averages (UPGMA) (Sneath & Sokal 1973) and SHAN clustering. All the analysis was done using the computer package NTSYS-PC-2.02e (Rohlf 1997). Resolving power (Rp) of the RAPD, ISSR and AFLP was calculated as per Prevost & 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-×P^2_i, P_i is the band frequency of the *i*th 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

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

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 on the clustering pattern of the analyzed populations. PCA was performed based on the RAPD, ISSR and AFLP data, for all the primers.

Results

Random Amplified Polymorphic DNA

All the 18 species produced distinct reproducible amplifications. The DNA profiles as observed in RAPD are represented in (Fig. 1). A total of 612 numbers of bands were amplified (summary in Tab.2). All the bands were found to be polymorphic. Maximum and minimum amplification was observed in the case of the species *Chamaecrista absus* L. (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 the case of OP D02 and OP D20 only two bands were found to be exclusive. In *Chamaecrista*

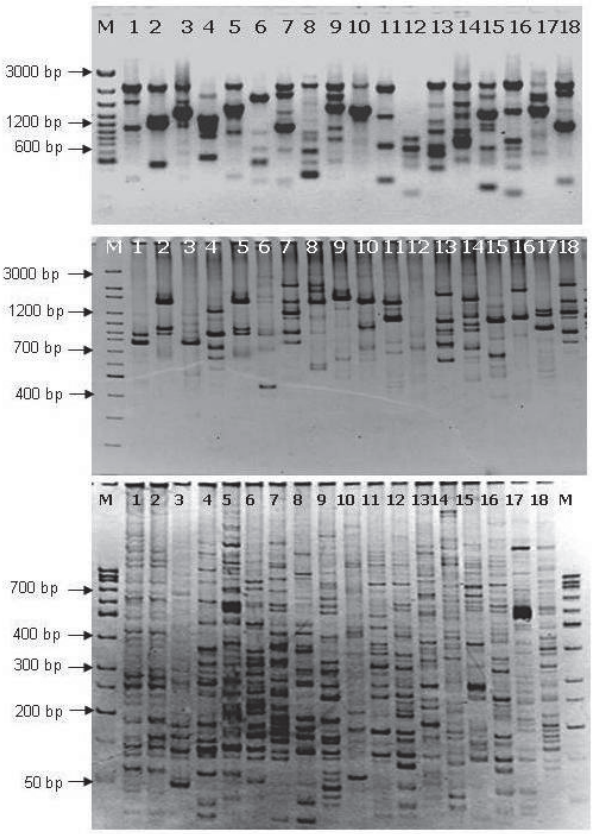

Figure 1. Banding pattern in different species of Cassiinae as seen in (from top to bottom) RAPD, ISSR and AFLP techniques

there were highest unique bands (20), only two bands were found to be unique in the case of *Ch. pumila* as well as for *C. grandis*. The dendrogram constructed using SHAN clustering shows three major groups among the 18 studied taxa (figure not shown).

Inter Simple Sequence Analysis

Six ISSR primers resulted in the amplification of 115 fragments. The ISSR banding pattern in Cassiinae is represented in Fig. 1. 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 was 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 *C. javanica* and the least number of amplicons were amplified for the species *Ch. mimosoides.* Details of ISSR banding pattern and bands amplified in different species with different primers have been represented in Tab. 3.

AFLP 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 polymorhic. Banding pattern in AFLP for 18 species of Cassiinae is shown in Fig. 1. 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 noted for *C. javanica* var. *indochinensis* for primer combination EACT/MCAG and the lowest for the primer combination EACC/MCTC (19) in the case of the species *C. javanica*. Bands resolved between 1500bp 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, whereas 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 Tab. 4.

Similarity for RAPD, ISSR and AFLP

Jaccard's similarity was calculated from the data generated from RAPD, ISSR and AFLP analysis. It was observed that *C. grandis* and *C. javanica* var. *indochinensis* were most closely related with Jaccard's similarity coefficient of 0.473, whereas *Ch. mimosoides* and *S. sulfurea* were distantly placed with the lowest Jaccard's similarity coefficient of 0.064. Among all the species, there was average similarity of 0.1834. Jaccard's similarity among the taxa is represented in Tab. 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 (Fig. 2). All the 18 taxa of Cassinae 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 *S. tora*, *S. occidentalis*, *S. alexandrina*, *S. siamea* and *S. pallida*, the second had *S. sulfurea*, *S. alata*, *S. spectabilis*,

Table 2. Details of RAPD analysis in Cassiinae.

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

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'	2700bp-300bp	19	0	13.33	7.2098
(GA)9T	5'GAG AGA GAG AGA GAG AGA T3'	2500bp-300bp	14	3	7.8888	5.6419
(GACA)4	5'GAC AGA CAG ACA GAC A3'	>3000bp-400bp	21	1	10.8889	8.1667
T(GA)9	5'TGAG AGA GAG AGA GAG AGA3'	2300bp-400bp	20	4	8.33333	6.006
$(GTG)_5$	5'GTG GTG GTG GTG GTG3'	>3000bp-400bp	20	0	12	7.272
$(CAA)_5$	5'CAA CAA CAA CAA CAA3'	>3000bp-100bp	21	0	5.78	4.969
Total			115	8		

Table 4. Details of AFLP analysis in Cassiinae.

Primer	Sequence of Primer	Total bands	Mnomorphic 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		

S. auriculata and *S. septemtrionalis* and both the subclusters shared 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 Tab. 6. The average cophenetic correlation between any two species was found to be 0.183392.

Bootstrap analysis

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

Principal coordinate 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 Fig. 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 *Cassia*, *Senna* and *Chamaecrista*. We observed similar type of result. However, the intrageneric relationship among species of *Senna* does not follow the sequence that Irwin and Barneby (1981) worked out. The three species represented here of 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 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 sect. *Grimaldia i.e. Ch. absus* got separated from the other two, which belong to sect. *Chamaecrista*. The infra-generic arrangement of species in *Chamaecrista* was in agreement with Irwin and Barneby (1981). The deviations with regard to intrageneric relationships may be due to selection of a small number of species from such a large taxon for the present investigation and amplification a small portion of the entire genome. Souza & 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.

In contrast, the species relationship in the genus *Cassia* as observed from RAPD tree was confusing. The distant placement of *C. javanica* and *C. roxburghii*, both belonging to the series *Obolospermae* (Irwin & Barneby 1981) and even separation of two varieties of *C. javanica* could not be suitably explained. Inclusion of more species and resorting

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

	S.tora	*S.occidentalis*	*Ch.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

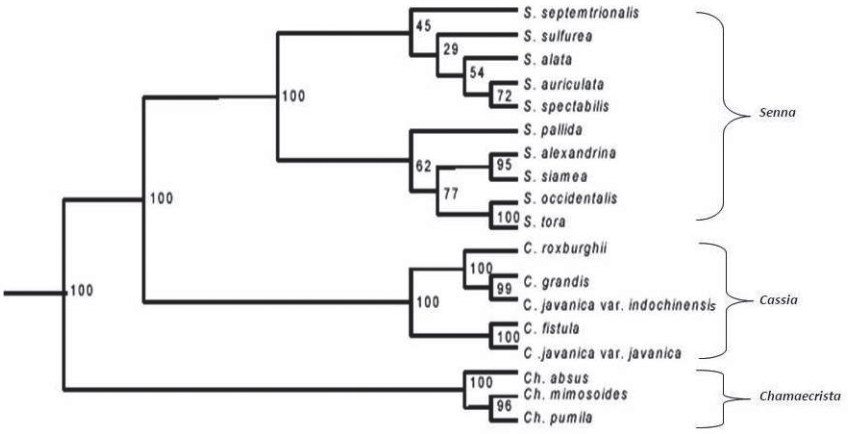

Figure 2. Dendrogram showing genomic relationship and bootstrap value among and within different clusters in Cassiinae as revealed from RAPD, ISSR and AFLP data.

to other molecular techniques in conjunction with RAPD are likely to alter the arrangement of taxa under the genus and bring about the expected order.

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 all the species of *Cassia* and *Senna*. The second clade was further bifurcated to two subclusters; one with all 10 species of *Senna* and the second subcluster had five species belonging to the genus *Cassia*. Thus, broadly there was a segregation of the subtribe Cassiinae into three genera. This was in conformity with the classification proposed by Irwin & Barneby (1981). Though the division of the subtribe on the basis of ISSR data appears justified, the clustering and arrangement of species under *Senna* and *Cassia* were not at par with the traditional grouping made earlier. As in the case of RAPD, in the genus *Chamaecrista*, *Ch. absus* that comes under sect. *Grimaldia* got separated from the remaining two and *Ch. mimosoides* and *Ch. pumila* formed a subcluster justifying their inclusion under 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*. But the other variety *C. javanica* var. *javanica* remained isolated and formed a clade with *C. fistula*. 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, segregation of species of *Senna* into traditionally recognized sections like *Psilorhegma, Peiranisia, Chamaefistula* and *Senna* also could not be done. 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 noted. 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 and 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 the remaining five species. All the five species of the first subcluster shared a common node at 34% similarity level; *S. tora* and *S. occidentalis* were the closest. The second subcluster consisted of *S. sulfurea*, *S. alata*, *S. spectabilis*, *S. auriculata* and *S. septemtrionalis* and shared a node with the first subcluster 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 phylogenetic relationships within *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

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

	S.tora	*S.occidentalis*	*Ch.absus*	*S.angustifolia*	*S.siamea*	*C.fistula*	*C.javanica*	*S.biflora*	*Ch.mimosoides*	*S.glauca*	*C.grandis*	*C.nodosa*	*S.alata*	*S.spectabilis*	*S.auriculata*	*C.roxburghii*	*Ch.pumila*	*S. laevigata*
S.tora	1.00																	
S.occidentalis	0.43	1.00																
Ch.absus	0.11	0.11	1.00															
S.angustifolia	0.27	0.27	0.11	1.00														
S.siamea	0.27	0.27	0.11	0.31	1.00													
C.fistula	0.14	0.14	0.11	0.14	0.14	1.00												
C.javanica	0.14	0.14	0.11	0.14	0.14	0.40	1.00											
S.biflora	0.24	0.24	0.11	0.24	0.24	0.14	0.14	1.00										
Ch.mimosoides	0.11	0.11	0.36	0.11	0.11	0.11	0.11	0.11	1.00									
S.glauca	0.23	0.23	0.11	0.23	0.23	0.14	0.14	0.23	0.11	1.00								
C.grandis	0.14	0.14	0.11	0.14	0.14	0.32	0.32	0.14	0.11	0.14	1.00							
C.nodosa	0.14	0.14	0.11	0.14	0.14	0.32	0.32	0.14	0.11	0.14	0.47	1.00						
S.alata	0.23	0.23	0.11	0.23	0.23	0.14	0.14	0.23	0.11	0.25	0.14	0.14	1.00					
S.spectabilis	0.23	0.23	0.11	0.23	0.23	0.14	0.14	0.23	0.11	0.25	0.14	0.14	0.28	1.00				
S.auriculata,	0.23	0.23	0.11	0.23	0.23	0.14	0.14	0.23	0.11	0.25	0.14	0.14	0.28	0.30	1.00			
C.roxburghii	0.14	0.14	0.11	0.14	0.14	0.32	0.32	0.14	0.11	0.14	0.40	0.40	0.14	0.14	0.14	1.00		
Ch.pumila	0.11	0.11	0.36	0.11	0.11	0.11	0.11	0.11	0.42	0.11	0.11	0.11	0.11	0.11	0.11	0.11	1.00	
S. laevigata	0.23	0.23	0.11	0.23	0.23	0.14	0.14	0.23	0.11	0.24	0.14	0.14	0.24	0.24	0.24	0.14	0.11	1.00

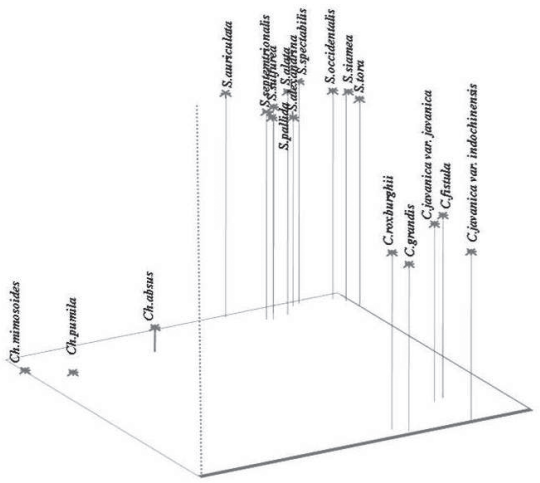

Figure 3. Principal co-ordinate analysis showing cluster arrangement in Cassiinae.

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 other authors who worked on the subtribe taking different morphological markers (Bhattacharya & Maheshwari, 1971; Lasseigne 1979); Malik & Krishna 1978; Shyam *et al.* 1983; Mathur 1985; Shyam & Vartak, 1985; Bhattacharya & Saha, 1992; Sahai *et al.* 1997). In the present study taking the molecular markers into account the trifurcation of the subtribe *Cassiinae* could be re-established but the intra-generic classification and phylogeny in different genera of the subtribe 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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