



# Journal of Biological Sciences

ISSN 1727-3048

**science**  
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## Interspecific Genetic Diversity in 15 Species of *Cassia* L. Evident by Chromosome and 4C Nuclear DNA Analysis

S. Mohanty and A.B. Das  
Cytogenetics Laboratory, Regional Plant Resource Centre,  
Nayapalli, Bhubaneswar-751015, Orissa, India

**Abstract:** In view of the vast changes in *Cassia* taxonomy in recent years, structural analysis of chromosomes and 4C DNA content of 15 species of *Cassia* has studied to aid in resolving phylogenetic problems. Root tip meristematic cells were pretreated in a aqueous solution of p-Dichlorobenzene and aesculine followed by fixation in Carnoy's-II solution for chromosome analysis and the method of cytophotometry was followed for the estimation of nuclear DNA content. Somatic chromosome number  $2n=28$  was observed in all the species except  $2n=16$  in *C. mimosoides*,  $2n=26$  in *C. biflora* and *C. tora*, respectively. In addition, aneuploid/polyploid chromosome number plates were also found in most of the species like *C. siamea* ( $2n=14, 28, 35$ ), *C. biflora* ( $2n=24, 26$ ), *C. mimosoides* ( $2n=16, 30$ ), *C. fistula* ( $2n=24, 28$ ), *C. tora* ( $2n=28, 52$ ). Minute details of the karyotype showed structural alteration of chromosomes in spite of the same diploid chromosome numbers in some species. In *C. mimosoides* the size of the chromosomes was quite large ( $2.06-3.34\ \mu\text{m}$ ); significant variations in the chromosome length were observed among different species from  $31.48\ \mu\text{m}$  in *C. roxburghii* to  $57.48\ \mu\text{m}$  in *C. tora*. Total chromosome volume also varied significantly among the studied species from  $12.38\ \mu\text{m}^3$  in *C. renigera* to  $24.02\ \mu\text{m}^3$  in *C. occidentalis*. Highest centromeric index percentage (45.96%) was noted in *C. grandis* and that of lowest (36.09%) in *C. mimosoides*. Significant variation in 4C nuclear DNA content was noted among the species that varied from 7.59 pg in *C. absus* to 35.63 pg in *C. siamea*. The correlation coefficients shows the various chromosomal and nuclear parameters are interdependent to some extent suggesting a interspecific relationship between structural and genetic changes of the genome architecture during evolution of speciation.

**Key words:** Legume, *Cassia*, interspecific relationship, chromosome number, karyotype, 4C nuclear DNA

### INTRODUCTION

The genus *Cassia* of the family Leguminosae represents one of the largest and diverse groups of flowering plants including herbs to trees that are well known for their beauty and utility. Besides their immense floricultural values, these find effective utilization in indigenous systems of medicine. The genus *Cassia* exhibits a great deal of diversity and is taxonomically complex; there has been considerable divergence of opinion concerning the delimitation and taxonomic status of this group of plants. The wide variability in habits ranging from tall trees to delicate annual herbs, number and size of leaflets, form and foliar has added difficulties to taxonomists in the delimitation of species or the intraspecific taxa (Singh, 2000). Identification of chromosomes and DNA markers constitutes the first step in understanding the genome

organization of the species. Study of chromosome number and structure have great value in revising and improving the classification of the plants that will add more evidences to understand the evolutionary sequences in the phylogeny of *Cassia* L. and synthesis of new classification of it. The number of chromosomes, their gross morphology, meiotic behavior, karyotypic differences and genomic DNA contents provide good material for delimitation of taxa up to the level of family and to determine evolutionary sequences. Each species has a characteristic set of chromosomes, which embody its basic set of genetic information, the genome that can be used to assess the genetic relatedness or distance between individuals. 4C DNA content gives an idea about the genome size of the species and variation of the individuals at the population level. Ohri *et al.* (1986) and George and Bhavanandan (1993, 1994) reported the chromosome numbers in some species of *Cassia* but there

is no detailed report on karyotype analysis. In order to ascertain precisely the importance of DNA in genetic diversity and phylogeny, an understanding of the genetic behavior at specific level, is necessary. Keeping in view to assess the function of chromosomes in genetic relatedness or evolution of the species, chromosome structure, abnormalities and variation in 4C DNA content has been studied in the fifteen species of *Cassia*.

## MATERIALS AND METHODS

Annual plant materials were collected from pastures and for perennial species seeds were collected from trees and germinated in the soil for fresh root tips for the study.

**Karyotype analysis:** For the chromosome study, fresh healthy root-tips were pretreated in 0.02 M hydroxyquinoline for 3 h at 14°C followed by overnight fixation in 1:3 propionic alcohol. The root-tips were stained in 2% propionic orcein after cold hydrolysis in 5 N HCl for 5 min and were squashed in 45% propionic acid. Ten well-scattered metaphase plates were selected for karyotype analysis of each species. Total genomic chromosome length was ascertained by adding the length of all chromosomes in the karyotype and the total chromosome volume of a karyotype was calculated by using the formula  $\pi r^2 h$ , where,  $r$  and  $h$  represents the radius and length of the chromosome, respectively. Form percentage (F%) of individual chromosomes was calculated and the total form percentage (F%) was the average of sum total F% of a karyotype (Das and Mallick, 1993). Mean values of total genomic chromosome length and total chromosome volume with standard errors were calculated.

**Interphase nuclear volume (INV):** For scoring of INV the root-tips of about 2-2.5 mm length were fixed in 1:3 acetic: ethanol for 24 h at 25°C, hydrolyzed in 1 N HCl at 4°C for 15 min. After thorough washing, the root-tips were put into Schiff's reagent for 1 h at 20°C and kept in the dark for staining. Squash preparation was done in 45% propionic acid. Ten randomly selected nuclei were scored from each root tip. Under oil immersion objectives mean of the two diameters of nuclei, obtained by measuring at right angles to each other, was used to calculate the value of INV using the formula  $4/3\pi r^3$ , where,  $r$  is the radius of the nuclei (Van't Hof, 1965).

**4C DNA content:** For Feulgen cytophotometric estimation of 4C DNA content, ten fixed root-tips from each species were hydrolyzed in 5 N HCl at 20°C for 1 h, washed in

distilled water and rinsed in SO<sub>2</sub> water and were stained in Schiff's reagent for 2 h. at 14°C, each root-tip squash was prepared in 45% propionic acid (Fox, 1969). Ten scorings were made from each slide and 4C DNA content was estimated from metaphase chromosomes using a Nikon Optiphot microscope fitted with a microspectrophotometer using monochromatic light at 550 nm following the method of Sharma and Sharma (1980). *In situ* DNA were obtained on the basis of optical density, which was converted to picograms (pg) using Vant Hof's (1965) 4C nuclear DNA values (67.1 pg) for *Allium cepa* var. Deshi as a standard.

**Statistical analysis:** Mean values of total genomic chromosome length and volume with standard error were calculated. The correlation coefficient analysis between different chromosomal parameters was done to find out the genomic characteristics. The ANOVA were performed (Sokal and Rohlf, 1973) among the nuclear DNA values following Duncan's Multiple Range Test (Harter, 1960). The correlation coefficient analysis was done between different chromosomal parameters to compare genomic characteristics. ANOVA was performed among the nuclear DNA values, using Duncan's Multiple Range Test (Harter, 1960).

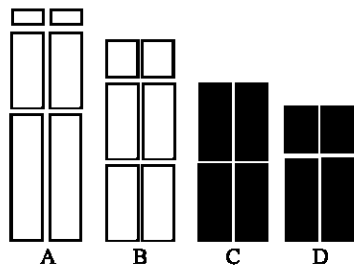
## RESULTS

**Chromosome numbers:** The diploid somatic chromosome number  $2n=16$  was observed in *C. mimosoides*,  $2n=26$  in *C. biflora* and *C. tora* and  $2n=28$  was noted in rest all of the species (Table 1 and Fig. 2-16). In addition, aneuploid/polyploid chromosome number were also observed in most of the species like *C. siamea* ( $2n=14, 28, 35$ ), *C. biflora* ( $2n=24, 26$ ), *C. mimosoides* ( $2n=16, 30$ ), *C. fistula* ( $2n=24, 28$ ), *C. tora* ( $2n=28, 52$ ) (Fig. 17-21).

**Chromosome characteristics:** *Cassia*, in general is characterized by small size chromosomes. Though most of the species have same number of diploid chromosomes, they differ in their minute details of the karyotype. The different species could be distinguished from one another on the basis of their variation in karyotype and Total Chromosome Length (TCL). The chromosomes are mostly median to sub median constricted in most of the species whereas subterminal constricted chromosomes were found in *C. spectabilis* and *C. roxburghii*. On the basis of the size of the chromosome and the position of the primary and secondary constrictions, a number of chromosome types were found to be common among the studied species that

Table 1: Somatic chromosome number, karyotype, chromosome length, chromosome volume and 4C-DNA content in the studied species of *Cassia*

Species	Somatic chromosome number (2n)	Karyotype formulae	Genomic chromosome length ( $\mu\text{m}$ )	Genomic chromosome volume ( $\mu\text{m}^3$ )	TF%	Interphase nuclear Volume ( $\mu\text{m}^3$ )	4C DNA content (pg)	Average chromosome length ( $\mu\text{m}$ )	Average chromosome volume ( $\mu\text{m}^3$ )
<i>Cassia javanica</i>	28	2A+2B+22C+2D	23.62 $\pm$ 2.13	7.36 $\pm$ 2.67	40.50	343.75 $\pm$ 4.30	15.04 $\pm$ 0.11	1.68	0.525
<i>C. mimosoides</i>	16	4A+2B+8C+2D	20.31 $\pm$ 2.22	11.76 $\pm$ 1.93	36.09	182.85 $\pm$ 3.25	18.71 $\pm$ 0.09	2.53	1.470
<i>C. occidentalis</i>	28	2A+22C+4D	26.74 $\pm$ 3.21	12.01 $\pm$ 1.98	42.95	292.41 $\pm$ 5.28	12.00 $\pm$ 0.18	1.91	0.857
<i>Cassia renigera</i>	28	2A+2B+18C+6D	19.77 $\pm$ 1.20	6.19 $\pm$ 2.44	40.74	325.51 $\pm$ 6.20	8.61 $\pm$ 0.19	1.41	0.440
<i>Cassia grandis</i>	28	22C+6D	17.74 $\pm$ 1.81	8.62 $\pm$ 3.12	45.96	271.16 $\pm$ 3.28	10.70 $\pm$ 0.11	1.26	0.615
<i>Cassia absus</i>	28	2A+4B+20C+2D	25.58 $\pm$ 2.67	8.80 $\pm$ 1.67	39.19	182.40 $\pm$ 2.57	7.59 $\pm$ 0.08	1.82	0.628
<i>Cassia biflora</i>	26	4A+22C	21.20 $\pm$ 2.09	9.25 $\pm$ 1.20	41.68	380.55 $\pm$ 2.36	25.42 $\pm$ 0.09	1.60	0.711
<i>C. spectabilis</i>	28	2A+2B+18C+6D	18.72 $\pm$ 2.45	7.09 $\pm$ 2.67	38.37	261.63 $\pm$ 2.74	8.08 $\pm$ 0.1	1.33	0.506
<i>Cassia siamea</i>	28	2A+6B+18C+2D	26.74 $\pm$ 1.14	8.07 $\pm$ 2.45	39.25	194.84 $\pm$ 4.26	35.63 $\pm$ 0.1	1.91	0.576
<i>Cassia obtusifolia</i>	28	4A+14C+10D	27.10 $\pm$ 1.76	9.65 $\pm$ 1.98	38.44	399.02 $\pm$ 2.91	25.77 $\pm$ 0.15	1.93	0.689
<i>Cassia tora</i>	26	2A+2B+14C+8D	28.74 $\pm$ 1.98	8.35 $\pm$ 2.13	39.72	419.89 $\pm$ 2.47	29.41 $\pm$ 0.12	2.21	0.642
<i>Cassia glauca</i>	28	6A+20C+2D	20.69 $\pm$ 2.44	10.65 $\pm$ 2.13	41.62	309.40 $\pm$ 2.47	22.56 $\pm$ 0.08	1.47	0.760
<i>Cassia alata</i>	28	2A+22C+4D	22.72 $\pm$ 2.13	7.14 $\pm$ 1.98	42.39	461.85 $\pm$ 3.11	33.99 $\pm$ 0.13	1.62	0.510
<i>Cassia fistula</i>	28	2B+24C+2D	24.15 $\pm$ 1.03	6.76 $\pm$ 1.03	42.60	233.09 $\pm$ 2.63	10.73 $\pm$ 0.14	1.72	0.482
<i>Cassia roxburghii</i>	28	4A+18C+6D	15.74 $\pm$ 3.21	8.63 $\pm$ 1.11	43.52	277.42 $\pm$ 5.04	23.59 $\pm$ 0.17	1.12	0.616

Fig. 1: Standard chromosome types found in *Cassia*

showed differences in number in each species. A general description of the representative types of chromosomes is given in Fig. 1.

Type A: Medium sized chromosomes with two constrictions in nearly median and nearly sub-terminal in positions.

Type B: Medium sized chromosomes with two constrictions one on median to nearly median and the other a satellite body on the long arm.

Type C: Medium sized chromosomes with median to nearly-median primary constriction.

Type D: Medium sized chromosomes with sub-median to nearly sub-median primary constriction.

The karyotype formula of all the species revealed definite differences in the chromosome structure (Table 1). Though all the 4 types of chromosomes i. e. Type A, B, C and D were present in *C. javanica*, *C. mimosoides*, *C. renigera*, *C. absus*, *C. spectabilis*, *C. siamea* and in *C. tora* they differ in their number and size (Fig. 22). All the species have the common type C and D chromosomes in different doses except in *C. biflora* that lack Type D chromosomes. Though median and sub median constricted Type C and D chromosomes were found in all the species, their dose differences were the most striking

features. In *C. tora* and *C. obtusifolia*, the two annual wild species have comparatively lower number of Type C chromosomes with nearly same numbers having  $2n=14$  and highest number of D type chromosomes. The tree species *C. fistula* has highest number (24) of Type C chromosomes. The perennial shrub *C. biflora* lacks Type B with secondary constricted chromosome. Secondary constricted chromosome number varied from 2 to 8 among the species. Secondary constricted chromosomes were absent in *C. grandis*. Secondary constricted Type A chromosomes were present in all the species except *C. fistula* and *C. grandis*. Secondary constricted Type B chromosomes were absent in *C. occidentalis*, *C. biflora*, *C. obtusifolia*, *C. glauca*, *C. alata* and *C. roxburghii*. Highest number of Type B chromosomes were found in *C. siamea* in which aneuploid number  $2n=14$  and  $2n=35$  number of chromosomes were also found in their somatic cells in addition to the normal  $2n=28$  chromosomes. Significant variations in chromosome length, volume and TF% were observed among the studied species of *Cassia*, details study in 15 species are presented in Table 1. In *C. mimosoides*, the size of the chromosomes was quite large (2.06-3.34  $\mu\text{m}$ ). Variations in the genomic chromosome length were observed among the studied species that range from 15.74  $\mu\text{m}$  in *C. roxburghii* to 28.74  $\mu\text{m}$  in *C. tora* (Table 1 and Fig. 2-16). Genomic chromosome volume also varied significantly among the studied species from 6.19  $\mu\text{m}^3$  in *C. renigera* to 12.01  $\mu\text{m}^3$  in *C. occidentalis* (Table 1). Highest centromeric index percentage (45.96%) was noted in *C. grandis* and that of lowest (36.09%) was found in *C. mimosoides*.

**INV and 4C nuclear DNA amount:** Interphase Nuclear Volume (INV) differed significantly in the studied species from 182.40 to 461.85  $\mu\text{m}^3$  in *C. absus* and *C. alata* (Table 1). Significant variation in 4C nuclear DNA content was observed among the species that varied from 7.59 pg

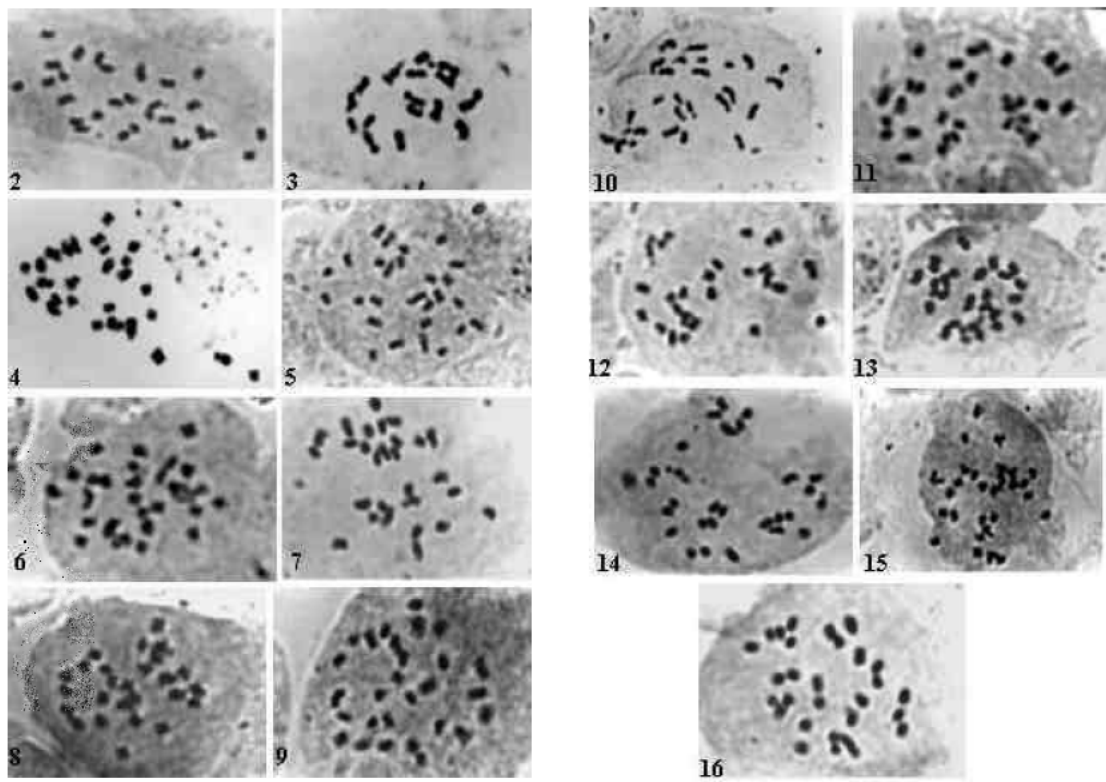


Fig. 2-16: Somatic chromosome plates of *Cassia* species

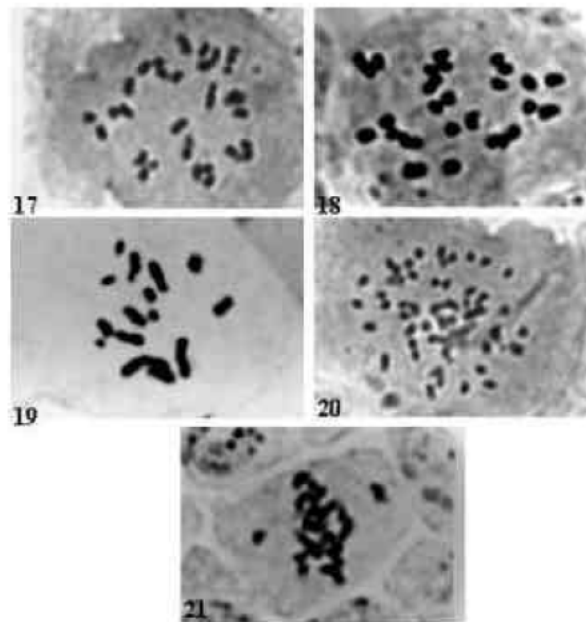


Fig. 17-21: Acuploid somatic chromosome numbers and chromosome abnormalities found in *Cassia siamea*. 17:  $2n=23$ , 18:  $2n=24$ , 19:  $2n=14$ , 20:  $2n=35$ , 21: metaphase cell showing early separated chromosomes

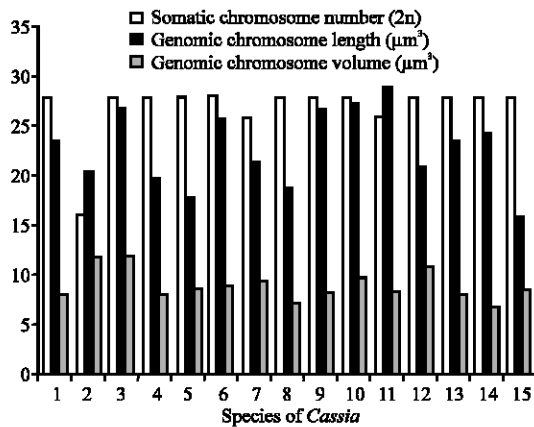


Fig. 22: Histogram representing comparative chromosome parameters in 15 species of *Cassia*.

- |                              |                              |
|------------------------------|------------------------------|
| 1 = <i>C. javanica</i> ,     | 2 = <i>C. mimosoides</i> ,   |
| 3 = <i>C. occidentalis</i> , | 4 = <i>C. renigera</i> ,     |
| 5 = <i>C. grandis</i> ,      | 6 = <i>C. absus</i> ,        |
| 7 = <i>C. biflora</i> ,      | 8 = <i>C. spectabilis</i> ,  |
| 9 = <i>C. siamea</i> ,       | 10 = <i>C. obtusifolia</i> , |
| 11 = <i>C. tora</i> ,        | 12 = <i>C. glauca</i> ,      |
| 13 = <i>C. alata</i> ,       | 14 = <i>C. fistula</i> ,     |
| 15 = <i>C. roxburghii</i>    |                              |

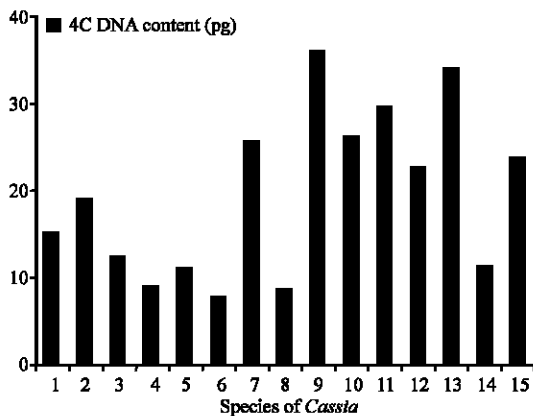


Fig. 23: Histogram representing variation in 4C DNA content in *Cassia*

in *C. absus* to 35.63 pg in *C. siamea* (Fig. 23). Though the total chromosome length, total chromosome volume, INV and 4C DNA content were correlated to each other but on the basis of average chromosome length and average chromosome volume and average 4C DNA content the variations were highly significant. The correlation coefficients showed the various chromosomal and nuclear parameters are interdependent to some extent suggesting an interspecific relationship

between structural and genetic changes of the genome architecture during evolution of speciation. The ANOVA showed significant variations in the nuclear DNA values among the species.

## DISCUSSION

The karyotypes of 15 species have analyzed, the detailed karyotype analysis was presented in Table 1. Intraspecific variability in the position and number of secondary constrictions was observed in all the species. All the species had symmetric karyotype morphology but differed in total complement size that varied from 31.48  $\mu\text{m}$  in *C. roxburghii* to 57.48  $\mu\text{m}$  in *C. tora*. There was symmetrical variation in the size of the chromosomes in different species suggesting changes in chromosome size during evolution have been homogenous along the complement. Total chromosome length in annual *Cassia* species is generally greater than for perennial species that suggests that the annual species might have derived from perennial species.

### Karyotype, genome length and nuclear DNA amount:

Karyotype studies of the 15 species of *Cassia* revealed some interesting facts. The metaphase chromosome number varied from  $2n=16$  to  $2n=28$  among the species studied with some polyploid chromosomes observed in *C. siamea* ( $2n=14, 28, 35$ ), *C. biflora* ( $2n=24, 26$ ), *C. mimosoides* ( $2n=16, 30$ ), *C. fistula* ( $2n=24, 28$ ), *C. tora* ( $2n=28, 52$ ). The number of secondary constricted A and B type chromosomes ranged from 2 to 8 among the species that is completely absent in *C. grandis* (Table 1). Numerical variation in different types of chromosomes were noticed in *Cassia* species-the dose variation in Type A, B, C and D chromosomes was the most important feature in different species. The number of Type C chromosomes was more compared to the Type D in all the species. Comparatively longer chromosomes with a lowest number of diploid chromosomes suggest a highly dynamic genome structure. The structural changes as well as the changes in the parts of heterochromatins might have played a vital role in interspecific differences (Das *et al.*, 1998, 1999).

The gradual increase of centromeric index (TF%) values from 36.09% in *C. mimosoides* to 45.96% in *C. grandis* might be due to the structural alterations in the genome (Table 1). The structural alteration in the morphology as well as variation in the secondary constricted chromosomes of different species might be due to duplication of chromosomes or translocation between the chromosomes with or without secondary

constrictions at a very early stage of evolution (Das *et al.*, 1999; Jena *et al.*, 2002). The variation in the average chromosome length from 1.12  $\mu\text{m}$  in *C. roxburghii* to 2.53  $\mu\text{m}$  in *C. mimosoides* (Table 1) suggest that *C. mimosoides* evolved not only merely by auto duplication but also by out breeding during speciation. The correlation coefficient between average chromosome length and average DNA content did not show significant relationship. This clearly suggests that the differences in length may be attributed to the differential condensation and spiralization of chromosome arm.

#### **Nuclear DNA amount in relation to genomic**

**chromosome volume and INV:** A detail analysis revealed significant variations in the total chromosome volume per chromosome ranging from 0.440  $\mu\text{m}^3$  in *C. renigera* to 1.470  $\mu\text{m}^3$  in *C. mimosoides* (Table 1). The polyploid species did not show any volume wise increase in chromosome size. The DNA content showed no significant correlation with average chromosome length but significant with average chromosome volume, since, in eukaryotic system chromosome volume is also determined not directly by DNA but by the basic and non basic proteins. The species specific compactness of DNA threads along with nucleosomes or the additional gene sequences (Das *et al.*, 1998, 1999, 1999; Jena *et al.*, 2002) with altered non-histone proteins in the chromosome played an important role for chromosomal architecture of the species. Average INV was significantly correlated with average DNA content and average chromosome volume whereas did not show any significant correlation with the average chromosome length of the species. Perhaps, the differential interaction of genomic characteristics lead to genomic DNA variation (Yamaguchi and Tsunoda, 1969; Das and Mallick, 1989).

**Diversification in DNA amount:** The estimated 4C DNA values showed significant differences at the interspecific level, such variations are in agreement with the findings of other researchers (Banerjee and Sharma, 1987; Das *et al.*, 1997). The constancy in the DNA amount at the species level in repeated experiments revealed the stable 4C DNA content in each species. The DNA amount, though differed significantly at the species level, the differences in the DNA content, however, greatly depended on the repetitive DNA amount (Flavell *et al.*, 1977). The minimum 4C DNA content (7.59 pg) in *C. absus* and the maximum (33.99 pg) was noted in *C. alata* with only Type A, C and D chromosomes, perhaps these three are the basic types of chromosomes and Type B chromosomes were derived in the evolution process.

The variability in the stable DNA content in different species might be attributed to the loss or addition of many repeats in the genomes through alteration of micro-and macro-environment during evolution of species (Price *et al.*, 1980). The variability in the DNA amount has often been attributed to loss or addition of highly repetitive DNA sequences in a genome which reached a certain level and got stabilized during micro-evolution and gradual selection.

#### **ACKNOWLEDGMENT**

Thankful to the Department of Science and Technology, Government of India (SR/FT/L-14/2003) for providing the financial assistance for this study.

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