MOLECULAR CYTOGENETICS OF *CROTALARIA* SPP. FROM BANGLADESH

PH.D. THESIS

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APRIL 2019

Dedicated

to

my beloved Parents,

Husband, Sons

and

my respected teacher

Dr. Sheikh Shamimul Alam

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Certificate

*This is to certify that the thesis entitled "**Molecular cytogenetics of Crotalaria spp. from Bangladesh**" submitted by Ashma Ahmed Warasy has been carried out under our combined supervision in the department of Botany, University of Dhaka. This is further to certify that it is an original work and suitable for submission for the award of doctor of philosophy in Botany.*

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– The Author

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List of abbreviations

BJRI	:	Bangladesh Jute Research Institute
CTAB	:	Cetyl trimethyl ammonium bromide
PVP	:	Polyvinyl pyrrolidone
EDTA	:	Ethylene diamine tetra acetic acid
PCR	:	Polymerase Chain Reaction
RAPD	:	Random Amplified Polymorphic DNA
SSR	:	Simple Sequence Repeat
AFLP	:	Amplified Fragment Length Polymorphism
RFLP	:	Restriction Fragment Length polymorphism
ISSR	:	Inter-simple sequence repeat
Kb	:	Kilo base
bp	:	Base pair
UPGMA	:	Unweighted Pair Group Method of Arithmetic Means
UV	:	Ultra violet
TE	:	Tris-EDTA
GHG	:	Green house gas
ddH_2O	:	Double distilled water
rpm	:	Revolution per minute
TAE	:	Tris-acetate-EDTA
FISH	:	Fluorescent *in situ* hybridization
MT	:	Metric Ton
t/ha	:	Ton/hectare
m	:	Metacentric
sm	:	Sub-metacentric
ac	:	Acrocentric
CI	:	Centromeric Index
RL	:	Relative length
RTs	:	Root tips
CMA	:	Chromomycin A_3
DAPI	:	4’-6-diamidino-2-phenylindole
GC	:	Guanine-Cytocine
AT	:	Adenine-Cytocine
GT	:	Grand Total
m	:	Minute

List of abbreviations

s	:	Second
h	:	Hour
1N	:	1 Normal
MW	:	Molecular weight
g	:	Gram
mg	:	Milligram
ng	:	Nanogram
µ	:	Micron
µm	:	Micrometer
µM	:	Micro molar
µl	:	Micro liter
µg	:	Microgram
$\bar{x}$	:	Mean
SD	:	Standard deviation
α	:	Alpha
β	:	Beta
γ	:	Gama
φ	:	Phi (small letter)
θ	:	Theta
ρ	:	Rho
ϕ	:	Phi (capital letter)
λ	:	Lambda
δ	:	Delta
Ω	:	Omega
%	:	Percentage
°C	:	Degree centigrade
et al.	:	et alii (and others)
i.e.	:	id est (that is)

ABSTRACT

ABSTRACT

Ten germplasm of *Crotalaria* species *viz. C. pallida* (Acc. No. 4250, 4803, 4805, 4806 and 4807), *C. incana* (Acc. No. 4790, 4801, 4804 and 4809) and *C. juncea* (Local) available in Bangladesh were investigated cytogenetically and at the molecular level using RAPD- and SSR- markers for their authentic characterization. The interphase nuclei and prophase chromosomes of these germplasm exhibited different types of orcein-staining patterns. Persistent nucleolus was observed in Acc. No. 4806 of *C. pallida* following orcein staining. Variable numbers of somatic chromosome numbers were found in *Crotalaria* species. In *C. pallida* 2n = 16 (Acc. No. 4803, 4805 and 4807) and 2n = 18 (Acc. No. 4250 and 4806) chromosomes were observed. In contrast, *C. incana* was found to possess 2n = 16 (Acc. No. 4801), 2n = 17 (Acc. No. 4790 and 4804) and 2n = 18 (Acc. No. 4809) chromosomes while 2n = 16 somatic chromosome number was observed in *C. juncea*. Somatic chromosome number found of 2n = 17 in Acc. No. 4790 and 4804 of *C. incana* might be originated either by intra-specific hybridization between 2n = 18 and 2n = 16 germplasm or by aneuploid origin that correlates with their phenotypic features like seedless pod formation. Thus it is suggested to avoid these two germplasm for further breeding programme. In ten germplasm of *Crotalaria* the variation of chromosomal length was almost negligible. Metacentric chromosomes were found in maximum germplasm indicate their symmetric nature of karyotype. In contrast, few submetacentric chromosomes were observed in several germplasm indicate relatively asymmetric nature of their karyotype. After CMA-banding, ten *Crotalaria* germplasm generated 31 centromeric and 34 terminal bands which indicated a tendency of accumulating GC-rich sequences at centromeric regions or chromosomal ends. The number of DAPI-bands was less than that of the CMA-band found in different germplasm of *Crotalaria*. Maximum terminal DAPI bands indicated a tendency of accumulating AT-rich repetitive sequences at the chromosomal ends. Few chromosomes could be used as marker of respective germplasm due to their unique DAPI-banding pattern. Fluorescent banding revealed the occurrence of genomic alteration within these germplasm. Further genomic DNA from the ten *Crotalaria* germplasm was studied using fourteen oligonucleotide primers and four microsatellite primer pairs for RAPD and SSR analysis, respectively. The fourteen RAPD primers generated 881 distinct bands with 95.57% polymorphisms indicating highly diverged nature of germplasm. In addition to polymorphism, 86 unique and 50 common RAPD bands were identified in ten *Crotalaria* germplasm. The four SSR primer pairs generated 107 distinct bands of which all were considered as polymorphic. Moreover, 19 unique SSR bands were identified among them. The dendrogram of RAPD and SSR showed that the Acc. No. 4803 of *C. pallida* and Acc. No. 4790 of *C. incana* were distinctly different from the rest and placed alone in a separate cluster that correlated with its phenotypic and cytogenetical features. Therefore, each germplasm of *Crotalaria* could be characterized authentically by cytogenetical and molecular analysis.

1. INTRODUCTION

1. INTRODUCTION

The genus *Crotalaria* belongs to legume family Fabaceae. It is a complex genus and is considered to be the third largest genus of Faboideae, with around 700 species distributed throughout the tropics and subtropics (Roxburgh 1832, Baker 1876, Munk 1962, Bisby and Polhill 1973, Polhill 1982, Lewis 1987, Nayar and Sastry 1987, Wu *et al.* 2003, Sibichen and Nampy 2007 and Ansari 2008). The genus *Crotalaria* is commonly known as rattlepods **(**Everist 1979). The common name rattlepod or rattlebox is derived from the fact that the seeds become loose in the pod as they mature, and rattle when the pod is shaken.

Initially Carolus Linnaeus described the genus *Crotalaria* with 13 species in his book "Species Plantarum" including *Crotalaria perfoliata* L., *C. sagitalis* L., *C. juncea* L., *C. triflora* L., *C. villosa* L., *C. verrucosa* L., *C. lotifolia* L., *C. lunaris* L., *C. laburnifolia* L., *C. micans* L., *C. albanand* L. *C. quinquefolia* L. (Linnaeus 1753). The number of the species within the genus was gradually increased upto around 700 as reported by several authors (Lamarck 1786, Mabberley 2008, Jianqiang *et al.* 2010 and Roux *et al.* 2013). In Bangladesh about 25 species of the genus *Crotalaria* were recorded *viz. C. acicularis, C. alata, C. albida, C. anagyroides, C. bracteata, C. calycina, C. cytisoides, C. dubia, C. ferruginea, C. incana* L., *C. juncea* L., *C. kurzii, C. laburnifolia* L., *C. mysorensis, C. nana, C. nana* Burn. Var. *patula, C. occulta, C. pallida, C. prostrate, C. quinquefolia* L., *C. retusa* L., *C. sessiliflora* L., *C. spectabilis, C. tetragona, C. verrucosa* L. (Ahmed *et al.* 2009). These plants were found in different districts of Bangladesh including Chattagram, Cox's Bazar, Bandarban, Rangamati, Khagrachari, Patuakhali, Rajshahi, Khulna, Comilla, Sylhet, Dhaka, Jessore, Dinajpur, Sherpur, Faridpur, etc (Hooker 1876, Datta and Mitra 1953, Sinclair 1955 and Khan *et al.* 1996). Among the 25 species, various germplasm belonging to three species namely *C. pallida*, *C. incana* and *C. juncea* are available in the gene bank of Bangladesh Jute Research Institute, Dhaka.

Crotalaria juncea L. is one of the most important species of *Crotalaria* genus (USDA 1999) since it is a fast growing plant and has great potential as an annually renewable, multi-purpose fibre crop. It is commonly known as Sunn hemp or Indian hemp (Kirtikar and Basu 1999). In Bangladesh, this plant is commonly known as "Shan Pat". *Crotalaria juncea* also

grows to a limited extent in a number of tropical countries (Anonymous 1933, Howard and Howard 1910, Mukherjee 1953). As one of the most widely grown green manure crops throughout the tropics, Sunn hemp is often grown in rotation with several different crop species (Kirby 1963, Kundu 1964, White and Haun 1965, Lai *et al.* 1967, Purseglove 1968, Srivastava and Pandit 1968, Barros Salgado *et al.* 1972, Mascarenhas *et al.* 1980, Rotar and Joy 1983, Sarkar and Ghoroi 2007, Sarkar and Ghoroi 2007 and Tripathi *et al.* 2013). Apart from the use as green manure crop, Sunn hemp is also used as fodder in Bangladesh as well as in India (Sarkar *et al.* 2015).

The stem of Sunn hemp has two fibres, the bast fibres and woody core fibres. The bast fibres are much longer than the core fibres and located in the outer bark. The fibre has more tensile strength and durability under exposure to humidity than that of jute (Chaudhury 1950, Mukherjee 1953, Nelson 1977). Sunn hemp fibre is of better quality than jute fibre since it has good lustre, higher tensile strength and better resistance to environmental exposure. The fibre has high cellulose content, low lignin and negligible ash (Tripathi *et al*. 2013). It has been identified as the suitable indigenous raw material for manufacturing of high quality tissue paper, cigarette paper, ropes, twines, net, handmade paper, tat-patties, canvas, fodder and also medicinal purposes (Chopra *et al.* 1956, Chawla *et al.* 1967, Ghosh *et al.* 1977, Ghani 2003, Bhatt *et al.* 2009 and Oruganti *et al.* 2014). It is used as blood purifier, abortificient, astringent, demulcent, emetic, purgative and in the treatment of anaemia, impetigo, menorrhagia and psoriasis (Bhatt *et al*. 2009). Hay of *C. juncea* is generally fed to livestock (Duke 1981).

Being a leguminous crop *Crotalaria* species also enriches the soil by fixing atmospheric nitrogen to the extent of 100 kg/ha (Pradhan *et al.* 1999 and Samba *et al.* 2002). It has high photosynthetic rate enabling it to trap atmospheric carbon dioxide thereby reducing green house gas (GHG) effect. Apart from these, it is also valued for its weed-suppressing (Rupper 1987), anthelminthic and soil erosion resistive-properties (Rotar and Joy 1983). On account of its ability to produce exceedingly high biomass coupled with little amount of lignin, it is also considered as a potential biofuel crop (Kumar and Dwivedi 2014). The sticks of Sunn hemp are used for staking beetle vines in many part of Bangladesh and also in India especially in Bundelkhand, Eastern Uttar Pradesh and West Bengal (Chaudhary *et al.* 2015

and 2016). Therefore, Sunn hemp is considered as a much valued crop for its economic and ecological importance. However, the crop has received little research attention. Consequently, the genetic improvement of this crop has been reported to be limited.

Crotalaria pallida and *C. incana* are another two important species of *Crotalaria* genus. They also have some economic importance. The plant of *C. pallida* is grown as a ground cover and a green manure crop, especially in the inter-rows of rubber trees, tea and coconut palms. It has given promising results in coconut plantations in Tamil Nadu (Ahmed *et al.* 2009). It yields a fibre similar to Sunn hemp (Ahmed *et al.* 2009). Flowers are eaten as a vegetable in Cambodia, where the seeds are roasted and grounded for use as a sort of coffee beverage. They were also used for making fermented preparations. The roots are sometimes chewed with betel nuts in Vietnam. In traditional medicine, the plant is used to treat urinary problems and fever, a poultice of the roots is applied to swelling of joints and an extract of the leaves is taken to expel intestinal worms (Chong *et al.* 2009). The seeds and leaves of *C. pallida* and *C. incana* contain alkaloids, integerrimine, nilgirine, usaramine and crotastriatine (Ahmed *et al.* 2009). Flowers and unripe fruits of *C. incana* are used as abortifacient in Paraguay, where the pods are seen as a magic cure for treating mute and stuttering children. *Crotalaria* species can be used as forage for horses and cattle owing to the large amounts of water soluble gums and proteins in their seeds (Purseglove 1981, Pandey and Srinivatsva 1990). They also grown for ornamental purpose (Ahmed *et al.* 2009).

Therefore, species of *Crotalaria* are very important for various purposes. It is regarded as a possible industrial and cash crop of Bangladesh. Bangladesh needs to develop special fibre yielding crops to increase the production of fibre to satisfy the ever increasing demands in these sectors of industry. Under these circumstances only improved germplasm would be required to increase the production as well as to create interest to the farmers in cultivating this crop. Different species of *Crotalaria* would be an alternative to jute developed through proper breeding programme. For such a programme, the genomic information is very necessary. Unfortunately very limited information is available about *Crotalaria* species since it is a virgin research field in Bangladesh. A good number *Crotalaria* species are available in the gene bank of BJRI (Bangladesh Jute Research Institution). These germplasm were collected randomly from different region and stored without proper identification and

characterization. These were only characterized on the basis of their morphological features. This type of characterization sometimes creates problem since phenotypic features are not always reliable. For this purpose, an authentic characterization of each germplasm is needed to choose the most useful parents for breeding programme and also for germplasm conservation. It is believed that these germplasm would be a wealth for the breeding programme if authentic characterization and genomic information be available.

Successful breeding program depends on the complete knowledge and understanding of the genetic diversity within and among genetic resources of the available germplasm. This will enable plant breeders to choose parental sources that generate diverse populations for selection (Esmail *et al.* 2008). The extent of genetic diversity in species is important in determining the selection to be performed and for the ability to adapt to variable environmental conditions. At the onset of a breeding program, information concerning genetic relationships can be used to improve the breeding population.

To obtain genetic information, different cytogenetical and molecular techniques could be applied. Several earlier scientists tried to characterize *Crotalaria* species with classical karyotype analysis (Senn 1938, Atchison 1950, Datta and Biswas 1963, Magoom *et al.* 1963, Datta and Choudhury 1966, Bir and Kumari 1977, Gupta and Gupta 1978a, Raina and Verma 1979, Roy and Mishra 1979, Mangotra and Koul 1979, Verma and Raina 1983, Verma *et al*. 1984, Palomino and Ricardo 1991). Thus several scientists reported multiple basic chromosome number such as x = 7, x = 8 for different *Crotalaria* species (Raghaven and Venkatasubban 1943, Atchison 1950 and Subramaniam and Pandey 2013). In *Crotalaria pallida*, 2n = 16 (Gupta and Gupta 1978a, Gupta and Gupta 1978b, Raina and Verma 1979, Verma and Raina1983, Li. 1988, Kumari and Bir 1990, Mangotra and Koul 1991, Olivera and Aguiar-Perecin 1999), 2n = 14 (Patil and Chennaveeraiah 1975, Verma and Raina 1991) and 2n = 8 (Verma and Raina 1978) were reported. For *C. incana*, 2n = 16 and 14 chromosomes were found (Patil and Chennaveeraiah 1975, Gupta and Gupta 1978b, Patil 1983, Palomino and Ricardo1991, Mangotra and Koul 1991, Olivera and Perecin 1999, Almada 2006, Flores 2006).

In contrast, 2n = 16, 14 and 8 chromosomes were reported in *C. juncea* (Bir and Kumari 1977, Verma and Raina 1978, Nadkarni 1982, Sarkar *et al.* 1982, Dnyansagar and Nadkarni 1983, Bairiganjan and Patnaik 1989, Kumari and Bir 1990, Verma and Raina 1991, Kar and Sen 1991, Jahan *et al.* 1994, Koul *et al.* 2000). These numbers were found in different species of *Crotalaria* representing broad morphological variation and a wide distribution in the tropics and subtropics (Palomino and Ricardo 1991). A summary of previous information regarding the chromosome count of *Crotalaria* species has been presented in Table 1.

Table 1. A chromosome count database of *Crotalaria* species.

Species	Chromosome number	References
C. pallida L.	2n = 16	Gupta and Gupta1978$_a$
		Gupta and Gupta1978$_b$
		Raina and Verma 1979
		Verma and Raina 1983
		Li 1988
		Kumari and Bir 1990
		Mangotra and Koul 1991
		Olivera and Aguiar-Perecin 1999
	2n = 14	Patil and Chennaveeraiah 1975
		Verma and Raina 1991
	2n = 8	Verma and Raina 1978
C. incana L.	2n = 16	Mangotra and Koul 1991
	2n = 14	Patil and Chennaveeraiah 1975
		Gupta and Gupta 1978
		Patil 1983
		Palomino and Vázquez 1991
		Olivera and Aguiar-Perecin 1999
		Almada 2006
		Flores 2006
C. juncea L.	2n = 16	Bir and Kumari 1977
		Sarkar *et al.* 1982
		Nadkarni1982
		Dnyansagar and Nadkarni 1983
		Bairiganjan and Patnaik 1989
		Kumari and Bir 1990
		Kar and Sen 1991
		Jahan *et al.* 1994
		Koul *et al.* 2000
	2n = 14	Verma and Raina 1991
	2n = 8	Verma and Raina 1978

Most of the earlier cytological investigations on *Crotalaria* was confined to 2n chromosome count. There was no detailed report on karyotypic analysis of different *Crotalaria* germplasm available in Bangladesh.

It is understood that karyotype is a stable character and specific for each specimen. However, conventional karyotype analysis is alone unable to express critically the differences among different germplasm of a species since the germplasm of a species possess similar 2n chromosomes numbers and even other karyotype parameters (Khatun and Alam 2010, Khatun *et al*. 2011, Sultana and Alam 2016a, Sultana *et al.* 2018). Moreover, the consideration of chromosome length, arm ratio, position and number of secondary constrictions are not always sufficient to differentiate individual chromosome. Minute deletion, inversion, tandem duplication etc. could not be possible to detect by conventional karyotype analysis. In such a case, a combination of modern cytogenetical and molecular techniques is necessary for comparative study among different germplasm of a species.

In addition, other karyomorphological parameters *viz*. staining property of interphase nuclei and prophase chromosomes should be considered to get more data about each germplasm. Tanaka (1971) classified the different types of interphase nuclei and prophase chromosomes on the basis of orcein staining property. Later different scientists tried to characterize interphase nuclei and prophase chromosomes by differential staining with orcein, CMA and DAPI (Alam and Kondo 1995, Fawzia and Alam 2011, Alam *et al.* 2011, Shahla and Alam 2011, Sultana and Alam 2016b). The outcome of these studies showed that various taxa including varieties of many plant species could be distinguished by their staining properties of interpahse nuclei and prophase chromosomes.

Staining with DNA-base specific banding with fluorochromes such as chromomycin A_3 (CMA) and 4´-6 diamidino-2-phenylindole (DAPI) is relatively recent method for karyotype study. Schweizer (1976) for the first time initiated this fluorescence technique for cytogenetical study. CMA binds with GC (Guanine-Cytosine)-rich repetitive sequences of the genome and gives characteristics yellow color bands. On the other hand, DAPI binds to AT (Adenine-Thymine)-rich repeats giving characteristic blue color (Schweizer 1976, Kondo and Hizume 1982, Alam and Kondo 1995, Jessy *et al.* 2005, Akhter and Alam 2005, Islam

and Alam 2011, Sultana *et al.* 2011, Manzum *et al.* 2014, Bonna *et al.* 2017, Dash *et al.* 2017). Thus it seems that fluorescent banding is quite satisfactory for detail and critical chromosome analysis such as identification of individual chromosome, determination of amount and site of AT- and GC-rich base pairs in chromosomes etc.

It is understood that karyomorphological analysis can provide information to characterize the available germplasm of *Crotalaria*. Moreover, molecular technique is required for characterization and to establish a relationship among them. Molecular markers have become important tools in studding genetic diversity (Bered *et al.* 2005). The utility of molecular markers are generally determined by the technology that is used to reveal DNA based polymorphism. Recent studies have shown that simple sequence repeats (SSR) and random amplification of polymorphic DNA (RAPD) could able to detect a certain degree of polymorphism in different plant species (He and Prakash 1997, Hopkins *et al.* 1999, Subrammanian *et al.* 2000, Raina *et al.* 2001, He *et al.* 2003, Alam *et al.* 2012, Sultana and Alam 2016c).

DNA fingerprinting by RAPD is one of the molecular methods for characterizing germplasm. The term DNA fingerprinting/profiling describes the combined use of several single locus detection systems. This method has been using as versatile tool for investigating various genomic aspects of organism. It includes characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy etc. The advantages of RAPD analysis over other methods are its low sample DNA requirement and the high frequency of polymorphic bands detected (Williams *et al.* 1990). Among the DNA markers Polymerase Chain Reaction (PCR) based marker using arbitrary primers, such as random amplified polymorphic DNA (RAPD) profiles offer a rapid and reliable identification and characterization of genotypes variation or genetic relatedness found in a population (Williams *et al.* 1990). The standardized RAPD technique can be successfully used for analyzing genetic diversity (Samarajeewa *et al.* 2002). It offers a simple efficient and economic means for cultivar identification and diversity analysis. Even duplicate accessions can be distinguished by using molecular markers (Virk *et al.* 1995). The RAPD technique generates molecular markers for comparative analysis that are quick, easy to use, free from environmental influences, unlimited in number and random but wide

coverage of the genome and have a relatively high level of polymorphism (Newbury and Ford-Lloyd 1993). Thus, molecular markers are very useful for studies regarding the phylogenetic relationship and characterization.

Simple sequence repeats (SSRs) are a class of molecular microsatellites markers based on tandem repeats of short (2-6 bp) DNA sequences (Litt and Lutty 1989). These repeat sequences are often highly polymorphic, even among closely related cultivars, due to slippage mutations during DNA replication causing variation in the number of repeating units. Different alleles of a given locus can readily be detected using primers designed from the conserved DNA sequences flanking the SSR and the polymerase chain reaction (PCR). SSR markers are generally reported to detect higher levels of polymorphism than RFLP, RAPD and AFLP (Powell *et al*. 1996b, Milbourne *et al.* 1997, Russel *et al.* 1997) and have been widely adopted for genetic analysis in plants (Panaud *et al.* 1996, Powell *et al.* 1996a). Thus it is believed that SSR markers will provide the molecular genetic differentiation to facilitate routine diversity analysis and molecular breeding applications.

RAPD and SSR molecular markers have been used for revealing genetic diversity among some germplasm and genotypes in *Crotalaria* (Wang *et al*. 2006). Moreover, genetic analysis using DNA markers have been used to reveal genetic bases of both qualitative and quantitative traits in different fibre yielding crop plants (Saha *et al.* 1998). These DNA markers have advantage over morphological traits as they are polymorph with no pleiotropic or epistatic effects and are not affected by environmental conditions thereby used to exploit the available *Crotalaria* gene pools and enhance germplasm resources (Cantrell *et al.* 1999).

Although some conventional karyotype and PCR based marker analysis of *Crotalaria* species had been undertaken, these were mostly scattered and not exactly used for characterization. There was no report on fluorescent banding for comparative karyotype analysis of *Crotalaria* germplasm. No attempt has been taken earlier to combine cytogenetical and molecular data for characterizing *Crotalaria* germplasm. Moreover, no cytogenetical and molecular analysis have yet been initiated for the *Crotalaria* germplasm available in Bangladesh. As a result, the germplasm obtained in Bangladesh have not yet been characterized. Such genomic characterization is necessary for developing successful breeding programme.

Information on genetic diversity would provide advanced information for understanding the genetic diversity of different *Crotalaria* germplasm for appropriate management of this species in breeding programme. Extent of polymorphism among *Crotalaria* germplasm can be determined using the banding pattern obtained through PCR with RAPD and SSR primers. Cluster analysis of these germplasm can be done and genetic distance can be estimated.

Considering the importance of studying the genetic variability of the germplasm in the present study, a combination of conventional cytogenetical method, fluorescent banding and molecular markers like RAPD and SSR will be used for an authentic characterization of *Crotalaria* germplasm. It may be pointed out here that this type of investigation is new and a pioneer research work in Bangladesh.

Therefore, a combination of cytogenetical and molecular analysis will be carried out for the first time to characterize ten *Crotalaria* germplasm with the following objectives:

i. Comparing the staining property of the interphase nuclei and prophase chromosomes following staining with orcein, CMA and DAPI.
ii. Preparation of conventional orcein-stained karyotype of each germplasm.
iii. Comparative analysis of the fluorescent banding pattern following staining with CMA- and DAPI- fluorochromes.
iv. Characterization of *Crotalaria* germplasm using the molecular technique of RAPD and SSR.
v. Determine the extent of polymorphism in *Crotalaria* germplasm.
vi. Establishment of the phylogenetic relationship among ten *Crotalaria* germplasm on the basis of cytogenetical and molecular data.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Plant materials

Ten germplasm belonging to three species of *Crotalaria* were investigated during this study. The seeds of various accessions of *Crotalaria* species were collected from Bangladesh Jute Research Institute (BJRI), Dhaka. These ten germplasm were maintained in the Botanical Garden, Department of Botany, University of Dhaka. A brief description of the ten germplasm of *Crotalaria* species has been presented in Table 2 and morphological features of these three species of *Crotalaria* are presented in Table 3. Photographs of the developing plants of *Crotalaria pallida* have been presented in Figures 1-5, *Crotalaria incana* in Figures 6-9 and *Crotalaria juncea* in Figure 10. Photographs of flowers, mature pods and seeds of three species have been shown in Figures 11-20, 21-30 and 31-40, respectively.

Table 2. Ten germplasm belonging to three species of *Crotalaria*.

Species	Acc. No./ Identity (Provided by BJRI)	Original Number	Country of origin
C. pallida L.	4250	NY/0231C	Tanzania
	4803	NY/105CR	Kenya
	4805	NY/143CR	Kenya
	4806	NY/160CR	Kenya
	4807	NY/209CR	Kenya
C. incana L.	4790	NY/045CR	Kenya
	4801	NY/102CR	Kenya
	4804	NY/126CR	Kenya
	4809	NY/261CR	Kenya
C. juncea L.	Local	-	BJRI

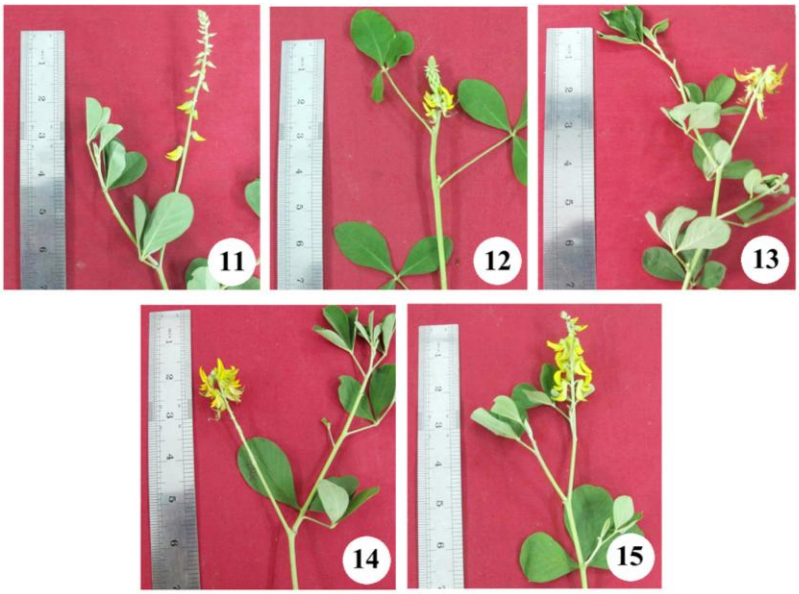
Table 3. Morphological features of three species of *Crotalaria*.

Features \ Species		*C. pallida*	*C. incana*	*C. juncea*
1.	Stem	An erect shrub, annual or short-lived perennial herb, about 1.5 m tall.	An erect, bushy annual or perennial herb, up to 1.8 m tall.	An erect, annual herb growing to a height of 2 m.
2.	Petiole	2-8.5 cm long	2.5-5 cm long	4-10 cm long
3.	Leaf	Leaves trifoliate, upper surface glabrous, lower surface appressed puberulous .	Leaves trifoliate, upper surface glabrous, lower surface thinly hairy.	Leaves trifoliate, hairy, both sides covered with reddish-brown silky hairs.
4.	Flower	Yellow, with reddish-brown veins.	Pale yellow with purple-brown veining.	Bright yellow, papillonaceous.
5.	Seeds	Seeds heart-shaped, greenish brown to blackish brown in color.	Seeds heart shaped or cordate, brown in color.	Seeds reniform, dark brown to black in color.
6.	Pod color	Brown	Black	Brown
7.	Pod size	2-4.5 x 1.8-2.0 cm	4-4.5 cm x 2.2-2.6 cm	1.8-2.0 cm x 2.3-2.7 cm
8.	Seeds per pod	15-35	12-18	2-5
9.	Seed size	2.5-3.0 mm long	3.5-4.0 mm long	6.0-7.0 mm long

Figs 1-5. Photographs showing developing plants of five germplasm of *Crotalaria pallida*. 1. Acc. No. 4250, 2. Acc. No. 4803, 3. Acc. No. 4805, 4. Acc. No. 4806, 5. Acc. No. 4807.

Figs 6-9. Photographs showing developing plants of four germplasm of *Crotalaria incana*. 6. Acc. No. 4790, 7. Acc. No. 4801, 8. Acc. No. 4804, 9. Acc. No. 4809.

Fig. 10. Photographs showing developing plants of *Crotalaria juncea*.



Figs 11-15. Photographs showing flowers of five germplasm of *Crotalaria pallida*. 11. Acc. No. 4250, 12. Acc. No. 4803, 13. Acc. No. 4805, 14. Acc. No. 4806, 15. Acc. No. 4807.

Figs 16-19. Photographs showing flowers of four germplasm of *Crotalaria incana*. 16. Acc. No. 4790, 17. Acc. No. 4801, 18. Acc. No. 4804, 19. Acc. No. 4809.

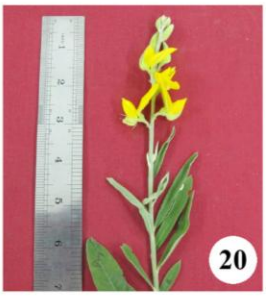
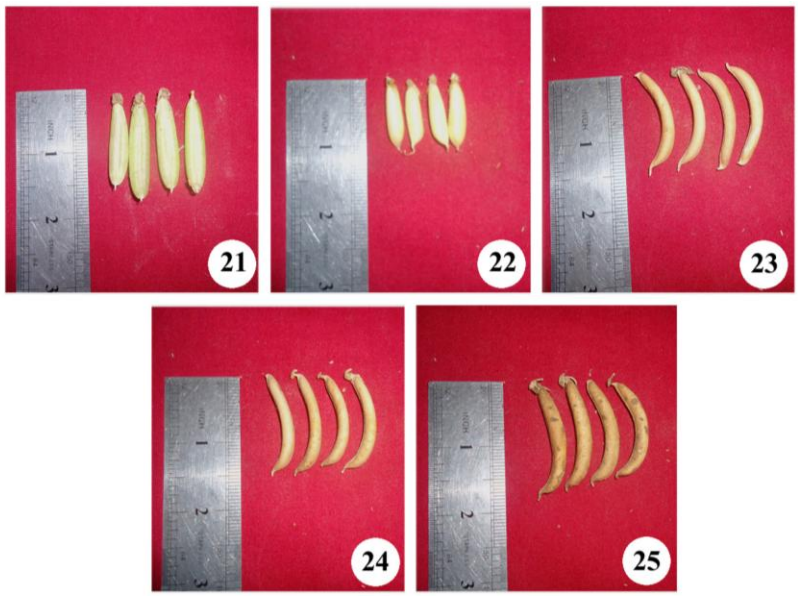


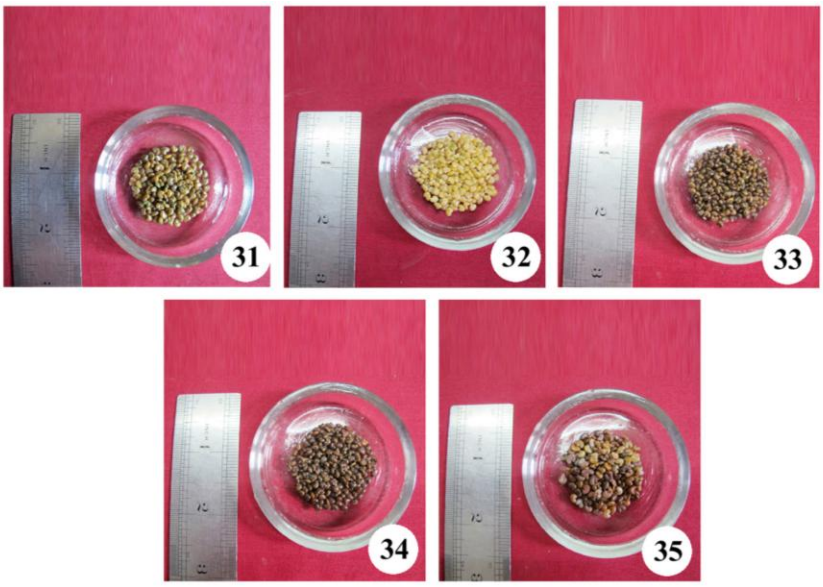
Fig. 20. Photographs showing flowers of *Crotalaria juncea*.



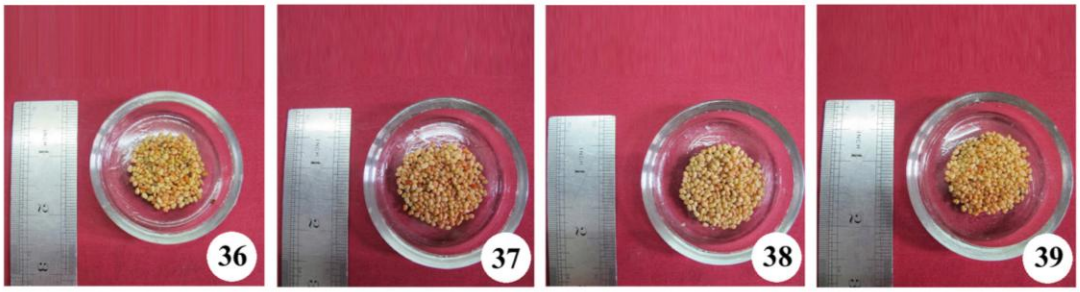
Figs 21-25. Mature pods of five germplasm of *Crotalaria pallida*. 21. Acc. No. 4250, 22. Acc. No. 4803, 23. Acc. No. 4805, 24. Acc. No. 4806, 25. Acc. No. 4807.

Figs 26-29. Mature pods of four germplasm of *Crotalaria incana*. 26. Acc. No. 4790 (seedless pod in inset), 27. Acc. No. 4801, 28. Acc. No. 4804 (seedless pod in inset), 29. Acc. No. 4809.

Fig. 30. Mature pods of *Crotalaria juncea*.



Figs 31-35. Seeds of five germplasm of *Crotalaria pallida*. 31. Acc. No. 4250, 32. Acc. No. 4803, 33. Acc. No. 4805, 34. Acc. No. 4806, 35. Acc. No. 4807.



Figs 36-39. Seeds of four germplasm of *Crotalaria incana*. 36. Acc. No. 4790, 37. Acc. No. 4801, 38. Acc. No. 4804, 39. Acc. No. 4809.

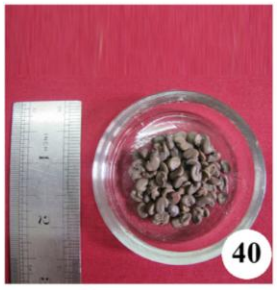


Fig. 40. Seeds of *Crotalaria juncea*.

2.2 Methods

This investigation was carried out using cytogenetical and molecular analysis of *Crotalaria* germplasm. The methods used in this study have been described under the following heads:

2.2.1 Cytogenetical studies

Various methods required for cytogenetical studies are mentioned below:

2.2.1.1 Preparation of reagents

Pre-fixative-8-Hydroxyquinoline (0.002 M)
0.29 gm of 8-Hydroxyquinoline crystal was thoroughly dissolved in a liter of distilled water by constant stirring and kept at below 20°C for future use.

Fixative (45% acetic acid)

Glacial acetic acid (45 ml) was mixed with 55 ml of distilled water.

Preservative (70% alcohol)

Absolute alcohol (70 ml) was mixed with 30 ml of distilled water.

Hydrolyzing agent (1N HCl)

HCl (36.5 ml) were thoroughly dissolved in a liter of distilled water by constant stirring and kept at room (27-28 °C) temperature for future use.

Hydrolyzing solution

Two parts of 1N HCl was mixed with one part of 45% acetic acid.

Stains

Aceto-orcein (1%)

1.0 g of orcein dye (Sigma, USA) was added to a flask containing 45 ml (100%) acetic acid. These were heated gently (not boiling) for about 20 h and messed volume up to 100

ml by adding distilled water. It was then filtered quickly and stored at room temperature (27-28 °C) for future use.

Chromomycin A_3 (CMA) (0.1 mg/ml)

1.0 mg of CMA (Sigma, USA) was thoroughly dissolved in 10 ml of McIlvaine's buffer (McIlvaine 1921) supplemented with Mg^{+2} by stirring and kept at -20 °C for future use.

4'-6 Diamidino-2-Phenylindole (DAPI) (0.01 mg/ml)

1.0 mg of DAPI (Sigma, USA) was thoroughly dissolved in 100 ml McIlvaine's buffer (without $Mg^{+2)}$ by stirring and kept at -20 °C for future use.

2.2.1.2 Preparation of Buffer

McIlvaine's buffer (pH 7.0)

About 59 (58.99) g of $Na_2HPO_4.12H_2O$ (FW 358.14) and 3.71 g citric acid were measured and mixed in one liter distilled water. It was then autoclaved for 10 m and kept at 4 °C for future use.

McIlvaine's buffer with Mg^{+2} (pH 7.0)

About 59 (58.99) g of $Na_2HPO_4.12H_2O$ (FW 358.14) and 3.71 g of citric acid were mixed in one liter of distilled water. After autoclaving, 2.46 g of $MgSO_4$ was added to it and kept at 4 °C for future use.

2.2.1.3 Preparation of Antibiotics

Distamycin A (0.1 mg/ml)

2.0 mg of distamycin A (Sigma, USA) thoroughly dissolved in 20 ml of McIlvaine's buffer by stirring and kept at -20 °C for future use.

Actinomycin D (0.25 mg/ml)

2.0 mg of Actinomycin D (Sigma, USA) thoroughly dissolved in 8 ml of McIlvaine's buffer by stirring and kept at -20 °C for future use.

2.2.1.4 Photography

An automatic digital Canon camera (8 mega pixels Canon power shoot PC1564 model), Magnification-8.9x, Mode-Auto and Canon PC1251 Magnification-7x, Mode- Portrate) was used.

2.2.1.5 Procedure for studying mitotic chromosomes

Collection of root-tips (RTs)

Roots were collected from the maintained area. The young healthy roots were cut 0.5 cm away from the tip by a clean blade.

Pre-treatment of root-tips (RTs)

The collected root tips (RTs) were soaked on a filter paper to remove surface water and pretreated with 8-hydroxyquinoline (0.002 M) for 1.15 h at room temperature (28-30 °C).

Fixation of root-tips (RTs)

RTs were fixed in 45% acetic acid for 15 m at 4 °C.

Preparation of slide for orcein-staining

The pretreated RTs were hydrolyzed for 10 s at 60 °C in a mixture of 1N HCl and 45% acetic-acid (2:1). Then the hydrolyzed RTs were soaked on a filter paper and taken on a clean slide. The meristematic region was cut with a fine blade. A drop of 1% aceto-orcein was added to the material and kept in an acetic acid chamber for 30-40 m. A clean cover glass was placed on the material. At first the materials were tapped gently by a tooth pick and then squashed by placing thumbs. During tapping and squashing care was taken so that the cover glass should not be moved because a minute displacement of it could damage the entire preparation. The slides were observed under Nikon (Eclipse 100) microscope.

2.2.1.6 Preparation of slide for fluorescent staining

Preparation of air-dried slides

After hydrolyzing and dissecting, the materials were tapped and squashed with 45% acetic-acid and kept in -80 °C freeze for 3 m. The cover glass was removed quickly and dried in air for at least 24 h before study.

2.2.1.6.1 CMA-staining

Method proposed by Alam and Kondo (1995) was followed with slight modifications. After 24 h of air drying, the slide was first pre-incubated in McIlvaine's buffer (pH 7.0) for 30 m. At once one drop of 0.1 mg/ml distamycin-A was added to the materials of slides and a cover glass placed on it. The slide was kept in humid chamber for 10 m. Then the slide was washed with distilled water in such a way that the cover glasses were removed. The slide was rinsed mildly in McIlvaine's buffer supplemented with $MgSO_4$ for 15 m. Then one drop of chromomycin A_3 (0.1 mg/ml) was added to the materials of slide and a clean cover glass was placed on it. The slide was kept in humid chamber for 12 m. The slide was washed with distilled water in such a way that the cover glasses were removed. The slides were treated again for 10 m in McIlvaine's buffer with Mg^{+2} and 10 m McIlvaine's buffer without Mg^{+2}. Slides were mounted in 50% glycerol and kept at 4 °C for overnight before observation. These were observed under Nikon (Eclipse 50i) fluorescent microscope with blue violet (BV) filter cassette.

2.2.1.6.2 DAPI-staining

For DAPI-staining method proposed by Alam and Kondo (1995) was followed after slight modifications. After 48 h of air drying, the slide was first pre-incubated in McIlvaine's buffer (pH 7.0) for 25 m. The slide was treated in 0.25 mg/ml actinomycin D for 10 m in a humid chamber. After antibiotic treatment, the slide was washed with distilled water in such a way that the cover glass removed. The slide was immersed again in McIlvaine's buffer (pH 7.0) for 15 m followed by treating in DAPI solution (0.1 mg/ml) for 12 m. After rinsing in McIlvaine's buffer (pH 7.0) for 10 m, the slide was mounted with 50% glycerol and kept at 4 °C. These were observed under a Nikon (Eclipse 50i) fluorescent microscope with ultra violet (UV) filter cassette.

2.2.1.7 Determination of centromeric type, relative length and centromeric index

Centromeric type

Procedure proposed by Levan *et al.* (1964) for determining centromeric types of chromosomes was followed with slight modification. In this study, the l/s arm ratio (l = length of the longer arm and s = length of the shorter arm) was considered in the following manner:

	Chromosome type (ct)	l/s arm ratio
(a)	Metacentric (m)	1.00- 1.50
(b)	Sub-metacentric (sm)	1.51- 3.00
(c)	Acrocentric (ac)	3.01- 7.00
(d)	Telocentric (t)	above 7.00

Relative length (RL) of chromosomes

The measurement of relative length was done by dividing the length of a particular chromosome with the total length of the diploid complements. It is represented as follows:

$$RL = \frac{\text{length of a particular chromosome}}{\text{total length of the diploid complements}}$$

Centromeric index (CI)

It was measured by the ratio of short arm to total length of that chromosome, expressed as per cent. It can be shown as below:

$$CI = \frac{\text{length of short arm}}{\text{total length of that chromosome}} \times 100$$

2.2.1.8 Classification of interphase nuclei and prophase chromosomes on the basis of Orcein-staining

The classification proposed by Tanaka (1971) was followed:

Interphase nuclei

(1) Diffuse type
(2) Simple chromocenter type
(3) Complex chromocenter type
(4) Rod pro-chromosome type
(5) Round pro-chromosome type

Prophase chromosomes

(1) Continuous type
(2) Interstitial type
(3) Gradient type
(4) Proximal type
(5) Tenuous type

2.2.1.9 Classification of fluorescent bands

According to the different fluorescent banding patterns observed in this study, bands were classified as follows:

α = Band in centromeric region.

γ = Band in whole chromosome

θ = Band in terminal region

∞ = Band in interstitial region

λ = Band in both terminal and centromeric region

ϕ = band on whole chromosome without centromere

β = Band in whole long arm

φ = Band in half of long arm

Ω = Band in whole short arm

δ = No band

2.2.1.10 Idiogram

To get an accurate measurement of lengths, chromosomes from at least three metaphase plates were measured for each germplasm. Their average arm length was used to prepare the idiogram. The chromosomes were arranged gradually from bigger to smaller in length. The short arm placed on the upper side of the axis and long arm on the lower side.

2.2.1.11 Calculation of Magnification

The photographs were taken by using 8 mega pixels canon power shoot A720 model with the magnification of 8.9× at auto mode. For measuring the magnification, at first the magnification was calculated by multiplying the magnification of objective (100×), tube length (1.25×) and camera lens (8.9×). From print the final magnification was calculated.

2.2.2 Molecular Study (RAPD and SSR marker analysis)

Methods used in RAPD and SSR marker analysis have been presented below:

2.2.2.1 Isolation of genomic DNA

To extract genomic DNA, young and actively growing fresh leaves were collected from each of the ten germplasms of *Crotalaria* spp. These leaves were cut apart with sterilized scissors and washed well initially in distilled water and then ethanol. These were dried on fresh tissue paper to remove spore of microorganisms and other sources of foreign DNA.

Preparation of different stock solutions and working solutions used for DNA isolation

For conducting the isolation procedures, the following stock solutions and working solutions were prepared:

1M stock solution of Tris-HCl pH 8.0 (100 ml)

12.14 g of Trizma base (MW=121.14) was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0 by adding about 5 ml of concentrated HCl in a fume hood. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4 $^{\circ}$C.

Stock solution of EDTA (0.5 M) pH 8.0 (100 ml)

18.61 g of EDTA (EDTA. $2H_2O$, MW = 372.24) was added to 75 ml of distilled water and stirred thoroughly with a magnetic stirrer. Approximately 2 g of NaOH pellets was added to adjust the final pH to 8.0. It may mention that EDTA alone will not dissolve, unless NaOH is added. The final volume of the solution was adjusted to 100 ml by adding sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4 °C.

Stock solution of NaCl (5 M) (100 ml)

29.22 g of sodium chloride (NaCl, MW = 58.44) was dissolved slowly (not at once) in 75 ml of distilled water. The total volume of the solution was adjusted to 100 ml with distilled water. The solution was then heated by oven for 15 s and stirred thoroughly on a magnetic stirrer to dissolve NaCl. It was then sterilized by autoclaving and stored at 4 °C.

β-Mercaptoethanol

β-Mercaptoethanol (Merck, Germany) was obtained as a 14.4 M solution from company and it was stored in a dark bottle at room temperature.

Ribonuclease-A stock solution

10 mg RNase-A (Merck, Germany) was dissolved in 1 ml of deionized distilled water and stored in -20 °C.

Tris-HCl saturated phenol

The crystal phenol was melted in a water bath at 65 °C for 30 m. Melted phenol (100 ml) was added to same volume of Tris-HCl (pH 8.0). It was mixed initially for at least 10 m with a magnetic stirrer and then kept in rest for 5 m. At this stage, two distinct phases were visible, colorless upper phase and colorful lower phase. With the help of a dropper, the upper phase was removed as much as possible. The same procedure was repeated until the pH of the lower phase rose up to 7.8. Repetition for several times was needed. In this experiment, six times repetitions were done which required about 3.5 h for obtaining the pH 7.75. After saturation, the phenol became the half of the initial volume.

Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) (100 ml)

50 ml of Phenol, 48 ml of Chloroform and 2 ml of Isoamyl alcohol were added and mixed properly by vortex mixture under a fume hood. The solution was then stored at 4 °C. The solution was shaken well before each use.

70% Ethanol (100 ml)

30 ml double distilled water (ddH_2O) was added in 70 ml absolute ethanol.

Stock Solution of TE (Tris-HCl EDTA) buffer pH 8.0 (100 ml)

1 ml of 1 M Tris-HCl was added to 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4 °C.

Sodium acetate (3 M) pH 5.2 (100 ml)

40.824 g of sodium acetate was mixed with 70 ml of ddH_2O, adjusted the final volume to 100 ml with ddH_2O and sterilized by autoclaving.

Extraction buffer (Homogenization buffer 100 ml)

To prepare extraction buffer the following components with proper concentrations were used. For the economic use of chemicals, different volumes of solutions were prepared as shown in Table 4.

Table 4. Volume of solutions prepared for extraction buffer.

Chemicals	Molecular weight	Stock con.	Working con.	Working volume	
				100 ml	1000 ml
CTAB	---	---	2%	2 g	20 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (pH 8)	372.24	0.5 M	20 mM	4 ml	40 ml
Trisbase (pH 8)	121.1	1.0 M	100 mM	10 ml	100 ml
β-Mercaptoethanol	---	14.4 M	100 mM	700 µl	7 ml

Steps of extraction buffer preparation (100 ml)

i. 10 ml of 1 M Tris HCl (pH 8.0) was taken in a 250 ml conical flask
ii. 28 ml 5 M NaCl was added to it
iii. 4 ml of 0.5 M EDTA (pH 8.0) was taken in the conical flask
iv. The mixture was then autoclaved
v. After autoclaving, 2 g CTAB was added and stirred very carefully.
vi. 700 µl β-mercaptoethanol was added prior use and mixed by glass rod under fume hood
vii. pH of all solutions were adjusted to 5 with HCl and make up to 100 ml by adding sterile de-ionized distilled water

Required equipments

1. Plant tissues (leaf tissues in this experiment)
2. Autoclave machine
3. Digital balance
4. Beaker
5. Centrifuge machine with 10,000-14,000 rpm
6. Conical flasks
7. Eppendorf tubes
8. Gloves
9. Ice machine
10. Water bath capable of maintaining 65 $^{\circ}$C
11. Micropipettes and nuclease free micropipette tips
12. Mortar and pestle
13. pH meter
14. Refrigerator
15. Water de-ionizer
16. Water distillation plant
17. Fume hood

Required Chemicals

1. Liquid nitrogen
2. 100% ethanol
3. Tris base
4. EDTA (Ethylenediaminetetraacetic acid)
5. NaCl (Sodium chloride)
6. CTAB (Cetyl Trimethyl Ammonium Bromide)
7. Sodium acetate
8. PVP (Polyvinyl pyrrolidone)
9. β-Mercaptoethanol
10. Phenol `
11. Chloroform
12. Isoamyl alchohol
13. Isopropanol
14. 70% ethanol

2.2.2.2 Protocol used for genomic DNA isolation

DNA was isolated using the mini preparation CTAB method (Doyle and Doyle 1987) with minor modification. The protocol was as follows:

2.2.2.3 Procedure of DNA isolation

1. Freshly harvested leaf tissue of 200 mg was grinded to fine powder in liquid nitrogen and taken in 2 ml centrifuge tube. 800 µL extraction buffers was added into 2 ml centrifuge tubes and vortexed for 5-10 s to mix the contents well. The tubes were put into 65 $^{\circ}$C of pre-heated water bath and invert every 5-10 m to allow mixing properly. The samples were then cooled down to room temperature.

2. The samples were centrifuged at 13,000 rpm for 10 m at room temperature to remove non soluble debris. The supernatant was transferred to fresh tubes.

3 The supernatant was mixed with equal volume of Phenol: Chloroform: Isoamyl alcohol solution (25:24:1) by inverting the tubes and centrifuged at 13,000 rpm for 10 m. This process was repeated 2-3 times.

4. The supernatant was taken into another fresh tubes and 0.1 vol. of 3 M sodium acetate (pH 5.2) was added followed by 0.6 vol. of 100% chilled Isopropanol. The solution was shaken slowly. In this step, DNA became visible as whitish fibre in the solution.

5. The solution was centrifuged for 10 m at 13,000 rpm at room temperature. The upper layer of the solution was discarded carefully by using adjustable micropipette.

6. The pellet was washed with 70% ice-cold ethanol. This washing step was repeated at least 2-3 times. Residual CTAB was removed by this step. The liquid was poured out and the tubes put on a paper towel for about 1 h at inverted position. Pellets should neither contain residual ethanol, nor allow for too dry. In both the cases, re-dissolving may be difficult.

7. The dried DNA was dissolved in 100 µl of TE buffer and treated with RNase A for 30 m at 37 $^{\circ}$C and store at -20 $^{\circ}$C.

2.2.2.4 Qualification and quantification of isolated DNA

Before PCR amplification it is important to know the concentration of genomic DNA because different DNA extraction methods produced DNA of widely different purity. It is necessary to optimize the amount of DNA for reproducibility and strong signal in PCR assay. Excessive genomic DNA may result smears lack of clearly defined bands on gel. On the other hand, too little DNA will give non-reproducible patterns (Williams *et al*. 1993). Measurement of isolated DNA concentration can be done by comparing DNA with the standard DNA on agarose gel electrophoresis or by estimating the absorbance of DNA by spectrophotometer at 260 nm wave lengths. Both the methods were carried out in this experiment.

2.2.2.4.1 Measurement of DNA concentration and quality by agarose gel electrophoresis

The following equipments and chemicals were used to conduct agarose gel electrophoresis:

1. A horizontal electrophoresis chamber and power supply
2. Gel casting tray and combs
3. Gel documentation system (BioSciTec, Gelscan 6.0 Professional, Germany)
4. Gloves
5. Pipette and tips
6. DNA ladder (1 kb)
7. Electrophoresis buffer (TAE)
8. 6X sample loading buffer
9. Agarose
10. DNA stain (ethidium bromide)

Preparation of stock solutions used for gel electrophoresis

For conducting the gel electrophoresis, the following stock solutions and other solutions were prepared:

TAE buffer (50X, pH 8.3, 1 litre)

242 g Trizma base (MW=121.14) was dissolved into 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (pH 8.0) was added to it. These were mixed well. The pH of the solution was adjusted by mixing concentrated HCl to pH 8.3. The final volume of the solution was adjusted to 1000 ml.

Loading dye (10X)

For making 10 ml of 10X loading dye, 40 mg of Bromophenol blue (i.e., the final concentration was 0.4%), 40 mg of Xylene cyanol FF (i.e., the final concentration was 0.4%) and 5 ml of 98% glycerol (i.e., the final concentration was 50%) were added to 4 ml of sterile de-ionized distilled water and mixed properly. The final volume was adjusted to 10 ml with sterile de-ionized distilled water and stored at 4 $^{\circ}$C.

Ethidium bromide solution

For preparing 1ml solution, ethidium bromide (10 mg) was added to 1 ml of sterile de-ionized distilled water. It was then mixed by hand shaking. The solution was then transferred to a dark bottle and stored at room temperature. Stock solution of 10 mg/ml can be purchased directly from companies.

2.2.2.4.2 Preparation of 1.3% Agarose Gel

For agarose gel electrophoresis, 100 ml of 1.3% agarose gel was prepared (100 ml was required for 15 ×15 cm tray with 0.5 cm thickness).

The following steps were followed during casting the gel-

1.3 g agarose powder was measured and put in a 250 ml conical flask. 100 ml of 1X TAE buffer was added to the flask. The agarose was melted in a microwave oven for several short intervals until the solution became clear. The solution did not allow boiling for long period. When the agarose solution was cooled to about 50 $^{\circ}$C (the flask was cooled enough to hold comfortably with bare hand), 8 µl (8mg/ml) of ethidium bromide was added and mixed well by gentle shaking.

2.2.2.4.3 Comb set-up

The ends of the gel casting tray was sealed or fitted with casting dams and the combs placed in the gel casting tray. The melted agarose solution was poured into the casting tray and let it to cool until solid. The comb was removed carefully from the solidified gel. The casting dams or gates were removed from the edges of the gel tray carefully so that the gel did not slide off the tray.

2.2.2.4.4 Preparation of DNA sample for electrophoresis

At first 1 µl 6X loading dye was placed on a piece of parafilm by a micropipette. 4 µl DNA sample (after thawing from frozen stock) was added to it and mixed 2-4 times with the adjustable micropipette.

2.2.2.4.5 Electrophoresis

The gel was placed in the electrophoresis chamber in such a way that the sample wells remained near to the cathode (negative end generally marked as black). DNA sample would migrate towards the anode (positive end generally marked as red) during electrophoresis.

The gel was covered by sufficient amount of 1X TAE buffer. The volume of the electrophoresis buffer should not above the maximum buffer mark on electrophoresis system. The prepared samples were loaded slowly and allowed to sink to the bottom of the wells. 4 µl of 1 kb DNA ladder (marker) was also loaded at least in one well (generally the first one). Electrophoresis was carried out at 50 volts and 100 mA for 1.5 hours. The separation process was monitored by the migration of the dye on the gel. When the first dye (bromophenol blue) had reached about three-fourths of the gel length, the electrophoresis was completed and stopped.

2.2.2.4.6 Documentation of the DNA sample

After electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed in gel documentation system (Cleaver Scientific Ltd. UK) for observing the DNA bands. The DNA was observed as band and photographed using gel documentation system. The electrophoregram of DNA samples of ten *Crotalaria* germplasms are shown in figure 191.

2.2.2.5 Quantification and qualification of DNA by spectrophotometer

For more confirmation, DNA was also quantified through spectrophotometer. Spectrophotometer is commonly used in laboratories for the measurement of DNA concentration and purity. The DNA concentration was obtained by multiplying the absorbance at 260 nm wave length by a constant. The DNA purity was measured by dividing the absorbance at 260 nm wave length with the absorbance at 280 nm wave length.

Good quality DNA should give the ratio (A260/A280) ranging from 1.8–2.0. The A260/A280 ratio higher than 2.0 and lower than 1.8 generally indicates RNA and protein contamination, respectively during extraction process. Following Sambrook *et al.* (1989),

the DNA concentration was calculated (1 O. D.) at 260 nm corresponds to 50 µl/ml of double standard DNA.

2.2.2.5.1 Set-up the spectrophotometer

To estimate genomic DNA concentration, absorbance readings of the DNA samples were recorded at 260 nm using a spectrophotometer (Analylikjena, Specord 50, Germany).

At first, the spectrophotometer UV-lamp was turned on. After warming up the wave length was set at 260 nm. After washing, the cuvette (the 'zero' or 'blank' cuvette) was filled with 2 ml sterile distilled water and placed on cuvette chamber. The absorbance reading was adjusted to zero for standardization.

2.2.2.5.2 Preparation of the DNA samples for spectrophotometry

The test samples were prepared by taking 2 µl of each DNA sample in the cuvette containing 1,998 µl sterile distilled water. The samples were mixed well by using an adjustable micropipette. The reading was taken by viewing the monitor of spectrophotometer at 260 nm and 280 nm wave length. After recording the absorbance readings, the cuvette was rinsed out with sterile distilled water, tamped out on a paper and wiped. The absorbance reading for each sample was recorded in the same way. The ratios of spectrophotometric absorption readings at 260 nm of different samples for quality determination of DNA are shown in the Table 5.

2.2.2.5.3 Calculation for the concentration of DNA

Before PCR, DNA concentrations were determined according to the following formula:

$$\text{DNA concentration} = \text{Dilution factor} \times \text{Conversion factor}$$

$$= A260 \times \frac{\text{Volume of distilled water (µl)}}{\text{Amount of DNA sample (µl)}} \times 50$$

$$= (\text{ng/µl})$$

$$= (\text{µg/ml}) [\text{since } 1\ \mu g = 10^{-3} ng \text{ i.e., } \mu g/ml = ng/\mu l]$$

A260 = spectrophotometric absorbance reading at 260 nm of DNA sample.
Dilution factor = the ratio of distilled water (µl) to amount of DNA sample (µl).

Conversion factor 50 = the 50 µg/ml of DNA contained in a solution which gives the spectrophotometric absorbance reading at 260 nm equal to 1.

Note: The calculated DNA concentration expressed in µg/ml gave a fraction. To avoid fraction it was converted into ng/µl (1µg = 10^{-3} ng) and therefore, multiplied with 1000.

Table 5. Spectrophotometric readings at 260 nm wave length and estimated concentration of DNA of ten germplasm of *Crotalaria* species.

Materials	Germplasm	Absorbance reading at 260 nm	Concentration of DNA (ng/µl)
C. pallida	4250	1.87	1900
	4803	1.91	2600
	4805	1.99	1700
	4806	1.72	3100
	4807	1.87	3000
C. incana	4790	1.36	3100
	4801	1.92	2200
	4804	1.90	1600
	4809	2.03	2000
C. juncea	Local	1.25	3100

2.2.2.6 Amplification of DNA by polymerase chain reaction (PCR) using RAPD and SSR primers

To perform the amplification of target DNA, RAPD and SSR primer sequences were mixed with genomic DNA in the presence of a thermostable DNA polymerase with suitable buffer and subjected to temperature cycling conditions typical for PCR.

2.2.2.6.1 Preparation of working solution (25 ng/µl) of DNA samples for PCR

Original stock solution concentration of each DNA sample was adjusted to a unique concentration (25 ng/µl) using the following formula:

$S_1 \times V_1 = S_2 \times V_2$
$V_1 = S_2 \times V_2 / S_1$
Where,
S_1= stock DNA concentration (ng/µl)
V_1= volume require (µl)
S_2= working DNA concentration (ng/µl)
V_2= working volume of DNA solution (µl)

Original stock DNA (2 µl) was taken in a 2 ml eppendorf tube and required amount of TE buffer calculated from the above formula added to it. Calculated required volume of TE buffer for each sample was shown in Table 6.

Table 6. Preparation of working DNA solution (25 ng/µl) for PCR reaction.

Materials	Germplasm	Working Solution (25 ng/µl) for PCR	
		TE buffer/ de-ionized water (µl) required	DNA (µl) required
C. pallida	4250	74	2
	4803	102	2
	4805	66	2
	4806	122	2
	4807	118	2
C. incana	4790	122	2
	4801	118	2
	4804	62	2
	4809	78	2
C. juncea	Local	122	2

2.2.2.6.2 Primer test

Eighteen (18) decamer primers were used for RAPD amplification of which fourteen primers exhibited good quality banding patterns and sufficient variability. These fourteen primers were selected for further analysis. The details of the fourteen primers were given in Table 7.

Five SSR primers were used to amplify simple sequence repeats of genomic DNA from ten germplasms of *Crotalaria* of which four primers exhibited good quality banding patterns and sufficient variability. Primers were evaluated on the basis of intensity or resolution of bands, repeatability of markers and consistency within individual and potential to differentiate germplasms (polymorphism). The details of the four primers were given in Table 8.

Table 7. Features of fourteen arbitrary RAPD primers used in the present study.

Sl. No.	Primer code	Sequence (5′—3′)	Annealing Temp. (°C)	GC-content (%)
1	OPA-4	AAT CGG GCT G	32	60.00
2	OPA-18	AGG TGA CCG T	32	60.00
3	OPAB-5	CCC GAA GCG A	34	70.00
4	OPAB-6	GTG GCT TGG A	32	60.00
5	OPC-10	TGT CTG GGT G	32	60.00
6	OPC-13	AAG CCT CGT C	32	60.00
7	OPC-14	TGC GTG CTT G	32	60.00
8	OPC-16	CAC ACT CCA G	32	60.00
9	OPC-26	CAC GTT ATC GCA	36	50.00
10	OPC-96	ACC AAG AAA GGG	36	50.00
11	OPF-22	AAG ATC AAA GAC	32	33.33
12	OPG-3	GAG CCC TCC A	34	70.00
13	OPG-6	GTG CCT AAC C	32	60.00
14	OPG-9	CTG ACG TCA C	32	60.00

Table 8. Features of five SSR primers used in the present study.

Primer code	Sequence (5′—3′)	Annealing Temp. (°C)	GC content (%)
AL-365892	F- CCTCCACATAGCTGGTCGAT R- GGTGTGTGCGGTTTAGGACT	58.0 59.3	55 55
AW-127626	F- CATTTTGAAGGAAGGAAGAA R- ATTTGGAAGCGGAATGTGAA	54.3 55.0	35 40
AW-584539	F-TTGATGGGCAATACATGTCG R- GTTGAAGGAAGGTGGTGGTG	55.2 57.7	45 55
MtSSRNFAW-142	F- AGGATCTAGCATGTTCTCAG R- AGGCCATGGAGGAGGACTA	56.5 58.0	45 55
MtSSRNFAW-152	F- ACGGATTTCACTTCCATTCA R- AGATCATGCAAGGCTTCAAA	55.6 56.8	40 40

2.2.2.6.3 Preparation of primers

The supplied primers were diluted to 100 µM. To make 100 µM of each primer, the following mathematical deduction was followed:

We know,

n = cv (where n= number of mole)

c= molarity *i.e.* concentration in molarity

v= volume.

Generally, 100 µM concentration of primer has to prepare as main stock solution.

In this case, c=100 µM since 53.4 nM of primer was present in the vial used in the investigation, supplied from the company, n= 53.4 nM.

v= required volume of TE buffer has to add in the supplied vial to make 100 µM main stocks, using the formula-

$$v = \frac{n}{c}$$

$$v = \frac{53.4\ \text{nmol}}{100\ \mu\text{mol}}$$

$$v = \frac{53.4 \times 10^{-9}\ \text{mol}}{100 \times 10^{-6}\ \text{mol}}$$

$$v = \frac{53.4 \times 10^{-5}\ \text{mol}}{\text{mol}}$$

$$v = \frac{53.4 \times 10^{-5}\ \text{mol}}{\text{molL}^{-1}}$$

$$v = \frac{53.4 \times 10^{-5}}{\text{L}^{-1}}$$

$$v = 53.4 \times 10^{-5}\text{L}$$

$$v = 53.4 \times 10^{-5} \times 100\ \text{ml}$$

$$v = 53.4 \times 10^{-2}\ \text{ml}$$

$$v = 53.4 \times 10^{-2} \times 1000\ \mu\text{l}$$

$$v = 534\ \mu\text{l}$$

Therefore, 534 µl of TE buffer was added to the vial to make 100 µM main stocks. Using the above deduction method, all primer pairs were diluted to 100µM main stock. All primers were diluted to 10 times *i.e.* 10 µM to make working solution for use.

2.2.2.6.4 Preparation of PCR reaction mixture/ PCR Cocktail

The following components were used to prepare PCR cocktail (Tables 9 and 10). The total volume of PCR cocktail was 25 µl per sample.

Table 9. Component of PCR cocktail (12 reactions) for RAPD primers.

Sl. No.	Reagents	Amount per sample	Total
1	Sterile de-ionized distilled water	18.8 µl	225.6 µl
2	*Taq* Buffer A 10X (Tris with 15 mM $MgCl_2$)	2.5 µl	30.0 µl
3	Primer (10 µM)	1.0 µl	12.0 µl
4	dNTPs (10 mM each)	0.5 µl	6.0 µl
5	*Taq* DNA Polymerase (5U/µl)	0.2 µl	2.4 µl
6	Template DNA (25 ng/µl)	2.0 µl	---
	Total	**25.0 µl**	

Table 10. Component of PCR cocktail (12 reactions) for SSR markers.

Sl. No.	Reagents	Amount per sample	Total
1	Sterile de-ionized distilled water	17.9 µl	214.8 µl
2	*Taq* buffer A 10X (Tris with 15 mM $MgCl_2$)	2.5 µl	30.0 µl
3	Primer forward (10 µM)	1.0 µl	12.0 µl
4	Primer reverse (10 µM)	1.0 µl	12.0 µl
5	dNTPs (10 mM each)	0.5 µl	6.0 µl
6	*Taq* DNA polymerase (5U/µl)	0.2 µl	2.4 µl
7	Template DNA (25 ng/µl)	2.0 µl	---
	Total	**25.0 µl**	

During the experiment, PCR buffer, dNTPs, primers and DNA sample solution were thawed from frozen stocks, mixed well by vortexing and kept on ice. Template DNA (25 ng/µl) were pipetted (2.0 µl) first into PCR tubes (0.5ml) compatible with the

thermocycler. For each DNA sample being tested, a pre-mix was prepared in the following order- buffer, dNTPs, DNA template and sterile distilled water. *Taq* DNA polymerase enzyme was added to the pre-mix. The pre-mix was then mixed well and aliquot into the tubes containing primers. The tubes were then sealed and placed in a thermocycler and the cycling started immediately.

2.2.2.6.5 PCR amplification

PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany). The optimum amplification cycle has been presented in Table 11 and 12.

Table 11. Arbitrary primer-PCR profile.

Denaturation/Extention/Annealing		Temperature(ºC)	Time
	Initial denaturation	95	5m
35 cycles	Denaturation at	95	45s
	Annealing at	32	30s
	Extension at	72	3m
	Final extension at	72	7m

Table 12. SSR marker-PCR profile.

Denaturation/Extention/Annealing		Temperature (°C)	Time
	Initial denaturation	95	5m
35 cycles	Denaturation at	95	45s
	Annealing at	56	45s
	Extension at	72	3m
	Final extension at	72	7m

After completion of cycling programme, the reactions were held at 4 °C.

2.2.2.6.6 Electrophoresis of the amplified products (RAPD and SSR) and documentation

The amplified products were resolved by electrophoresis on 1.3% agarose gel for RAPD and 2% agarose gel for SSR. The gel was prepared using 1.0g agarose for RAPD and 2.0

g agarose for SSR with 8 µl (8 mg/ml) ethidium bromide and 100 ml 1X TAE buffer at 50 volts and 100 mA for 1.0 h. 1 kb DNA ladder was electrophoresed alongside the RAPD and SSR product as marker. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system for both the markers.

2.2.2.7 RAPD and SSR marker data analysis

The PCR products were analyzed after gel electrophoresis. The photographs were critically discussed on the basis of presence (1) or absence (0), size of bands and overall polymorphism of bands. The scores obtained using all primers in the RAPD and SSR markers analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei's (1972) gene diversity, genetic distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the germplasm using computer program "POPGENE 32" (Version 1.32).

3. RESULTS

3. RESULTS

3.1 Appropriate season for obtaining maximum number of dividing cells from the root-tips

Although few dividing cells were observed throughout the year, the maximum number of dividing cells (about ninety per cent) were found in the root tip cells (RTCs) studied during the month of May to November. The numbers of dividing cells were very poor when the temperature was either very low or very high.

3.2 Appropriate time in obtaining maximum number of dividing cells from the root tips

Root tips of *Crotalaria* species were collected regularly at 9.00, 9.30, 10.00, 10.30, 11.00 and 11.30 a.m. It was found that 10.00 a.m. to 11.00 a.m. was the optimum time for obtaining maximum number of dividing cells (about ninety per cent).

3.3 Appropriate chemical for pretreatment

Different chemicals like 8-hydroxyquinoline, colchicine and para dichloro benzene (PDB) were tried for pretreatment of dividing cells to find out proper contraction and intact morphology of chromosomes. It was found that only 8-hydroxyquinoline solution (0.002 M) for 1 h gave the best result.

3.4 Interphase nuclei

Classical cytological analysis with orcein-staining and fluorescent banding technique with CMA and DAPI were used to find out the staining property of interphase nuclei of ten *Crotalaria* germplasm. For every staining, at least 50 interphase nuclei were observed for each germplasm.

3.4.1 Orcein staining

In *C. pallida,* more or less uniformly stained nuclei was observed in Acc. No. 4250 and 4807 (Figs 41 and 45). On the other hand, three to five darkly stained large heterochromatic regions were found at the peripheral region of the interphase nuclei in Acc. No. 4805 (Fig. 43). In contrast, a number of darkly stained small heterochromatic regions were observed in the interphase nuclei of Acc. No. 4803 and 4806 (Figs 42 and

44). In addition to this a prominent nucleolus was also observed in Acc. No. 4803 and 4806 (Figs 42 and 44).

In case of *C. incana*, five to seven darkly stained large heterochromatic regions were found in Acc. No. 4790, 4801 and 4809 (Figs 46, 47 and 49). On the other hand, more or less homogeneous stained nuclei were observed in Acc. No. 4804 (Fig. 48). A prominent nucleolus was visible in Acc. No. 4790, 4801 and 4809 (Figs 46, 47 and 49).

C. juncea was found to possess scatterdly arranged numerous small heterochromatic regions around the interphase nuclei and also exhibited a prominent nucleolus (Fig. 50).

3.4.2 CMA-staining

Two bright and prominent CMA-bands were observed in the interphase nuclei of all germplasm of *C. pallida* except Acc. No. 4805. In Acc. No. 4805, several small CMA-bands were scatterdly distributed throughout the nucleus (Figs 51, 52, 53, 54 and 55).

In *C. incana*, two prominent CMA- bands were found in the interphase nuclei of Acc. No. 4804 and 4809 (Figs 58 and 59). However, in addition to two prominent bands several CMA-bands were also observed in Acc. No. 4790 (Fig. 56). On the other hand, 10-12 CMA-bands were present in the interphase nuclei of Acc. No. 4801 (Fig. 57).

In case of *C. juncea,* two CMA-positive bands were found in interphase nuclei of which one was comparatively bigger (Fig. 60).

3.4.3 DAPI-staining

Few faintly stained DAPI-bands were found in interphase nuclei of Acc. No. 4803, 4805 and 4807 of *C. pallida* (Figs 62, 63 and 65). Two bright DAPI-bands were found in Acc. No. 4250 whereas several DAPI-bands were present in Acc. No. 4806 (Figs 61 and 64). A non-staining region was observed in the interphase nuclei of Acc. No. 4250, 4805, 4806 and 4807 (Figs 61, 63, 64 and 65).

In *C. incana*, a number of scatterdly distributed DAPI-bands were observed in the four germplasm (Figs 66, 67, 68 and 69). Non staining area was also found in the nucleus of Acc. No. 4801 and 4804 (Figs 67 and 68).

In *C. juncea*, numerous small dot like DAPI-bands alone with a relatively bigger band was observed in the interphase nucleus. A non-staining region was also observed in this nucleus (Fig. 70).

3.5 Prophase chromosomes

To find out the staining property of prophase chromosomes of ten *Crotalaria* germplasm classical cytological analysis with Orcein-staining and fluorescent banding technique with CMA and DAPI were used. For every staining, at least 50 prophase chromosome were observed for each germplasm.

3.5.1 Orcein staining

In *C. pallida*, prophase chromosomes of five germplasm were stained with orcein in two different ways. Prophase chromosomes of Acc. No. 4250 and 4805 stained darkly in one end and gradually faint to the other end (Figs 71 and 73). Therefore, one end of these chromosomes was much darker than other end. Prophase chromosomes of Acc. No. 4803, 4806 and 4807 were stained uniformly along their entire length. A prominent nucleolus was observed in Acc. No. 4803, 4806 and 4807 (Figs 72, 74 and 75).

In case of *C. incana*, the prophase chromosomes stained homogeneously with orcein. A prominent nucleolus was found in four germplasm of *C. incana* (Figs 76, 77, 78 and 79).

On the other hand, in *C. juncea* most of the prophase chromosomes were stained darker in one end and gradually faint to the other end. In this case no prominent nucleolus was observed (Fig. 80).

3.5.2 CMA-staining

In prophase chromosome, two prominent and bright CMA-positive bands were found in all the materials of *C. pallida* except Acc. No. 4806. In addition, few more bright bands were observed in Acc. No. 4803, 4805 and 4807 (Figs 81, 82, 83, 84 and 85).

On the other hand, several CMA-positive bands were found in the prophase chromosome of each germplasm of *C. incana* (Figs 86, 87, 88 and 89).

In *C. juncea*, several dot like CMA-positive bands were found (Fig. 90).

3.5.3 DAPI-staining

No prominent DAPI-positive band was found in the prophase chromosome in any of the germplasm of *C. pallida* (Figs 91, 92, 93, 94 and 95).

Few DAPI-positive bands were found to be scattered among the prophase chromosome of Acc. No. 4790 and 4801 of *C. incana* (Figs 96 and 97). No prominent DAPI-band was also observed in Acc. No. 4804 and 4809 (Figs 98 and 99).

In contrast, several dot like DAPI fluoresced bands were scatterdly distributed around the nucleus of *C. juncea* (Fig. 100).

No prominent nucleolus was observed in any of the germplasm of three *Crotalaria* species.

3.6 Metaphase chromosomes

Orcein, CMA and DAPI-staining were used for metaphase chromosome observation of ten *Crotalaria* germplasm (Figs 101-130). For every staining, at least 50 metaphase stages were observed for each germplasm.

3.6.1 Orcein-stained mitotic metaphase chromosomes

For each germplasm three clear metaphase plates were considered for chromosome measurement (Figs 101-110). Total length, arm ratio, centromeric index, relative length and centromeric type of each chromosome of ten *Crotalaria* germplasm were presented in Tables 13-22.

3.6.2 CMA-stained mitotic metaphase chromosomes

The number of CMA-positive band, total length of CMA-positive band and percentage of CMA-positive banded regions was measured for each germplasm by considering three clear CMA-stained mitotic metaphase plate (Figs 111-120; Table 24).

3.6.3 DAPI-stained mitotic metaphase chromosomes

Three clear DAPI-stained mitotic metaphase plate for each germplasm were considered for counting the number of DAPI-band, total length of DAPI-positive band and percentage of DAPI-positive banded regions (Figs 121-130; Table 25).

3.7 Karyotypes and Idiograms

On the basis of overall length and centromeric position, somatic chromosomes of *C. pallida* could be assembled in 8 pairs (Acc. No. 4803, 4805 and 4807) and in 9 pairs (Acc. No. 4250 and 4806) (Figs 131-135 and 161-165; Tables 13-17). However, the somatic chromosomes of all germplasm of *C. incana* could be tallied in 8 pairs (Acc. No. 4790, 4801 and 4804) and 9 pairs (Acc. No. 4809) (Figs 136-139 and 166-169; Tables 18-21). On the other hand, *C. juncea* could be assembled in 8 pairs (Fig. 140 and 170; Table 22). From the data obtained, idiograms were prepared supplementing the karyotypes (Figs 161-170).

3.7.1 Karyotype and Idiogram prepared from orcein-stained mitotic metaphase chromosomes

Orcein stained karyptype of ten *Crotalaria* germplasm expressed their somatic chromosome number, total length of chromosome complements, arm ratio, relative length, centromeric index and karyotypic formula. Karyotypic features of each germplasm after orcein-staining has been described below:

3.7.1.1 Karyotype of *C. pallida*

Acc. No. 4250 of *C. pallida* was found to possess 2n = 18 chromosomes (Figs 101a-101c). Total length of diploid chromosome complements in this germplasm was 21.69 ± 0.62 µm. Range of relative length of individual chromosome was 0.04 to 0.08. Individual chromosome length ranged was from 0.77 ± 0.08 to 1.70 ± 0.03 µm. The centromeric formula was recorded 8m + 10sm for this germplasm (Figs 131 and 161; Tables 13 and 23).

Somatic chromosome number (2n = 16) was observed in Acc. No. 4803 (Figs 102a-102c).Total length of diploid chromosome complements in Acc. No. 4803 was 17.07 ± 1.86 µm. Relative length of individual chromosome ranged from 0.04 to 0.08 whereas individual chromosomal length ranging from 0.67 ± 0.06 to 1.45 ± 0.12 µm. The centromeric formula of this germplasm was 10m + 6sm (Figs 132 and 162; Tables 14 and 23).

2n = 16 chromosomes was found in case of Acc. No. 4805 (Figs 103a-103c). The total length of 2n chromosome complements was 15.25 ± 1.89 µm. This length was smallest among the five germplasm of *C. pallida*. The relative length of the individual chromosomes was ranging from 0.05 to 0.08. The individual chromosome length ranged from 0.71 ± 0.04 to 1.20 ± 0.21 µm. The centromeric formula of this germplasm was 16m (Figs 133 and 163; Tables 15 and 23).

In Acc. No. 4806, total chromosome number was 2n = 18 (Figs 104a-104c). Total length of diploid chromosome complements was 20.50 ± 2.51 µm. Range of relative length of individual chromosome was 0.04 to 0.08. Individual chromosome length ranged was from 0.74 ± 0.14 to 1.69 ± 0.41 µm. The centromeric formula of this germplasm was 16m + 2sm (Figs 134 and 164; Tables 16 and 23).

Acc. No. 4807 was found to possess 2n = 16 chromosomes (Figs 105a-105c). Total length of diploid chromosome complements was 24.58 ± 1.76 µm. This length was highest among the five germplasm of *C. pallida.* Range of relative length of individual chromosome was 0.04 to 0.10. Individual chromosome length ranged was from 0.91 ± 0.18 to 2.47 ± 0.30 µm. The centromeric formula of this germplasm was 14m + 2sm (Figs 135 and 165; Tables 17 and 23).

3.7.1.2 Karyotype of *C. incana*

Acc. No. 4790 of *C. incana* was found to possess 2n = 17 chromosomes (Figs 106a-106c). Total length of diploid chromosome complements was 19.89 ± 0.70 µm. This length was found to be smallest among the four accessions of *C. incana.* Range of relative length of individual chromosome was 0.04 to 0.07. Individual chromosome length ranged was from 0.74 ± 0.11 to 1.47 ± 0.04 µm. The centromeric formula of this germplasm was determined as 17m (Figs 136 and 166; Tables 18 and 23).

2n = 16 chromosomes was found in Acc. No. 4801 (Figs 107a-107c). Total length of diploid chromosome complements was 20.74 ± 1.93 µm. Range of relative length of individual chromosome was 0.04 to 0.09. Individual chromosome length ranged was from 0.82 ± 0.19 to 1.85 ± 0.12 µm. The centromeric formula of this germplasm was 16m (Figs 137 and 167; Tables 19 and 23).

In Acc. No. 4804, 2n = 17 chromosomes was observed (Figs 108a-108c). The total length of diploid chromosome complements was 22.21 ± 1.12 µm. Range of relative length of individual chromosome was 0.04 to 0.07. Individual chromosome length ranged was from 0.82 ± 0.12 to 1.63 ± 0.21 µm. The centromeric formula was determined as 15m + 2sm in this germplasm (Figs 138 and 168; Tables 20 and 23).

Somatic chromosome number was found to be 2n = 18 in Acc. No. 4809 (Figs 109a-109c). The total length of diploid chromosome complements was 23.65 ± 0.36 µm. This length was highest among the four germplasm of *C. incana.* Range of relative length of individual chromosome was 0.04 to 0.08. Individual chromosome length ranged was from 0.81 ± 0.01 to 1.71 ± 0.02 µm The centromeric formula was 4m + 14sm for this germplasm of *C. incana* (Figs 139 and 169; Tables 21 and 23).

3.7.1.3 Karyotype of *C. juncea*

In case of *C. juncea,* 2n chromosome number was 16 (Figs 110a-110c). Total length of diploid chromosome complements was 20.49 ± 0.71 µm. Range of relative length of individual chromosome was 0.04 to 0.08. Individual chromosome length was ranged from 0.78 ± 0.12 to 1.72 ± 0.07 µm. The centromeric formula of this germplasm was 16m (Figs 140 and 170; Tables 22 and 23).

No satellite or secondary constriction was found in any of the germplasm following orcein-staining.

3.7.2 Karyotype and Idiogram prepared from CMA-stained mitotic metaphase chromosomes

Ten germplasm of *Crotalaria* species showed different types of CMA-banding pattern. The distinct characters of each germplasm following CMA-staining has been described below:

3.7.2.1 Karyotype of *C. pallida*

A total of twelve CMA-positive bands were found in different chromosomes of Acc. No. 4250. Both the member of chromosome pair II, III, VI and VIII were found to possess terminal CMA-positive band on short arm. On the other hand, one member of pair V showed terminal band on short arm while its homologue member did not possess any

band. However, a member of pair IX was entirely fluoresced with CMA whereas the other member had terminal CMA-band on short arm. In addition, only centromeric band was present in a member of pair IV (Figs 141 and 171). The total length of CMA-banded region was 4.05 ± 0.05 µm which occupied about 18.26% of the total chromatin length. The CMA-positive karyotypic formula was 1α + 10θ + 1γ + 6δ in case of this germplasm (Table 24).

Highest number of CMA-positive bands (16) among five germplasm of *C. pallida* were observed in Acc. No. 4803. Both members of pair I, III, IV, V and VII were found to possess centromeric CMA-positive band. Terminal CMA-band on short arm was present in both member of pair II and VI. Chromosomes pair VIII had a centromeric band in one member and terminal band on short arm in other member (Figs 142 and 172). The total length of CMA-banded region was 3.97 ± 0.04 µm which covered about 26.08% of the total chromatin length. The CMA-banded karyotypic formula of this Acc. No. was 11α + 5θ (Table 24).

Four CMA-positive bands were found in Acc. No. 4805. Both the members of pair II showed terminal CMA-positive band on short arm. A member of pair V and VIII also had a terminal CMA-positive band on short arm while their homologue member did not possess such bands (Figs 143 and 173). The total length of CMA-banded region was 0.80 ± 0.02 µm which occupied about 5.62% of the total chromatin length. The number of CMA-band, total length and percentage of CMA-banded region is smallest in this germplasm. The CMA-positive karyotype formula of this germplasm was 4θ + 12δ (Table 24).

In Acc. No. 4806, four CMA-positive bands were found. CMA-band along with whole length was found in both the member of chromosome pair VIII. However, one member of pair IX was entirely fluoresced with CMA whereas its homologous had terminal band on short arm (Figs 144 and 174). The total length of CMA-banded region was 2.77 ± 0.04 µm which was covered about 14.87% of the total chromatin length. The CMA-positive karyotypic formula of this germplasm was 1θ + 3γ + 14δ (Table 24).

In Acc. No. 4807, seven CMA-positive bands were found. A pair of CMA-positive bands was found at the terminal region of the short arm in each member of pair I. Only a member of pair III had a terminal CMA-positive band on short arm. However, no such

band was found in its homologue. Both the members of pair IV and VI showed CMA-bands along the whole length (Figs 145 and 175). The total length of CMA-banded region was 7.21 ± 0.05 µm, which covered about 31.57% of the total chromatin length. The total length and percentage of GC-rich repeats was highest in this germplasm. The CMA-positive karyotypic formula of this germplasm was 4γ + 3θ + 9δ (Table 24).

3.7.2.2 Karyotype of *C. incana*

In Acc. No. 4790, five CMA-positive bands were found. A terminal CMA-positive band was observed on short arm of both the members of pair IV. A centromeric CMA-positive band was present in one member of pair I while no CMA-positive band was found on its homologue. Two entirely fluoresced CMA-positive bands were observed in both member of pair VII (Figs 146 and 176). The total length of CMA-banded region was 2.73 ± 0.04 µm which occupied about 14.23% of the total chromatin length. The CMA-positive karyotypic formula of this germplasm was 2θ + 1α + 2γ + 12δ (Table 24).

Total nine CMA-positive bands were found in Acc. No. 4801. Both members of pair II and IV possessed centromeric CMA-positive band. Terminal band on short arm was observed in both chromosome of pair VIII and one chromosome of pair VI. However, two entirely fluoresced chromosomes were found in pair VII (Figs 147 and 177). The total length of CMA-banded region was 3.57 ± 0.06 µm which was about 17.07% of the total chromatin length. The CMA-positive karyotypic formula of this specimen was 3θ + 4α + 2γ + 7δ (Table 24).

In Acc. No. 4804, three CMA-positive bands were found. Both the members of pair VII had terminal CMA-positive band on short arm. In contrast, a CMA-band was observed at the interstitial region of short arm in chromosome pair I while its homologue did not showed any band (Figs 148 and 178). The total length of CMA-banded region was 0.65 ± 0.03 µm which covered about 2.88% of the total chromatin length. The CMA-positive karyotypic formula of this germplasm was 2θ + 1ρ + 14δ (Table 24).

Acc. No. 4809 was found to possess three CMA-positive bands. One member of chromosome pair IX showed terminal CMA-positive band on short arm while the other member had CMA-positive band on whole short arm. In contrast, one member of chromosome pair VIII possess terminal CMA-positive band. However, no such band was

found in its homologue (Figs 149 and 179). The total length of CMA-banded region was 0.60 ± 0.05 µm which occupied about 2.53% of the total chromatin length. The CMA-positive karyotypic formula of this specimen was 2θ + 1Ω + 15δ (Table 24).

3.7.2.3 Karyotype of *C. juncea*

In case of *C. juncea*, all the chromosome showed CMA-positive band. Both the members of chromosome pair I, II, IV, V, VI, VII and VIII had centromeric CMA-positive band. On the other hand, both the members of pair III showed terminal CMA-positive band on the short arm (Figs 150 and 180). The total length of CMA-banded region was 3.22 ± 0.04 µm which was about 16.06% of the total chromatin length. The CMA-positive karyotypic formula of this germplasm was 14α + 2θ (Table 24).

No satellite or secondary constriction was observed in any germplasm of *Crotalaria* species after CMA-banding.

3.7.3 Karyotype and Idiogram prepared from DAPI-stained mitotic metaphase chromosomes

Ten germplasm of *Crotalaria* species showed different types of DAPI-banding pattern and karyotypic features. The distinct characteristics of each germplasm after DAPI-staining have been described below:

3.7.3.1 Karyotype of *C. pallida*

Five DAPI-positive bands were found in different chromosomes of Acc. No. 4250. In chromosome pair IV one member showed entirely fluoresced DAPI- positive band while other member had DAPI-positive band in the long arm. Both the members of pair VIII showed entirely fluoresced DAPI- positive band. One member of pair VII showed terminal DAPI-positive band on the short arm while it's homologue showed no DAPI-positive band (Figs 151 and 181). The total length of DAPI-banded region was 4.20 ± 0.10 µm which occupied about 18.44% of the total chromatin length. The total length and percentage of DAPI-banded region was highest in this germplasm. The DAPI-positive karyotypic formula of this germplasm was 1θ + 3γ + 1β + 13δ (Table 25).

In case of Acc. No. 4803, only three chromosomes showed DAPI-positive bands. Both the members of pair III showed DAPI-positive band at the half of long arm. In contrast, a

chromosome of pair V was entirely fluoresced with DAPI except the centromeric region while the other chromosome of this pair had no DAPI-positive band (Figs 152 and 182). The total length of DAPI-banded region was 1.35 ± 0.13 µm which covered about 8.50% of the total chromatin length. The number of DAPI-band, total length and percentage of DAPI-banded region is smallest in this germplasm. The DAPI banded karyotypic formula of this germplasm was 2φ + 1ϕ + 13δ (Table 25).

No DAPI-positive band was observed in Acc. No. 4805 and 4807 (Figs 153, 155, 183 and 185). DAPI-banded karyotypic formulae of these germplasm were 16δ (Table 25).

In Acc. No. 4806, six DAPI-positive bands were found. The number of DAPI-band was highest in this germplasm. Terminal DAPI-positive band was observed on short arms of both members of pair III and IV. On the other hand, one member of chromosome pair IX showed a terminal DAPI- positive band on short arm while entirely fluoresced DAPI-positive band was found on its homologue (Figs 154 and 184). The total length of DAPI-banded region was 1.69 ± 0.03 µm which covered about 9.0% of the total chromatin length. The DAPI-positive karyotypic formula of this germplasm was 5θ + 1γ + 12δ (Table 25).

3.7.3.2 Karyotype of *C. incana*

In Acc. No. 4790, six DAPI-positive bands were found. Both the members of pair I and IV showed terminal DAPI-positive band on the short arm. One member of pair VI showed terminal DAPI-positive band on short arm while no DAPI-positive band was found on its homologue. On the other hand, one member of pair VII was entirely fluoresced with DAPI while no DAPI-positive band was found in its homologue (Figs 156 and 186). The total length of DAPI-banded region was 2.47 ± 0.03 µm which occupied about 12.87% of the total chromatin length. The DAPI-positive karyotypic formula of this germplasm was 5θ + 1γ + 11δ (Table 25).

Four DAPI-bands were found in Acc. No. 4801. Both the members of pair I was found to possess terminal DAPI-positive bands on short arm. Terminal DAPI-positive band was observed on short arms of a member of pair IV while other member had no DAPI-positive band. On the other hand, one member of pair VIII showed DAPI-positive band on whole short arm while no DAPI- positive band was found on its homologue (Figs 157

and 187). The total length of DAPI-banded region was 2.49 ± 0.03 µm which covered about 11.73% of the total chromatin length. The DAPI-positive karyotypic formula of this germplasm was 3θ + 1Ω + 12δ (Table 25).

In Acc. No. 4804, nine DAPI-positive bands were found. Four centromeric DAPI-positive bands were observed in both the members of pairs II and VI. Entirely fluoresced DAPI-positive bands were found in a member of pair IV and both the members of pair V. Other member of pair IV had no DAPI-positive band. On the other hand, both the members of pair I showed DAPI- positive band in upper terminal region along with another band at centromeric position (Figs 158 and 188). The total length of DAPI-banded region was 5.35 ± 0.04 µm which covered about 24.49% of the total chromatin length. The number of DAPI-band, total length and percentage of DAPI-banded region is highest in this germplasm. The DAPI-positive karyotypic formula of this germplasm was 4α + 3γ + 2λ + 8δ (Table 25).

In Acc. No. 4809, two DAPI-positive bands were observed. Both the member of chromosome pair VII showed terminal DAPI-positive bands on short arm (Figs 159 and 189). The total length of DAPI-banded region was 0.58 ± 0.05 µm which occupied about 2.45% of the total chromatin length. The number of DAPI-band, total length and percentage of DAPI-banded region is smallest in this germplasm. The DAPI-positive karyotypic formula of this specimen was 2θ + 16δ (Table 25).

3.7.3.3 Karyotype of *C. juncea*

No DAPI-positive band was observed in *C. juncea* revealing absence of AT-rich repetitive sequences in its genome (Figs 160 and 190). The DAPI-positive karyotypic formula of this germplasm was 16δ (Table 25).

Table 13. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. pallida* L. (Acc. No-4250).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centrom-eric index (CI)	Centrom-eric type (CT)
I	1.12 ± 0.02	0.58 ± 0.00	1.70 ± 0.03	1.93	0.08	24.12	sm
	1.16 ± 0.01	0.46 ± 0.02	1.62 ± 0.01	2.52	0.07	28.40	sm
II	0.79 ± 0.11	0.59 ± 0.13	1.38 ± 0.02	1.34	0.06	42.75	m
	0.80 ± 0.10	0.58 ± 0.13	1.38 ± 0.02	1.38	0.06	42.03	m
III	0.91 ± 0.01	0.45 ± 0.04	1.36 ± 0.04	2.02	0.06	33.09	sm
	0.90 ± 0.03	0.44 ± 0.03	1.35 ± 0.05	2.05	0.06	32.84	sm
IV	0.85 ± 0.06	0.48 ± 0.03	1.33 ± 0.08	1.77	0.06	36.09	sm
	0.83 ± 0.06	0.47 ± 0.01	1.31 ± 0.08	1.77	0.06	36.15	sm
V	0.76 ± 0.04	0.47 ± 0.01	1.24 ± 0.04	1.62	0.06	38.21	sm
	0.75 ± 0.07	0.47 ± 0.01	1.22 ± 0.06	1.60	0.06	38.52	sm
VI	0.71 ± 0.03	0.45 ± 0.04	1.16 ± 0.01	1.58	0.05	38.79	sm
	0.71 ± 0.03	0.45 ± 0.04	1.16 ± 0.01	1.58	0.05	38.79	sm
VII	0.56 ± 0.02	0.50 ± 0.02	1.06 ± 0.02	1.12	0.05	47.17	m
	0.54 ± 0.01	0.50 ± 0.02	1.05 ± 0.01	1.08	0.05	48.08	m
VIII	0.51 ± 0.00	0.44 ± 0.03	0.95 ± 0.03	1.16	0.04	46.32	m
	0.49 ± 0.03	0.38 ± 0.03	0.88 ± 0.06	1.29	0.04	46.68	m
IX	0.41 ± 0.06	0.36 ± 0.02	0.77 ± 0.08	1.14	0.04	46.75	m
	0.41 ± 0.06	0.36 ± 0.02	0.77 ± 0.08	1.14	0.04	46.75	m
		GT=	**21.69 ± 0.62**	**µm**			

m = Metacentric chromosome
sm = Sub-metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 14. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. pallida* L. (Acc. No-4803).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centrom-eric index (CI)	Centrom-eric type (CT)
I	0.77 ± 0.10	0.68 ± 0.07	1.45 ± 0.12	1.13	0.08	46.90	m
	0.75 ± 0.10	0.67 ± 0.04	1.42 ± 0.13	1.12	0.08	47.18	m
II	0.75 ± 0.05	0.63 ± 0.09	1.38 ± 0.12	1.19	0.08	45.65	m
	0.71 ± 0.08	0.60 ± 0.12	1.31 ± 0.20	1.18	0.08	45.80	m
III	0.77 ± 0.08	0.47 ± 0.07	1.24 ± 0.15	1.64	0.07	37.90	sm
	0.76 ± 0.09	0.44 ± 0.08	1.20 ± 0.16	1.73	0.07	36.67	sm
IV	0.73 ± 0.09	0.42 ± 0.04	1.15 ± 0.12	1.74	0.07	36.52	sm
	0.68 ± 0.12	0.45 ± 0.05	1.13 ± 0.09	1.51	0.07	40.71	sm
V	0.63 ± 0.05	0.42 ± 0.15	1.05 ± 0.11	1.50	0.06	40.00	m
	0.58 ± 0.11	0.40 ± 0.18	0.98 ± 0.15	1.45	0.06	40.82	m
VI	0.62 ± 0.07	0.31 ± 0.07	0.93 ± 0.12	2.00	0.05	33.33	sm
	0.61 ± 0.08	0.31 ± 0.09	0.92 ± 0.12	1.97	0.05	33.70	sm
VII	0.45 ± 0.08	0.32 ± 0.10	0.76 ± 0.12	1.41	0.05	41.56	m
	0.45 ± 0.08	0.32 ± 0.10	0.76 ± 0.12	1.41	0.05	41.56	m
VIII	0.39 ± 0.06	0.31 ± 0.05	0.69 ± 0.09	1.26	0.04	44.29	m
	0.40 ± 0.07	0.27 ± 0.05	0.67 ± 0.06	1.48	0.04	40.30	m
GT=17.07 ± 1.86 µm							

m = Metacentric chromosome
sm = Sub-metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 15. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. pallida* L. (Acc. No-4805).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centrom-eric type (CT)
I	0.61 ± 0.12	0.59 ± 0.09	1.20 ± 0.21	1.03	0.08	49.17	m
	0.62 ± 0.09	0.57 ± 0.11	1.19 ± 0.20	1.19	0.08	47.90	m
II	0.61 ± 0.07	0.53 ± 0.12	1.14 ± 0.17	1.15	0.07	46.49	m
	0.59 ± 0.09	0.53 ± 0.12	1.12 ± 0.20	1.11	0.07	47.31	m
III	0.55 ± 0.10	0.48 ± 0.09	1.04 ± 0.18	1.15	0.07	46.15	m
	0.55 ± 0.10	0.48 ± 0.09	1.04 ± 0.18	1.15	0.07	46.15	m
IV	0.50 ± 0.07	0.48 ± 0.09	0.98 ± 0.15	1.04	0.06	48.98	m
	0.50 ± 0.07	0.48 ± 0.09	0.98 ± 0.15	1.04	0.06	48.98	m
V	0.49 ± 0.05	0.44 ± 0.06	0.93 ± 0.11	1.11	0.06	47.31	m
	0.48 ± 0.03	0.44 ± 0.06	0.92 ± 0.08	1.09	0.06	47.83	m
VI	0.44 ± 0.03	0.44 ± 0.03	0.87 ± 0.05	1.00	0.06	50.57	m
	0.43 ± 0.04	0.42 ± 0.04	0.85 ± 0.07	1.02	0.06	49.41	m
VII	0.42 ± 0.05	0.37 ± 0.05	0.79 ± 0.07	1.14	0.05	46.84	m
	0.42 ± 0.05	0.35 ± 0.03	0.78 ± 0.05	1.20	0.05	44.87	m
VIII	0.38 ± 0.02	0.33 ± 0.02	0.71 ± 0.04	1.15	0.05	46.48	m
	0.38 ± 0.02	0.33 ± 0.02	0.71 ± 0.04	1.15	0.05	46.48	m
GT=15.25 ± 1.89 µm							

m = Metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 16. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. pallida* L. (Acc. No-4806).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centrom-eric type (CT)
I	0.92 ± 0.15	0.77 ± 0.27	1.69 ± 0.41	1.19	0.08	45.56	m
	0.89 ± 0.18	0.74 ± 0.21	1.63 ± 0.38	1.20	0.08	45.40	m
II	0.80 ± 0.09	0.61 ± 0.09	1.42 ± 0.18	1.31	0.07	42.96	m
	0.77 ± 0.09	0.60 ± 0.07	1.37 ± 0.17	1.28	0.07	43.80	m
III	0.71 ± 0.02	0.59 ± 0.12	1.30 ± 0.14	1.20	0.06	45.38	m
	0.71 ± 0.01	0.55 ± 0.08	1.27 ± 0.09	1.29	0.06	43.31	m
IV	0.68 ± 0.03	0.53 ± 0.10	1.21 ± 0.08	1.28	0.06	43.80	m
	0.66 ± 0.02	0.52 ± 0.08	1.18 ± 0.07	1.27	0.06	44.07	m
V	0.58 ± 0.03	0.50 ± 0.05	1.08 ± 0.08	1.16	0.05	46.30	m
	0.58 ± 0.03	0.49 ± 0.06	1.07 ± 0.09	1.18	0.05	45.79	m
VI	0.57 ± 0.03	0.47 ± 0.06	1.04 ± 0.08	1.21	0.05	45.19	m
	0.57 ± 0.02	0.47 ± 0.06	1.04 ± 0.07	1.21	0.05	45.19	m
VII	0.53 ± 0.01	0.45 ± 0.08	0.98 ± 0.09	1.18	0.05	45.92	m
	0.52 ± 0.03	0.43 ± 0.09	0.95 ± 0.12	1.21	0.05	45.26	m
VIII	0.55 ± 0.02	0.34 ± 0.11	0.90 ± 0.10	1.62	0.04	37.78	sm
	0.54 ± 0.02	0.32 ± 0.13	0.86 ± 0.13	1.69	0.04	37.21	sm
IX	0.40 ± 0.09	0.38 ± 0.10	0.77 ± 0.17	1.05	0.04	49.35	m
	0.38 ± 0.07	0.36 ± 0.08	0.74 ± 0.14	1.06	0.04	48.65	m
		GT=	**20.50 ± 2.51 µm**				

m = Metacentric chromosome
sm = Sub-metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 17. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. pallida* L. (Acc. No-4807).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centrom-eric type (CT)
I	1.29 ± 0.13	1.18 ± 0.17	2.47 ± 0.30	1.09	0.10	47.77	m
	1.29 ± 0.13	0.95 ± 0.17	2.23 ± 0.29	1.36	0.09	42.60	m
II	1.07 ± 0.10	0.80 ± 0.17	1.87 ± 0.26	1.34	0.08	42.78	m
	1.04 ± 0.15	0.78 ± 0.18	1.83 ± 0.32	1.33	0.07	42.62	m
III	1.01 ± 0.18	0.69 ± 0.14	1.71 ± 0.31	1.46	0.07	40.35	m
	0.92 ± 0.15	0.75 ± 0.16	1.67 ± 0.31	1.23	0.07	44.91	m
IV	0.89 ± 0.17	0.70 ± 0.11	1.59 ± 0.28	1.27	0.06	44.03	m
	0.88 ± 0.18	0.69 ± 0.09	1.57 ± 0.27	1.28	0.06	43.95	m
V	0.95 ± 0.07	0.50 ± 0.12	1.45 ± 0.17	1.90	0.06	34.48	sm
	0.94 ± 0.08	0.51 ± 0.11	1.45 ± 0.17	1.84	0.06	35.17	sm
VI	0.75 ± 0.06	0.62 ± 0.12	1.37 ± 0.17	1.21	0.06	45.26	m
	0.70 ± 0.02	0.60 ± 0.12	1.31 ± 0.12	1.17	0.05	45.80	m
VII	0.63 ± 0.03	0.54 ± 0.04	1.17 ± 0.05	1.17	0.05	46.15	m
	0.56 ± 0.04	0.53 ± 0.07	1.09 ± 0.11	1.06	0.04	48.62	m
VIII	0.48 ± 0.09	0.44 ± 0.09	0.91 ± 0.17	1.09	0.04	48.35	m
	0.47 ± 0.10	0.44 ± 0.09	0.91 ± 0.18	1.07	0.04	48.35	m
GT=24.58 ± 1.76 µm							

m = Metacentric chromosome
sm = Sub-metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 18. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. incana* L. (Acc. No-4790).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centrom-eric type (CT)
I	0.86 ± 0.09	0.67 ± 0.02	1.47 ± 0.04	1.28	0.07	45.58	m
	0.82 ± 0.01	0.65 ± 0.03	1.46 ± 0.04	1.26	0.07	44.52	m
II	0.81 ± 0.11	0.62 ± 0.10	1.43 ± 0.21	1.31	0.07	43.36	m
	0.74 ± 0.02	0.66 ± 0.02	1.40 ± 0.04	1.12	0.07	47.14	m
III	0.74 ± 0.02	0.60 ± 0.05	1.33 ± 0.06	1.23	0.07	45.11	m
	0.74 ± 0.02	0.60 ± 0.05	1.33 ± 0.06	1.23	0.07	45.11	m
IV	0.72 ± 0.00	0.51 ± 0.02	1.23 ± 0.02	1.41	0.06	41.46	m
	0.69 ± 0.04	0.51 ± 0.02	1.21 ± 0.06	1.35	0.06	42.15	m
V	0.66 ± 0.02	0.52 ± 0.02	1.18 ± 0.04	1.27	0.06	44.07	m
	0.63 ± 0.05	0.54 ± 0.02	1.17 ± 0.04	1.17	0.06	46.15	m
VI	0.60 ± 0.01	0.51 ± 0.01	1.12 ± 0.00	1.18	0.06	45.54	m
	0.60 ± 0.02	0.49 ± 0.02	1.09 ± 0.05	1.22	0.05	44.95	m
VII	0.55 ± 0.02	0.46 ± 0.02	1.01 ± 0.04	1.20	0.05	45.54	m
	0.54 ± 0.00	0.44 ± 0.02	0.98 ± 0.02	1.23	0.05	44.90	m
VIII	0.48 ± 0.04	0.43 ± 0.02	0.91 ± 0.05	1.12	0.05	47.25	m
	0.43 ± 0.07	0.41 ± 0.05	0.84 ± 0.12	1.05	0.04	48.81	m
	0.37 ± 0.06	0.37 ± 0.06	0.74 ± 0.11	1.00	0.04	50.00	m
GT=19.89 ± 0.70 µm							

m = Metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 19. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. incana* L. (Acc. No-4801).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centrom-eric type (CT)
I	1.09 ± 0.06	0.76 ± 0.07	1.85 ± 0.12	1.43	0.09	41.08	m
	1.07 ± 0.07	0.77 ± 0.06	1.84 ± 0.10	1.39	0.09	41.85	m
II	0.95 ± 0.06	0.73 ± 0.08	1.68 ± 0.14	1.30	0.08	43.45	m
	0.92 ± 0.08	0.73 ± 0.08	1.65 ± 0.15	1.26	0.08	44.24	m
III	0.81 ± 0.12	0.67 ± 0.02	1.47 ± 0.13	1.21	0.07	45.58	m
	0.80 ± 0.11	0.67 ± 0.03	1.46 ± 0.14	1.19	0.07	45.89	m
IV	0.71 ± 0.12	0.60 ± 0.10	1.31 ± 0.21	1.18	0.06	45.80	m
	0.70 ± 0.11	0.60 ± 0.10	1.30 ± 0.20	1.17	0.06	46.15	m
V	0.64 ± 0.09	0.56 ± 0.08	1.19 ± 0.12	1.14	0.06	47.06	m
	0.62 ± 0.07	0.56 ± 0.08	1.18 ± 0.12	1.11	0.06	47.46	m
VI	0.58 ± 0.08	0.52 ± 0.04	1.09 ± 0.11	1.12	0.05	47.71	m
	0.56 ± 0.06	0.49 ± 0.05	1.06 ± 0.09	1.14	0.05	46.23	m
VII	0.52 ± 0.09	0.45 ± 0.06	0.97 ± 0.15	1.16	0.05	46.39	m
	0.52 ± 0.09	0.45 ± 0.06	0.97 ± 0.15	1.16	0.05	46.39	m
VIII	0.49 ± 0.11	0.41 ± 0.08	0.91 ± 0.17	1.01	0.04	45.05	m
	0.43 ± 0.12	0.40 ± 0.08	0.82 ± 0.19	1.01	0.04	48.78	m
GT=20.74 ± 1.93 µm							

m = Metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 20. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. incana* L. (Acc. No-4804).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centrom-eric type (CT)
I	0.93 ± 0.17	0.71 ± 0.05	1.63 ± 0.21	1.31	0.07	43.56	m
	0.87 ± 0.08	0.70 ± 0.06	1.57 ± 0.12	1.24	0.07	44.59	m
II	0.92 ± 0.16	0.61 ± 0.07	1.53 ± 0.14	1.51	0.07	40.40	sm
	0.91 ± 0.11	0.60 ± 0.07	1.51 ± 0.10	1.52	0.07	40.54	sm
III	0.75 ± 0.10	0.68 ± 0.02	1.42 ± 0.08	1.10	0.06	47.89	m
	0.73 ± 0.07	0.63 ± 0.02	1.36 ± 0.05	1.16	0.06	46.32	m
IV	0.71 ± 0.05	0.59 ± 0.01	1.30 ± 0.07	1.20	0.06	45.38	m
	0.67 ± 0.04	0.62 ± 0.05	1.29 ± 0.09	1.08	0.06	48.06	m
V	0.65 ± 0.10	0.57 ± 0.02	1.22 ± 0.13	1.14	0.05	46.72	m
	0.62 ± 0.04	0.49 ± 0.05	1.11 ± 0.05	1.27	0.05	44.14	m
VI	0.59 ± 0.08	0.48 ± 0.08	1.07 ± 0.09	1.23	0.05	44.86	m
	0.54 ± 0.08	0.46 ± 0.05	1.00 ± 0.07	1.17	0.05	46.00	m
VII	0.51 ± 0.05	0.42 ± 0.08	0.93 ± 0.11	1.21	0.04	45.16	m
	0.51 ± 0.05	0.42 ± 0.08	0.93 ± 0.11	1.21	0.04	45.16	m
VIII	0.44 ± 0.08	0.40 ± 0.06	0.84 ± 0.14	1.10	0.04	47.62	m
	0.43 ± 0.07	0.40 ± 0.06	0.82 ± 0.12	1.08	0.04	48.78	m
	0.43 ± 0.07	0.40 ± 0.06	0.82 ± 0.12	1.08	0.04	48.78	m
GT=22.21 ± 1.12 µm							

m = Metacentric chromosome
sm = Sub-metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 21. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. incana* L. (Acc. No-4809).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centrom-eric type (CT)
I	1.19 ± 0.01	0.52 ± 0.01	1.71 ± 0.02	2.29	0.08	30.41	sm
	1.17 ± 0.01	0.45 ± 0.01	1.62 ± 0.01	2.60	0.08	27.78	sm
II	0.92 ± 0.04	0.54 ± 0.00	1.46 ± 0.04	1.70	0.07	36.99	sm
	0.92 ± 0.03	0.54 ± 0.09	1.46 ± 0.12	1.70	0.07	36.99	sm
III	0.97 ± 0.02	0.47 ± 0.03	1.44 ± 0.04	2.60	0.07	32.64	sm
	0.90 ± 0.05	0.52 ± 0.02	1.42 ± 0.05	1.73	0.07	36.62	sm
IV	0.94 ± 0.05	0.48 ± 0.02	1.42 ± 0.06	1.96	0.06	33.80	sm
	0.85 ± 0.06	0.56 ± 0.04	1.41 ± 0.07	1.52	0.06	39.72	sm
V	0.88 ± 0.01	0.45 ± 0.01	1.33 ± 0.01	1.96	0.06	33.83	sm
	0.88 ± 0.01	0.45 ± 0.01	1.33 ± 0.02	1.96	0.06	33.83	sm
VI	0.76 ± 0.01	0.51 ± 0.00	1.27 ± 0.01	1.49	0.05	40.16	m
	0.72 ± 0.01	0.54 ± 0.00	1.26 ± 0.01	1.33	0.05	42.86	m
VII	0.72 ± 0.01	0.54 ± 0.00	1.26 ± 0.01	1.33	0.05	42.86	m
	0.72 ± 0.00	0.54 ± 0.01	1.26 ± 0.01	1.33	0.05	42.86	m
VIII	0.74 ± 0.02	0.48 ± 0.01	1.22 ± 0.03	1.54	0.05	39.34	sm
	0.72 ± 0.02	0.36 ± 0.04	1.08 ± 0.03	2.00	0.05	33.33	sm
IX	0.63 ± 0.00	0.27 ± 0.00	0.90 ± 0.00	2.33	0.04	30.00	sm
	0.58 ± 0.01	0.23 ± 0.00	0.81 ± 0.01	2.52	0.04	28.40	sm
GT=23.65 ± 0.36 µm							

m = Metacentric chromosome
sm = Sub-metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 22. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. juncea* L. (Local).

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
I	0.97 ± 0.04	0.74 ± 0.04	1.72 ± 0.07	1.31	0.08	43.02	m
	0.97 ± 0.04	0.73 ± 0.02	1.70 ± 0.05	1.33	0.08	42.94	m
II	0.84 ± 0.03	0.65 ± 0.02	1.49 ± 0.05	1.29	0.07	43.62	m
	0.82 ± 0.05	0.65 ± 0.02	1.47 ± 0.06	1.26	0.07	44.22	m
III	0.74 ± 0.04	0.64 ± 0.00	1.39 ± 0.04	1.16	0.07	46.04	m
	0.72 ± 0.05	0.64 ± 0.00	1.36 ± 0.06	1.13	0.07	47.06	m
IV	0.71 ± 0.03	0.64 ± 0.00	1.35 ± 0.03	1.11	0.07	47.41	m
	0.71 ± 0.03	0.64 ± 0.00	1.35 ± 0.03	1.11	0.07	47.41	m
V	0.72 ± 0.03	0.52 ± 0.02	1.24 ± 0.00	1.38	0.06	41.94	m
	0.71 ± 0.03	0.51 ± 0.04	1.23 ± 0.02	1.39	0.06	41.46	m
VI	0.66 ± 0.04	0.51 ± 0.04	1.18 ± 0.03	1.29	0.06	43.22	m
	0.64 ± 0.07	0.54 ± 0.05	1.18 ± 0.03	1.19	0.06	45.76	m
VII	0.57 ± 0.04	0.53 ± 0.02	1.10 ± 0.06	1.08	0.05	48.18	m
	0.54 ± 0.06	0.51 ± 0.04	1.06 ± 0.09	1.06	0.05	48.11	m
VIII	0.46 ± 0.05	0.43 ± 0.03	0.89 ± 0.07	1.07	0.04	48.31	m
	0.39 ± 0.06	0.39 ± 0.06	0.78 ± 0.12	1.00	0.04	50.00	m
GT=20.49 ± 0.71 µm							

m = Metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 23. Comparative orcein-stained karyotype analysis of ten germplasm of *Crotalaria* species.

Species	Acc. No /Identity	2n	Range of chromosomal length (µm) ($\bar{x}$ ± SD)	Total length of 2n chromosome complements (µm)	Centromeric formulae	Karyotype (Stebbins 1971)
C. pallida	4250	18	0.77 ± 0.08–1.70 ± 0.03	21.69 ± 0.62	8m + 10sm	Relatively Asymmetric
	4803	16	0.67 ± 0.06–1.45 ± 0.12	17.07 ± 1.86	10m + 6 sm	Relatively Asymmetric
	4805	16	0.71 ± 0.04 –1.20 ± 0.21	15.25 ± 1.89	16m	Symmetric
	4806	18	0.74 ± 0.14–1.69 ± 0.41	20.50 ± 2.51	16m + 2 sm	Relatively Asymmetric
	4807	16	0.91 ± 0.18–2.47 ± 0.30	24.58 ± 1.76	14m + 2sm	Relatively Asymmetric
C. incana	4790	17	0.74 ± 0.11–1.47 ± 0.04	19.89 ± 0.70	17m	Symmetric
	4801	16	0.82 ± 0.19–1.85 ± 0.12	20.74 ± 1.93	16m	Symmetric
	4804	17	0.82 ± 0.12–1.63 ± 0.21	22.21 ± 1.12	15m + 2sm	Relatively Asymmetric
	4809	18	0.81 ± 0.01–1.71 ± 0.02	23.65 ± 0.36	4m + 14sm	Relatively Asymmetric
C. juncea	Local	16	0.78 ± 0.12–1.72 ± 0.07	20.49 ± 0.71	16m	Symmetric

m = Metacentric chromosome
sm = Sub-metacentric chromosome
GT = Grand Total
$\bar{x}$ = Mean
SD = Standard Deviation

Table 24. Comparative CMA-banding analysis of ten germplasm of *Crotalaria* species.

Species	Acc. No. /Identity	No. of CMA-bands	Total length of CMA-positive banded region (µm) ($\bar{x} \pm SD$)	% of CMA-positive banded region (µm)	CMA- banded karyotypic formulae
C. pallida	4250	12	4.05 ± 0.05	18.26	1α + 10θ + 1γ + 6δ
	4803	16	3.97 ± 0.04	26.08	11α + 5θ
	4805	4	0.80 ± 0.02	5.62	4θ + 12δ
	4806	4	2.77 ± 0.04	14.87	1θ + 3γ + 14δ
	4807	7	7.21 ± 0.05	31.57	4γ + 3θ + 9δ
C. incana	4790	5	2.73 ± 0.04	14.23	2θ + 1α + 2γ + 12δ
	4801	9	3.57 ± 0.06	17.07	3θ + 4α + 2γ + 7δ
	4804	3	0.65 ± 0.03	2.88	2θ + 1 ρ + 14δ
	4809	3	0.60 ± 0.05	2.53	2θ + 1 Ω + 15δ
C. juncea	Local	16	3.22 ± 0.04	16.06	14α + 2θ

Classification of CMA-positive bands

α = Band in centromeric region
γ = Band in whole chromosome
θ = Band in terminal region
ρ = Band in interstitial region
Ω = Band in whole short arm
δ = No band

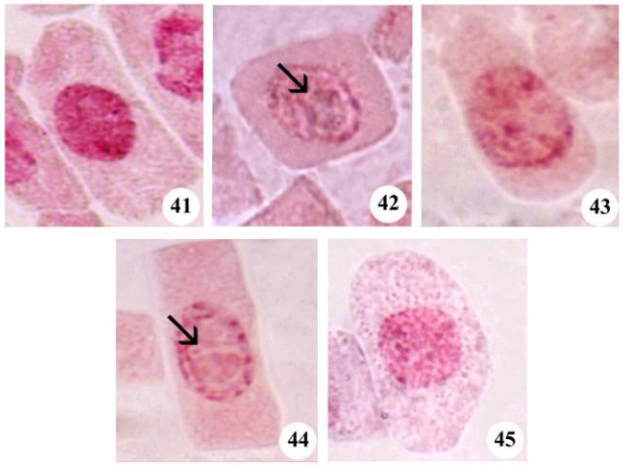
Table 25. Comparative DAPI-banding analysis of ten germplasm of *Crotalaria* species.

Species	Acc. No. /Identity	No. of DAPI-bands	Total length of DAPI-positive banded region (µm) ($\bar{x}$ ± SD)	% of DAPI - positive banded region (µm)	DAPI - banded karyotypic formulae
C. pallida	4250	5	4.20 ± 0.10	18.44	1θ+3γ+1β+13δ
	4803	3	1.35 ± 0.13	8.50	2φ+1ϕ+13δ
	4805	-	-	-	16δ
	4806	6	1.69 ± 0.03	9.0	5θ + 1γ + 12δ
	4807	-	-	-	16δ
C. incana	4790	6	2.47 ± 0.03	12.87	5θ + 1γ + 11δ
	4801	4	2.49 ± 0.03	11.73	3θ + 1 Ω + 12δ
	4804	9	5.35 ± 0.04	24.49	4α + 3γ + 2λ + 8δ
	4809	2	0.58 ± 0.05	2.45	2θ + 16δ
C. juncea	Local	-	-	-	16δ

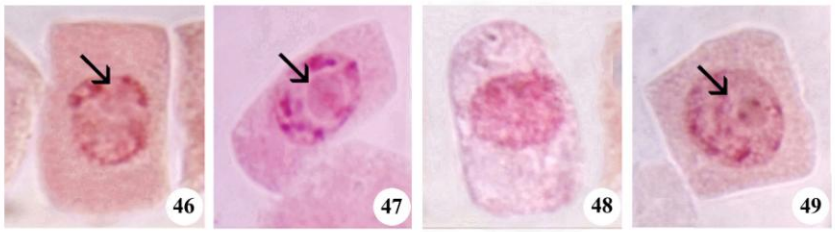
Classification of DAPI-positive bands

α = Band in centromeric region
γ = Band in whole chromosome
θ = Band in terminal region
λ = Band in both terminal and centromeric region
ϕ = band on whole chromosome without centromere
β = Band in whole long arm
φ = Band in half of long arm
Ω = Band in whole short arm
δ = No band

Results



Figs 41-45. Orcein-stained mitotic interphase nuclei of five germplasm of *Crotalaria pallida*. 41. Acc. No. 4250, 42. Acc. No. 4803, 43. Acc. No. 4805, 44. Acc. No. 4806, 45. Acc. No. 4807. Bar = 5 µm.

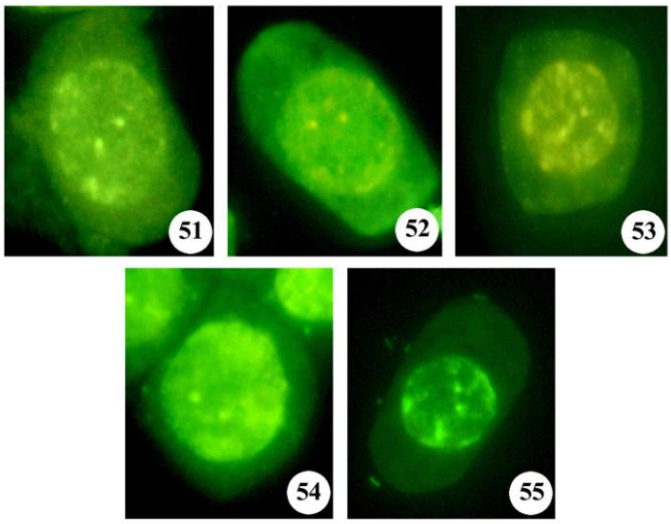


Figs 46-49. Orcein-stained mitotic interphase nuclei of four germplasm of *Crotalaria incana*. 46. Acc. No. 4790, 47. Acc. No. 4801, 48. Acc. No. 4804, 49. Acc. No. 4809. Bar = 5 µm.

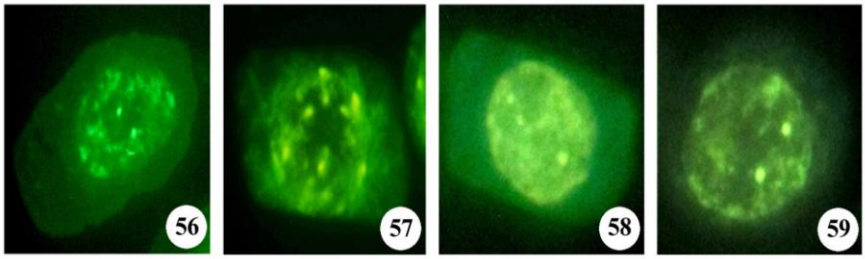
Fig. 50. Orcein-stained mitotic interphase nucleus of *Crotalaria juncea*. Bar = 5 µm.

Arrow (→) indicates nucleolus.

Results



Figs 51-55. CMA-stained mitotic interphase nuclei of five germplasm of *Crotalaria pallida*. 51. Acc. No. 4250, 52. Acc. No. 4803, 53. Acc. No. 4805, 54. Acc. No. 4806, 55. Acc. No. 4807. Bar = 5 µm.



Figs 56-59. CMA-stained mitotic interphase nuclei of four germplasm of *Crotalaria incana*. 56. Acc. No. 4790, 57. Acc. No. 4801, 58. Acc. No. 4804, 59. Acc. No. 4809. Bar = 5 µm.

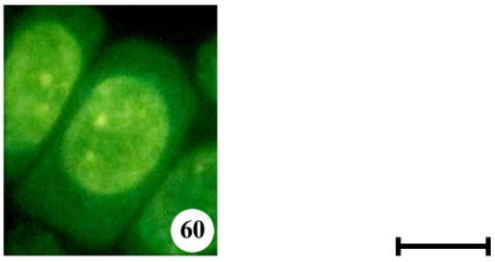
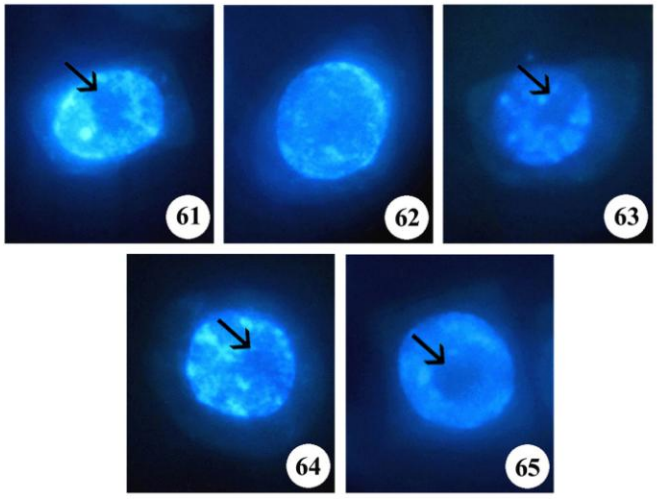
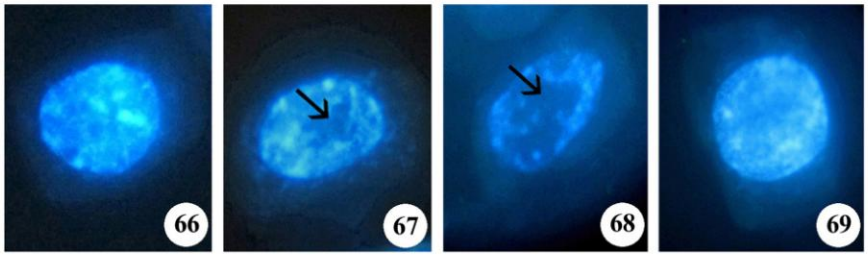


Fig. 60. CMA-stained mitotic interphase nucleus of *Crotalaria juncea*. Bar = 5 µm.

Results



Figs 61-65. DAPI-stained mitotic interphase nuclei of five germplasm of *Crotalaria pallida*. 61. Acc. No. 4250, 62. Acc. No. 4803, 63. Acc. No. 4805, 64. Acc. No. 4806, 65. Acc. No. 4807. Bar = 5 µm.



Figs 66-69. DAPI-stained mitotic interphase nuclei of four germplasm of *Crotalaria incana*. 66. Acc. No. 4790, 67. Acc. No. 4801, 68. Acc. No. 4804, 69. Acc. No. 4809. Bar = 5 µm.

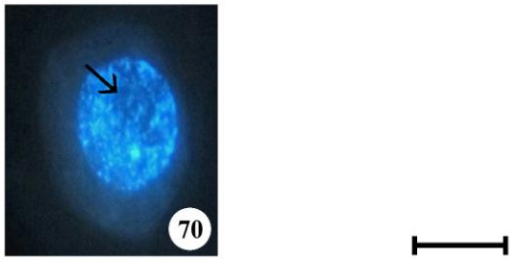
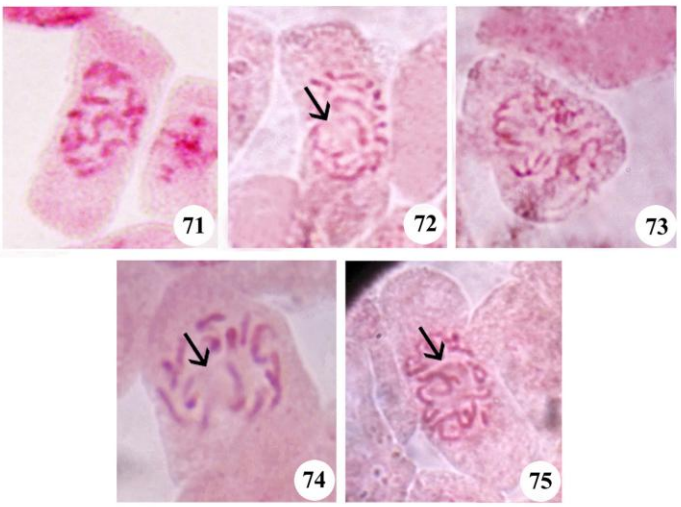
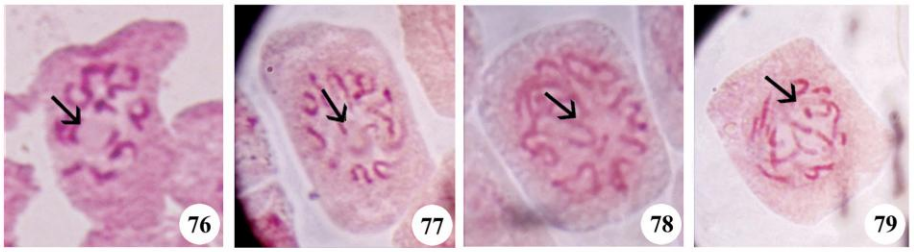


Fig. 70. DAPI-stained mitotic interphase nucleus of *Crotalaria juncea*. Bar = 5 µm. Arrow (⟶) indicates big non-staining area.

Results



Figs 71-75. Orcein-stained mitotic prophase chromosomes of five germplasm of *Crotalaria pallida*. 71. Acc. No. 4250, 72. Acc. No. 4803, 73. Acc. No. 4805, 74. Acc. No. 4806, 75. Acc. No. 4807. Bar = 5 µm.



Figs 76-79. Orcein-stained mitotic prophase chromosomes of four germplasm of *Crotalaria incana*. 76. Acc. No. 4790, 77. Acc. No. 4801, 78. Acc. No. 4804, 79. Acc. No. 4809. Bar = 5 µm.

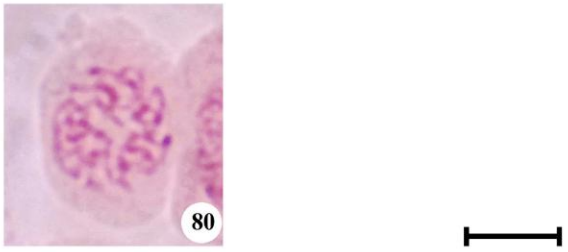
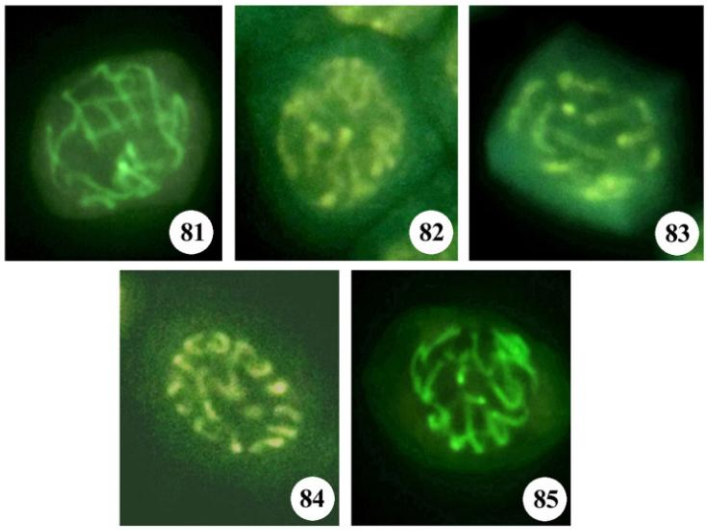


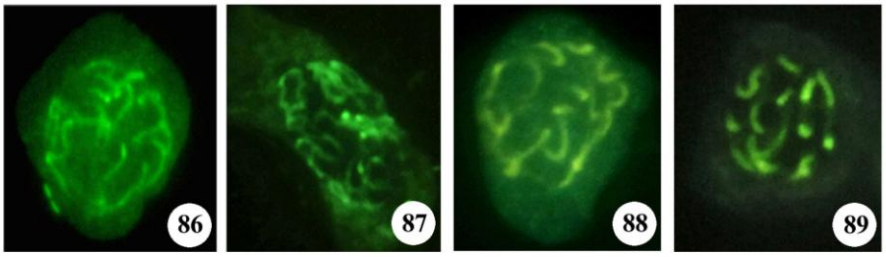
Fig. 80. Orcein-stained mitotic prophase chromosomes of *Crotalaria juncea*. Bar = 5 µm.

Arrow (⟶) indicates nucleolus.

Results



Figs 81-85. CMA-stained mitotic prophase chromosomes of five germplasm of *Crotalaria pallida*. 81. Acc. No. 4250, 82. Acc. No. 4803, 83. Acc. No. 4805, 84. Acc. No. 4806, 85. Acc. No. 4807. Bar = 5 μm.



Figs 86-89. CMA-stained mitotic prophase chromosomes of four germplasm of *Crotalaria incana.* 86. Acc. No. 4790, 87. Acc. No. 4801, 88. Acc. No. 4804, 89. Acc. No. 4809. Bar = 5 μm.

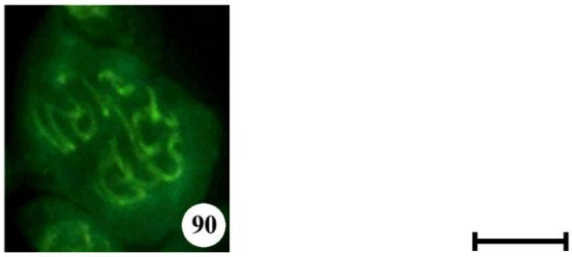
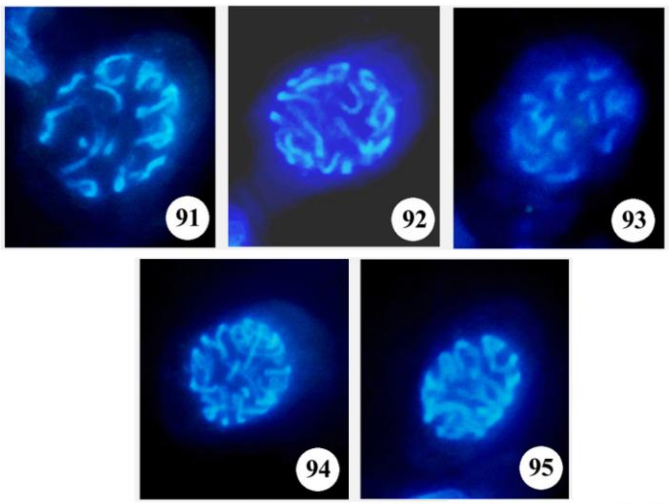
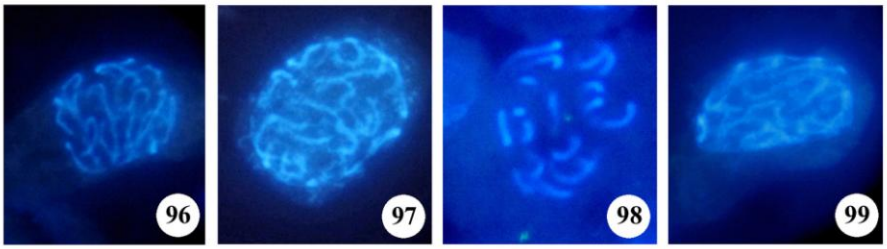


Fig. 90. CMA-stained mitotic prophase chromosomes of *Crotalaria juncea.* Bar = 5 μm.

Results



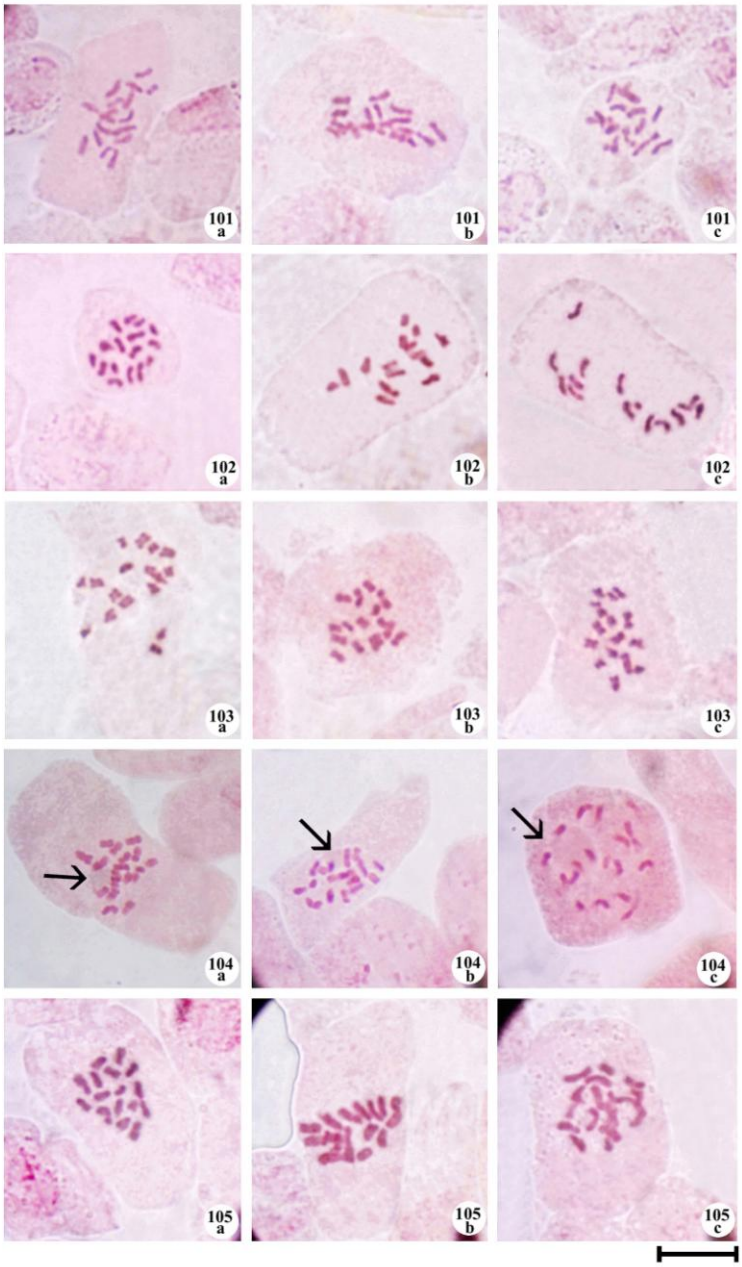
Figs 91-95. DAPI-stained mitotic prophase chromosomes of five germplasm of *Crotalaria pallida*. 91. Acc. No. 4250, 92. Acc. No. 4803, 93. Acc. No. 4805, 94. Acc. No. 4806, 95. Acc. No. 4807. Bar = 5 µm.



Figs 96-99. DAPI-stained mitotic prophase chromosomes of four germplasm of *Crotalaria incana*. 96. Acc. No. 4790, 97. Acc. No. 4801, 98. Acc. No. 4804, 99. Acc. No. 4809. Bar = 5 µm.

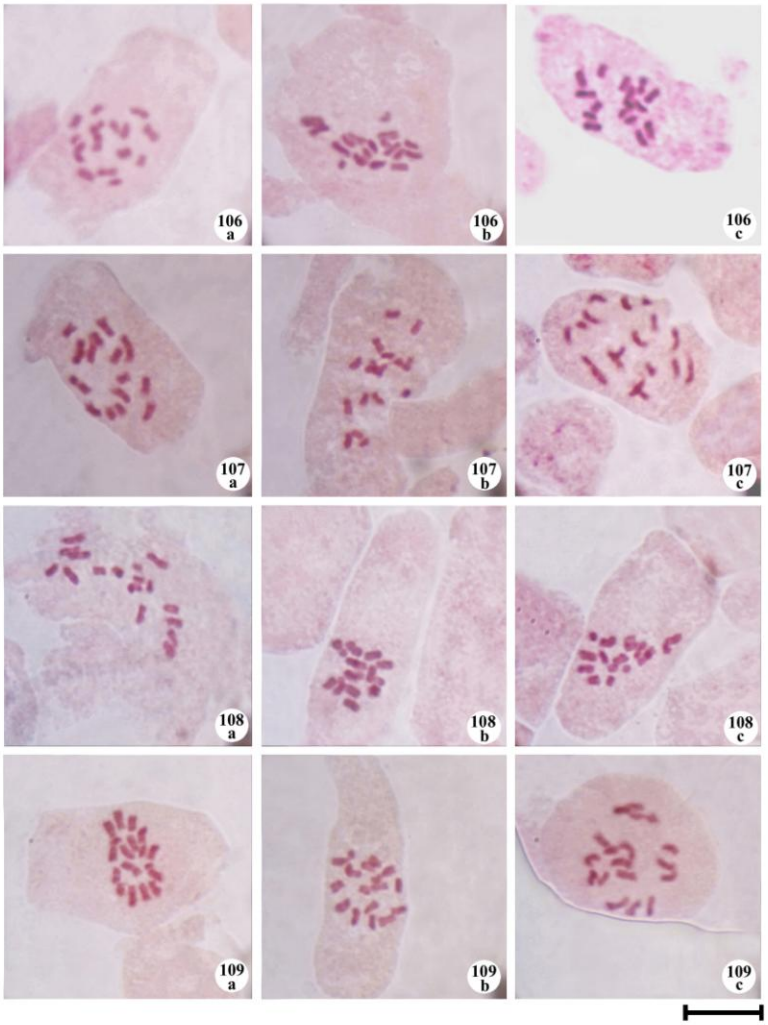
Fig. 100. DAPI-stained mitotic prophase chromosomes of *Crotalaria juncea*. Bar = 5 µm.

Results



Figs 101-105. Orcein-stained mitotic metaphase chromosomes of five germplasm (three replicates of each showing in a, b and c) of *Crotalaria pallida.* 101. Acc. No. 4250, 102. Acc. No. 4803, 103. Acc. No. 4805, 104. Acc. No. 4806, 105. Acc. No. 4807. Bar = 5 µm. Arrow (⟶) indicates nucleolus.

Results



Figs 106-109. Orcein-stained mitotic metaphase chromosomes of four germplasm (three replicates of each showing in a, b and c) of *Crotalaria incana.* 106. Acc. No. 4790, 107. Acc. No. 4801, 108. Acc. No. 4804, 109. Acc. No. 4809. Bar = 5 µm.

Results

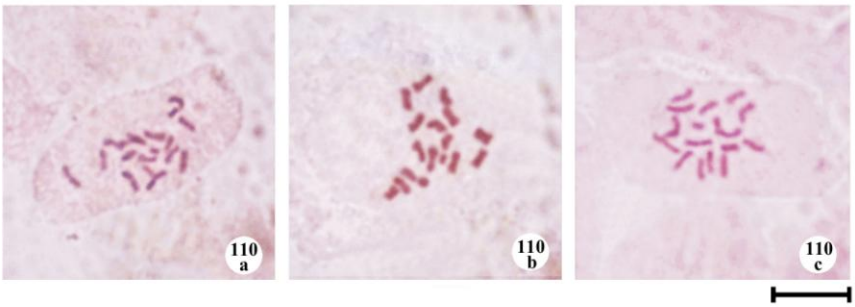
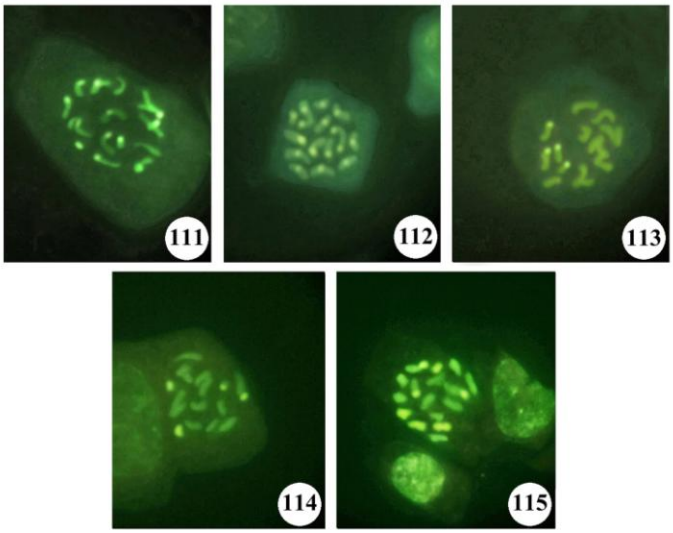
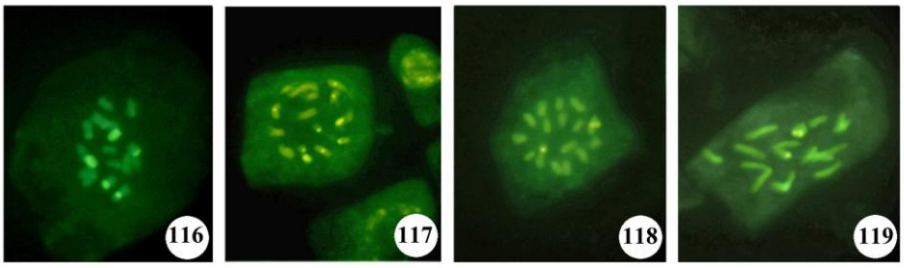


Fig. 110. Orcein-stained mitotic metaphase chromosomes of *Crotalaria juncea* (three replicates showing in a, b and c). Bar = 5 μm.

Results



Figs 111-115. CMA-stained mitotic metaphase chromosomes of five germplasm of *Crotalaria pallida*. 111. Acc. No. 4250, 112. Acc. No. 4803, 113. Acc. No. 4805, 114. Acc. No. 4806, 115. Acc. No. 4807. Bar = 5 µm.



Figs 116-119. CMA-stained mitotic metaphase chromosomes of four germplasm of *Crotalaria incana*. 116. Acc. No. 4790, 117. Acc. No. 4801, 118. Acc. No. 4804, 119. Acc. No. 4809. Bar = 5 µm.

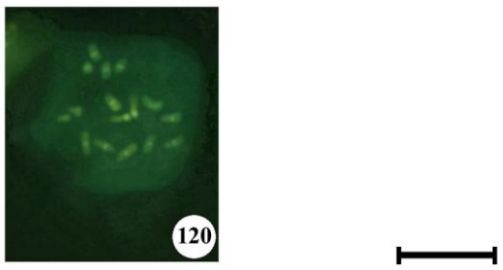
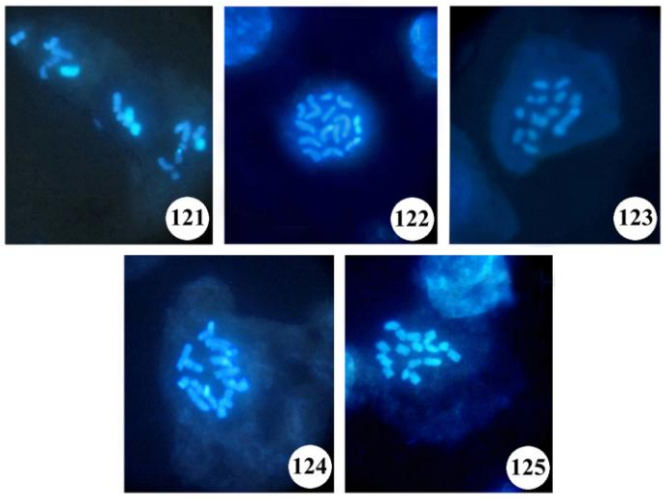
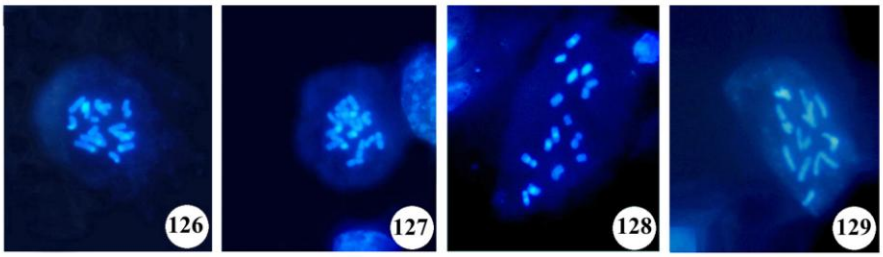


Fig. 120. CMA-stained mitotic metaphase chromosomes of *Crotalaria juncea*. Bar = 5 µm.

Results



Figs 121-125. DAPI-stained mitotic metaphase chromosomes of five germplasm of *Crotalaria pallida.* 121. Acc. No. 4250, 122. Acc. No. 4803, 123. Acc. No. 4805, 124. Acc. No. 4806, 125. Acc. No. 4807. Bar = 5 µm.



Figs 126-129. DAPI-stained mitotic metaphase chromosomes of four germplasm of *Crotalaria incana.* 126. Acc. No. 4790, 127. Acc. No. 4801, 128. Acc. No. 4804, 129. Acc. No. 4809. Bar = 5 µm.

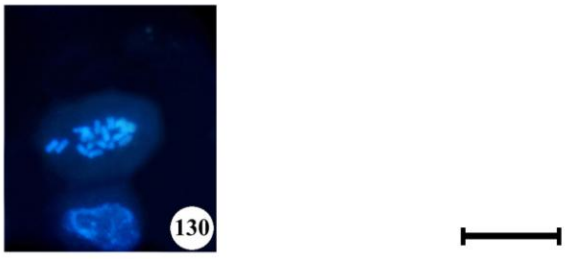
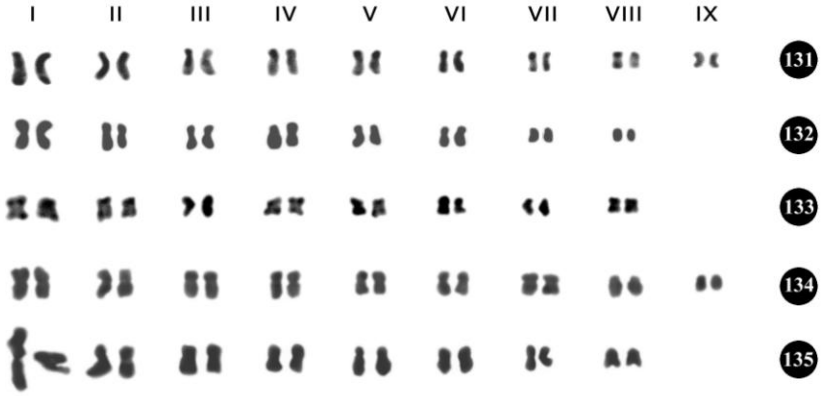
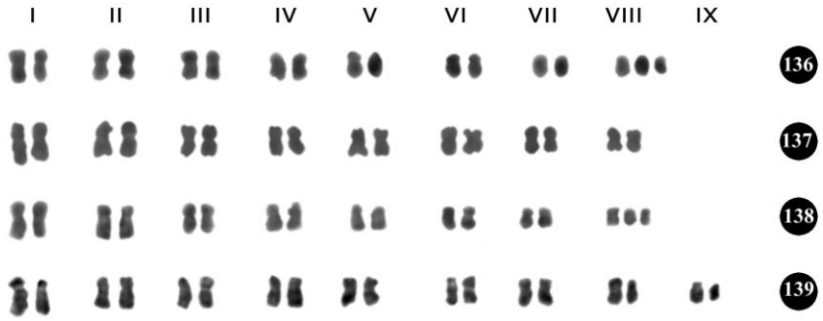


Fig. 130. DAPI-stained mitotic metaphase chromosomes of *Crotalaria juncea.* Bar = 5 µm.

Results



Figs 131-135. Karyotypes prepared from orcein-stained mitotic metaphase chromosomes of five germplasm of *Crotalaria pallida.* 131. Acc. No. 4250, 132. Acc. No. 4803, 133. Acc. No. 4805, 134. Acc. No. 4806, 135. Acc. No. 4807. Bar = 5 µm.



Figs 136-139. Karyotypes prepared from orcein-stained mitotic metaphase chromosomes of four germplasm of *Crotalaria incana.* 136. Acc. No. 4790, 137. Acc. No. 4801, 138. Acc. No. 4804, 139. Acc. No. 4809. Bar = 5 µm.

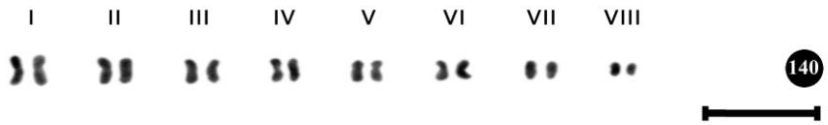
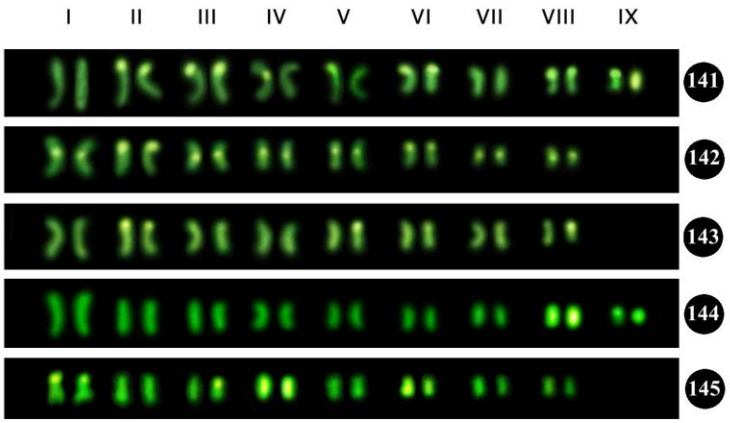
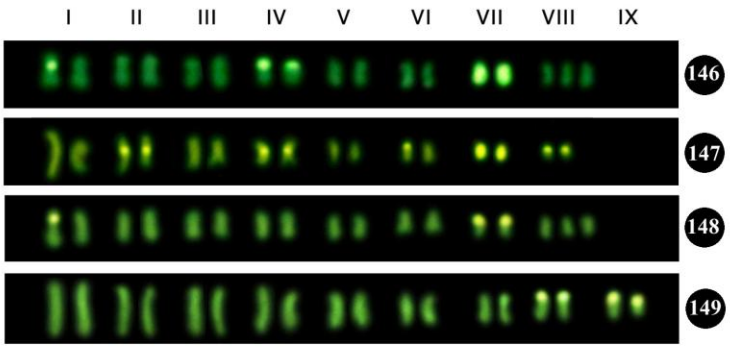


Fig.140. Karyotype prepared from orcein-stained mitotic metaphase chromosomes of *Crotalaria juncea*. Bar = 5 µm.

Results



Figs 141-145. Karyotypes prepared from CMA-stained mitotic metaphase chromosomes of five germplasm of *Crotalaria pallida*. 141. Acc. No. 4250, 142. Acc. No. 4803, 143. Acc. No. 4805, 144. Acc. No. 4806, 145. Acc. No. 4807. Bar = 5 µm.



Figs 146-149. Karyotypes prepared from CMA-stained mitotic metaphase chromosomes of four germplasm of *Crotalaria incana*. 146. Acc. No. 4790, 147. Acc. No. 4801, 148. Acc. No. 4804, 149. Acc. No. 4809. Bar = 5 µm.

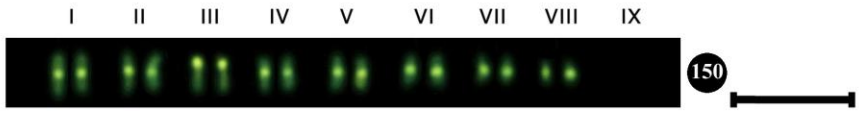
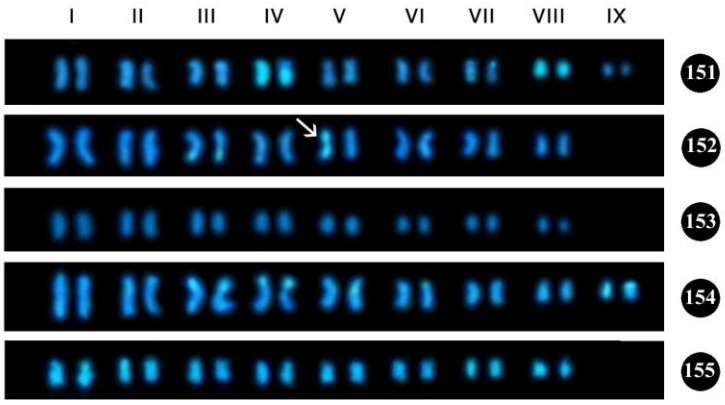
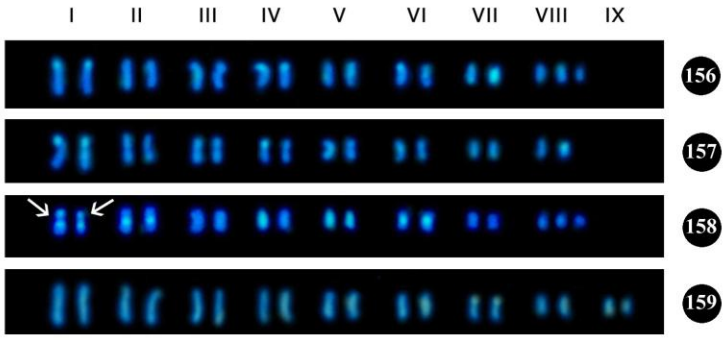


Fig. 150. Karyotype prepared from CMA-stained mitotic metaphase chromosomes of *Crotalaria juncea*. Bar = 5 µm.

Results



Figs 151-155. Karyotypes prepared from DAPI-stained mitotic metaphase chromosomes of five germplasm of *Crotalaria pallida*. 151. Acc. No. 4250, 152. Acc. No. 4803, 153. Acc. No. 4805, 154. Acc. No. 4806, 155. Acc. No. 4807. Bar = 5 µm.



Figs 156-159. Karyotypes prepared from DAPI-stained mitotic metaphase chromosomes of four germplasm of *Crotalaria incana*. 156. Acc. No. 4790, 157. Acc. No. 4801, 158. Acc. No. 4804, 159. Acc. No. 4809. Bar = 5 µm.

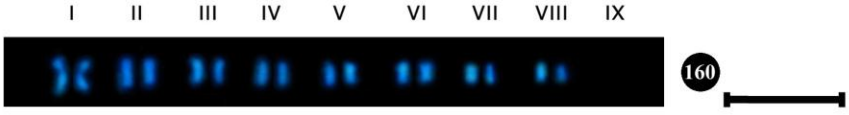
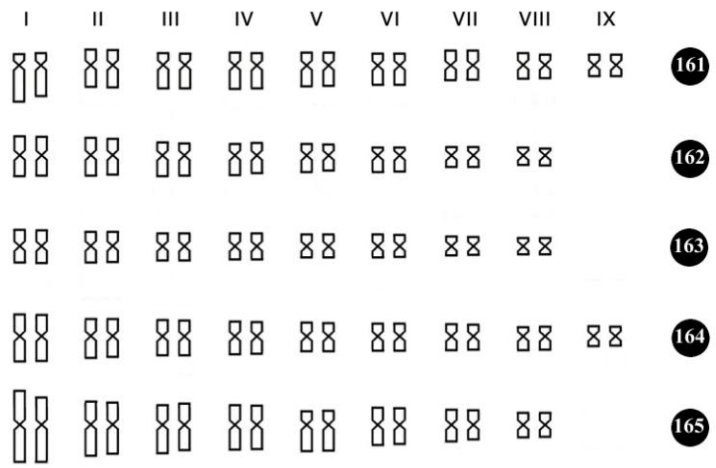


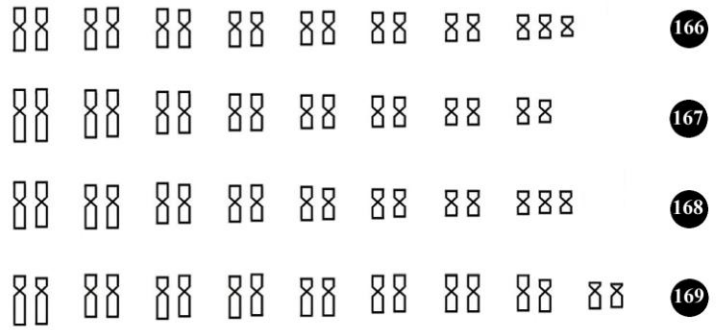
Fig. 160. Karyotype prepared from DAPI-stained mitotic metaphase chromosomes of *Crotalaria juncea*. Bar = 5 µm.

Arrow (→) indicates marker chromosome.

Results



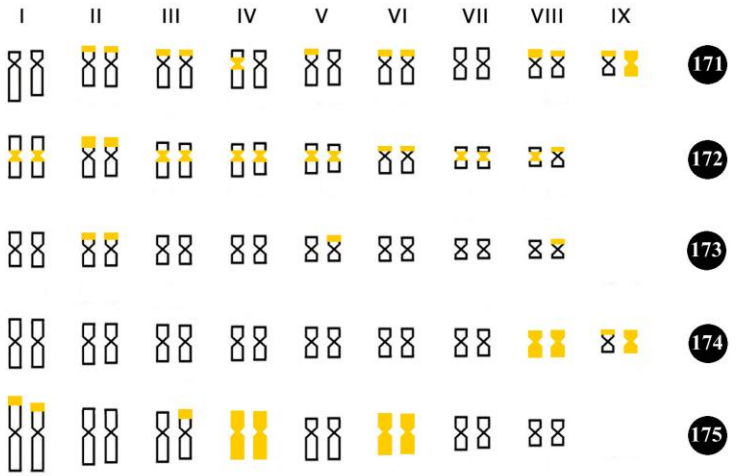
Figs 161-165. Idiograms prepared from orcein-stained mitotic metaphase chromosomes of five germplasm of *Crotalaria pallida.* 161. Acc. No. 4250, 162. Acc. No. 4803, 163. Acc. No. 4805, 164. Acc. No. 4806, 165. Acc. No. 4807. Bar = 5 µm.



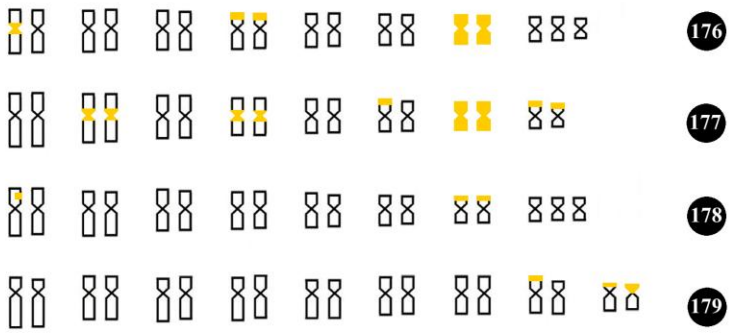
Figs 166-169. Idiograms prepared from orcein-stained mitotic metaphase chromosomes of four germplasm of *Crotalaria incana.* 166. Acc. No. 4790, 167. Acc. No. 4801, 168. Acc. No. 4804, 169. Acc. No. 4809. Bar = 5 µm.

Fig. 170. Idiogram prepared from orcein-stained mitotic metaphase chromosomes of *Crotalaria juncea.* Bar = 5 µm.

Results



Figs 171-175. Idiograms prepared from CMA-stained mitotic metaphase chromosomes of five germplasm of *Crotalaria pallida*. 171. Acc. No. 4250, 172. Acc. No. 4803, 173. Acc. No. 4805, 174. Acc. No. 4806, 175. Acc. No. 4807. Bar = 5 µm.

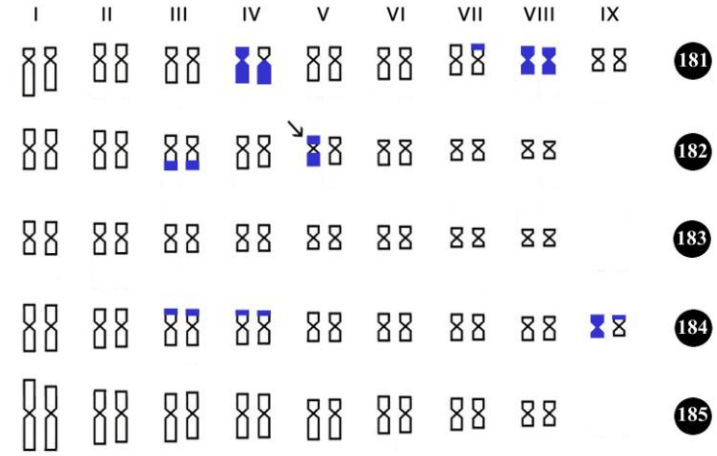


Figs 176-179. Idiograms prepared from CMA-stained mitotic metaphase chromosomes of four germplasm of *Crotalaria incana*. 176. Acc. No. 4790, 177. Acc. No. 4801, 178. Acc. No. 4804, 179. Acc. No. 4809. Bar = 5 µm.

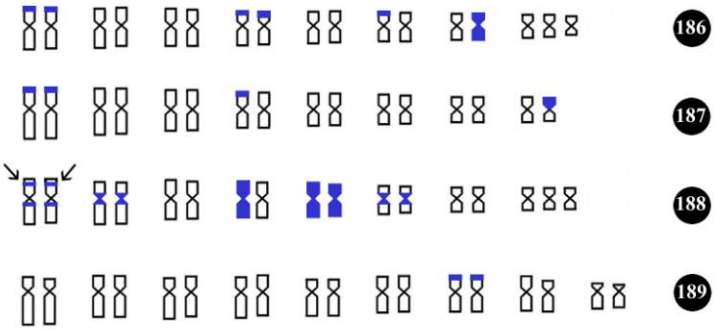
Fig. 180. Idiogram prepared from CMA-stained mitotic metaphase chromosomes of *Crotalaria juncea*. Bar = 5 µm.

= CMA-positive banded region

Results



Figs 181-185. Idiograms prepared from DAPI-stained mitotic metaphase chromosomes of five germplasm of *Crotalaria pallida*. 181. Acc. No. 4250, 182. Acc. No. 4803, 183. Acc. No. 4805, 184. Acc. No. 4806, 185. Acc. No. 4807. Bar = 5 µm.



Figs 186-189. Idiograms prepared from DAPI-stained mitotic metaphase chromosomes of four germplasm of *Crotalaria incana*. 186. Acc. No. 4790, 187. Acc. No. 4801, 188. Acc. No. 4804, 189. Acc. No. 4809. Bar = 5 µm.

Fig. 190. Idiogram prepared from DAPI-stained mitotic metaphase chromosomes of *Crotalaria juncea*. Bar = 5 µm.

Arrow (→) indicates marker chromosome.

▬ = DAPI-positive banded region

3.8 RAPD analysis

Initialy, eighteen arbitrary primers were used for RAPD amplification of which fourteen primers exhibited good quality banding patterns with sufficient variability. Therefore, fourteen primers were used for further analysis. Description of primers used in this study was given in Materials and Methods section (Table 7). The primer wise RAPD profiles and banding patterns of *Crotalaria* germplasm are given below:

3.8.1 Primer OPA-4

The primer OPA-4 generated a total of 71 RAPD bands within the ten germplasm of *Crotalaria* species.

Among five germplasm of *C. pallida*, highest number of bands (3000, 2700, 1900, 1500, 1200, 1000, 900, 650, 500 and 250 bp) were observed in Acc. No. 4807 of which bands of 900 and 250 bp could be considered as unique bands since they were absent in other germplasm. Acc. No. 4805 and 4806 generated similar banding pattern and showed seven bands (1900, 1500, 1200, 1000, 800, 650 and 500 bp). Five RAPD bands were found in Acc. No. 4803 (2500, 1400, 1200, 1000 and 800 bp). Acc. No. 4250 was found to possess two bands (1000 and 800 bp). 1000 bp was common in all germplasm of *C. pallida* (Fig. 192; Table 26).

In *C. incana*, highest number of bands (15 bands) were observed in Acc. No. 4790 (3000, 2700, 2500, 2400, 2100, 1750, 1500, 1300, 1200, 1000, 850, 700, 600, 400 and 300 bp) of which 1750, 1300, 850, 600 and 400 bp were unique in nature since they were absent in other germplasm. Acc. No. 4801 had ten bands (3500, 2700, 2400, 1500, 1400, 1200, 1000, 800, 650 and 300 bp). Eight bands (3500, 2800, 2400, 1500, 1400, 1200, 1000 and 800 bp) were present in Acc. No. 4804 of which 2800 bp was found to be unique. Lowest bands (2100, 1900, 1500, 1200, 1000 800 and 650 bp) were found in Acc. No. 4809 among the four germplasm of *C. incana* (Fig. 192; Table 26).

No band was found to develop in *C. juncea* with this primer (Fig. 192; Table 26).

Among the total 71 bands found in different germplasm of *C. pallida* and *C. incana*, eight were found to be unique in nature. There was no common band found in ten germplasm.

Therefore, all bands were polymorphic and thus 100% polymorphism was obtained (Fig. 192; Table 26).

3.8.2 Primer OPA-18

The five germplasm of *C. pallida* showed almost identical banding profile with a little deviation. Although no common bands were observed in these five germplasm, three germplasm (Acc. No. 4805, 4806 and 4807) shared seven common DNA fragments (1200, 1100, 1000, 750, 700, 500 and 450 bp). Six bands were found in Acc. No. 4803 (2000, 1400, 1100, 1000, 600 and 300 bp) of which 1400, 600 and 300 bp were unique since it was absent in other germplasm of *C. pallida*. Five bands were found in Acc. No. 4250 (2000, 1200, 1100, 1000 and 450 bp). Fragment size 1100 and 1000 bp were common in all germplasm of *C. pallida* (Fig. 193; Table 27).

In *C. incana*, Acc. No. 4790, 4801 and 4809 generated germplasm specific RAPD banding pattern whereas no band was observed in Acc. No. 4804. Six bands were found in Acc. No. 4790 (1200, 1100, 1000, 850, 730 and 500 bp) of which 730 bp was unique in nature. Similarly, Acc. No. 4801 also showed six bands (1200, 1100, 850, 750, 550 and 350 bp). Acc. No. 4809 was found to possess eight bands (2000, 1600, 1200, 1000, 750, 550, 350 and 200 bp) of which 200 bp was unique in nature (Fig. 193; Table 27).

C. juncea was found to possess five bands (1600, 1200, 750, 500 and 400 bp) of which one was found to be unique (400 bp). Band of 750 bp in case of *C. juncea* was prominently brighter than all other bands generated by three *Crotalaria* species (Fig. 193; Table 27).

The primer OPA-18 generated a total of 57 RAPD bands for the ten germplasm of *Crotalaria* species. There was no common band found in ten germplasm. Therefore, all the 57 bands were polymorphic in nature producing 100% polymorphism (Fig. 193; Table 27).

3.8.3 Primer OPAB-5

The primer OPAB-5 produced 53 RAPD bands within the ten germplasm of *Crotalaria* species.

In *C. pallida*, Acc. No. 4806 and 4807 showed similar RAPD-banding pattern with primer OPAB-5 and possessed six bands with molecular weight of 1200, 850, 700, 650, 450 and 200 bp. On the other hand, similar RAPD-banding profile (850, 700 and 200 bp) were also found in case of Acc. No. 4250 and 4803 using this primer. The rest one germplasm showed a single band of 850 bp. No unique band was found in these germplasm. Moreover, these germplasm showed a common DNA fragment of 850 bp (Fig. 194; Table 28).

In case of *C. incana*, Acc. No. 4801 and 4804 shared identical DNA fragments (1300, 1100, 850, 650 and 400 bp). However, an additional fragment of 2500 bp was observed in Acc. No. 4804 and two fragments of 700 and 250 bp were present in Acc. No. 4801 of which 250 bp was found to be unique. Seven RAPD bands (1400, 1100, 1000, 850, 700, 650 and 400 bp) were found in Acc. No. 4790. On the other hand, Acc. No. 4809 were also found to possess seven RAPD bands such as 2500, 1500, 1300, 1100, 1000, 850 and 400 bp of which 1500 bp was unique in nature (Fig. 194; Table 28).

In case of *C. juncea* seven bands of 1400, 1300, 1100, 850, 650, 550 and 400 bp were found to produce of which band of 550 bp was unique among all the other bands (Fig. 194; Table 28).

One bright band of 850 bp was common in all germplasm of *Crotalaria* species. Therefore, out of 53 RAPD bands 43 were polymorphic and the percentage of polymorphisms was 81.13 % (Fig. 194; Table 28).

3.8.4 Primer OPAB-6

A total of 49 RAPD bands were observed within the ten germplasm of *Crotalaria* species using the primer OPAB-6.

The five accessions such as Acc. No. 4250, 4803, 4805, 4806 and 4807 of *C. pallida* showed almost similar banding pattern (1100, 1050, 950 and 600 bp) except Acc. No. 4250. Fragment size 1100 bp was absent in this germplasm (Fig. 195; Table 29).

In *C. incana*, Acc. No. 4801 and 4804 showed similar banding pattern (1200, 850, 700, 550, 450, 400, 300, 280 and 250 bp). A number of different sized bands were found in Acc. No. 4790 (1050, 850, 650, 550, 500, 450 and 400 bp of which 650 and 500 bp were

unique). Acc. No. 4809 was found to possess five bands such as 1200, 700, 550,450, 400 bp (Fig. 195; Table 29).

No band was found in *C. juncea* with this primer (Fig. 195; Table 29).

No common fragment was shared within these ten germplasm of *Crotalaria* and thus exhibited 100% polymorphism with this primer (Fig. 195; Table 29).

3.8.5 Primer OPC-10

Total 71 RAPD bands were found in ten germplasm amplified through the RAPD primer OPC-10.

A number of bands of different size were obtained in each germplasm of *C. pallida* using this primer. Five bands were found in Acc. No. 4250 (2000, 1600, 1100, 850 and 600 bp). In contrast, seventeen bands were found in Acc. No. 4803 (2400, 2000, 1750, 1600, 1500, 1400, 1100, 1050, 850, 650, 600, 500, 400, 350, 300, 270 and 250 bp of which 1750 and 270 bp were unique) and Acc. No. 4805 (3500, 1600, 1500, 1400, 1100, 1050, 900, 850, 750, 700, 650, 600, 500, 400, 350, 300 and 250 bp of which 3500 and 900 bp were unique). On the other hand, six bands were observed in Acc. No. 4806 (1600, 1400, 550, 500, 300 and 250 bp) and nine bands found in Acc. No. 4807 such as 2400, 2000, 1600, 1100, 950, 850, 600, 500 and 400 bp (Fig. 196; Table 30).

In case of *C. incana,* comparatively few bands were found in different germplasm. Four bands (1600, 950, 850 and 700 bp) were found in Acc. No. 4790, seven bands (2100, 2000, 1600, 800, 550, 400 and 300 bp) in Acc. No. 4801 and three bands (2100, 1600 and 800 bp) in Acc. No. 4804. On the other hand, Acc. No. 4809 was also found to possess three bands (2100, 1600 and 750 bp) with this primer (Fig. 196; Table 30).

No band was found in *C. juncea* with this primer (Fig. 196; Table 30).

All the bands showed polymorphism with primer OPC-10 due to the absence of any common band (Fig. 196; Table 30).

3.8.6 Primer OPC-13

Primer OPC-13 generated lowest number (12) of bands among ten germplasm of *Crotalaria* species.

Bands of different size were found in each germplasm of *C. pallida.* Acc. No. 4250 were found to possess three bands of 1600, 1200 and 700 bp of which 700 bp was unique. Two bands (1200 and 900 bp) were observed in Acc. No. 4803 of which one band was appeared to be unique in nature (900 bp). Two bands at 1600 and 1200 bp were also found in Acc. No. 4805. On the other hand, Acc. No. 4806 and 4807 shared a similar DNA fragment of 1600 bp (Fig. 197; Table 31).

A single band of 1350 bp was found in all germplasm of *C. incana* except Acc. No. 4790 where no band was observed (Fig. 197; Table 31).

C. juncea did not show any band with this primer (Fig. 197; Table 31).

All the 12 bands were polymorphic due to absence of any common band among these ten germplasm of *Crotalaria* (Fig. 197; Table 31).

3.8.7 Primer OPC-14

This primer generated a total of 102 RAPD bands within ten germplasm of *Crotalaria* species.

More or less similar banding pattern was found in all germplasm of *C. pallida.* With this primer, eleven similar DNA fragments (2400, 1700, 1500, 1400, 1350, 1100, 900, 800, 700, 550 and 300 bp) were found in Acc. No. 4805, 4806 and 4807. Eleven bands were also found in Acc. No. 4803 with a little difference (2400, 2100, 1900, 1700, 1350, 1100, 900, 800, 700, 550 and 400 bp of which 2100 bp was unique in nature). In contrast, eight bands (1700, 1500, 1350, 1100, 900, 800, 700 and 550 bp) were present in Acc. No. 4250 (Fig. 198; Table 32).

In case of *C. incana,* similar banding pattern (1700, 1500, 1400, 1350, 1100, 900, 800, 700, 550 and 350 bp) was found in Acc. No. 4801 and 4804. Almost similar banding pattern was observed in Acc. No. 4790 with a little difference. Fragment size 550 was absent and an additional band of 1900 bp was present in this germplasm. Eight bands (1800, 1700, 1500, 1400, 1100, 900, 800 and 350 bp) were present in Acc. No. 4809 (Fig. 198; Table 32).

C. juncea were found to possess 12 bands (1900, 1800, 1500, 1400, 1350, 1100, 800, 700, 600, 500, 400 and 350 bp) of which 600 bp was unique because it is totally absent in other germplasm (Fig. 198; Table 32).

Two bands (1100 and 800 bp) were common in all germplasm of *Crotalaria* species. Therefore, out of 102 bands 82 bands were polymorphic which generated 80.39% polymorphism (Fig. 198; Table 32).

3.8.8 Primer OPC-16

A total of 63 RAPD bands were observed within ten germplasm of *Crotalaria* species in this primer.

Among the five germplasm of *C. pallida*, Acc. No. 4806 and 4807 showed almost similar banding pattern with a little difference. These two germplasm showed eleven similar bands (2450, 2000, 1600, 1450, 1300, 1100, 1000, 900, 750, 600 and 450 bp). Besides these bands, a unique band (2800 bp) was present in Acc. No. 4806. Three bands (2000, 1600 and 750 bp) were found in Acc. No. 4805. Other two germplasm (Acc. No. 4250 and 4803) did not show any band with this primer (Fig. 199; Table 33).

In case of *C. incana*, a number of different sized bands were found in each germplasm. In contrast, no band was observed in Acc. No 4804. Eleven bands were found in Acc. No. 4790 (2450, 1600, 1100, 1000, 850, 700, 550, 500, 350, 300 and 200 bp). Thirteen bands were present in Acc. No. 4801 (1600, 1250, 1100, 1000, 850, 750, 700, 550, 500, 450, 300, 250 and 200 bp of which 1250 bp was unique since absent in the rest germplasm). On the other hand, eight bands (1600, 850, 700, 550, 400, 350, 300 and 250 bp) were found in Acc. No. 4809 with a unique fragment of 400 bp (Fig. 199; Table 33).

Five bands (1600, 1100, 850, 800 and 750 bp) were found in *C. juncea* of which 800 bp was absent in other germplasm, thus considered as unique band (Fig. 199; Table 33).

There was no common band found with this primer in the ten germplasm. Therefore, the polymorphism was 100 % (Fig. 199; Table 33).

3.8.9 Primer OPC-26

Highest numbers of RAPD bands (109) were found in ten *Crotalaria* germplasm using the primer OPC-26.

Among the five germplasm of *C. pallida*, Acc. No. 4805, 4806 and 4807 showed similar banding pattern. These three germplasm shared seven identical bands (2150, 1900, 1450, 950, 850, 550 and 400 bp). Acc. No. 4250 showed almost similar banding pattern with previous three germplasm. In addition a fragment of 800 bp was also found in Acc. No. 4250.The rest one germplasm (Acc. No. 4803) was found to possess nine bands (3000, 2150, 1900, 1650, 1350, 800, 550, 500 and 400 bp) of which 1650, 1350 and 500 bp were unique as these bands were absent in other germplasm. A single bright band of 550 bp was common in all germplasms (Fig. 200; Table 34).

Among the four germplasm of *C. incana*, Acc. No. 4801, 4804 and 4809 showed more or less similar banding pattern. Fragment size 3000, 2000, 1800, 1400, 1200, 1000, 900, 800, 750, 650 and 550 bp were common in these three germplasm. Besides these two more fragments (1500 and 200 bp), (400 and 250 bp) were present in Acc. No. 4801 and 4809, respectively. On the other hand, six more fragments (1500, 1300, 450, 400, 250 and 200 bp) were present in Acc. No. 4804. Nine bands (2350, 2050, 1500, 1300, 1150, 900, 800, 700 and 200 bp) were found in Acc. No. 4790 of which 2350, 2050 bp were unique in nature (Fig. 200; Table 34).

In case of *C. juncea* nineteen bands (2600, 1900, 1800, 1500, 1450, 1300, 1150, 1100, 1000, 950, 800, 700, 600, 550, 450, 380, 300, 230 and 100 bp) were found of which seven bands (2600, 1100, 600, 380, 300, 230 and 100 bp) were unique since these bands were not found in other germplasm (Fig. 200; Table 34).

There was no common band found with this primer in these ten *Crotalaria* germplasm. Therefore, all were polymorphic bands and polymorphism was 100 % (Fig. 200; Table 34).

3.8.10 Primer OPC-96

Total 85 RAPD bands were observed within the ten germplasm of *Crotalaria* species with this primer.

Among the five germplasm of *C. pallida*, Acc. No. 4805 and 4806 showed similar banding pattern. These two germplasm shared eight similar bands (1600, 1300, 900, 800, 700, 650, 550 and 250 bp). Different banding patterns were found in other three germplasm. Five bands (1600, 900, 800, 700 and 550) were found in Acc. No. 4250 and 14 bands (2500, 2100, 1900, 1600, 1500, 1400, 1200, 900, 800, 700, 600, 500, 400 and 250 bp) were present in Acc. No. 4803 of which six were unique (2500, 2100, 1900, 1500, 600 and 400 bp). The rest one germplasm Acc. No. 4807 was found to possess three bands at 1600, 800 and 700 bp (Fig. 201; Table 35).

More or less similar banding pattern was found in all germplasm of *C. incana*. Nine bands (2000, 1600, 1300, 1100, 800, 700, 500, 350 and 250 bp) were common in Acc. No. 4801, 4804 and 4809. Total 12 bands (2000, 1600, 1400, 1200, 950, 900, 800, 700, 500, 450, 300 and 250 bp) were present in Acc. No. 4790 of which three (900, 450 and 300 bp) were unique in nature (Fig. 201; Table 35).

In *C. juncea,* eight bands (2400, 1600, 1250, 1100, 800, 700, 650 and 350 bp) were found of which 3 bands (2400, 1250 and 750 bp) were found to be unique (Fig. 201; Table 35).

With this primer fragment size 1600 and 700 bp was common in all the ten germplasm which generated 76.47 % polymorphism (Fig. 201; Table 35).

3.8.11 Primer OPF-22

The primer OPF-22 showed a total of 56 RAPD bands within the ten germplasm.

Few RAPD bands were found in the germplasm of *C. pallida*. A single band of 750 bp was found in Acc. No. 4250. In contrast, four bands were found in Acc. No. 4803 (1950, 750, 600 and 350 bp) and 4805 (850, 750, 600 and 400 bp). Three bands were observed in Acc. No. 4806 at 850, 750 and 600 bp and Acc. No. 4807 at 750, 600 and 350 bp (Fig. 202; Table 36).

With this primer six bands (1600, 1400, 1250, 950, 700 and 500 bp) were found in Acc. No. 4790, ten bands (1950, 1600, 1450, 1050, 950, 750, 700, 550, 400 and 250 bp) were observed in Acc. No. 4801, nine bands (1950, 1400, 1250, 1050, 950, 700, 550, 350 and 250 bp) were present in Acc. No. 4804 and eight bands (1600, 1250, 1050, 950, 700, 550, 400 and 250 bp) were found in Acc. No. 4809 of *C. incana* (Fig. 202; Table 36).

In *C. juncea,* eight bands (2200, 1450, 1400, 950, 750, 600, 500 and 300 bp) were observed of which one band (2200bp) was unique in nature (Fig. 202; Table 36).

No common band was found in these germplasm. Therefore, all of 56 bands were polymorphic and the percentage of polymorphism was 100% (Fig. 202; Table 36).

3.8.12 Primer OPG-3

A total of 76 RAPD bands were found within the ten germplasm of *Crotalaria* species with the primer OPG-3.

Acc. No. 4803, 4805, 4806 and 4807 of *C. pallida* showed almost similar banding profile with little difference. Fragment size 1500, 1100, 750, 600, 500 and 400 bp were common in these three germplasm while one more band at 300 bp was present in Acc. No. 4807 and two more bands (300 and 200 bp of which 200 bp was unique in nature) were found in Acc. No. 4806. No band was found in other germplasm (Acc. No. 4250) of *C. pallida* (Fig. 203; Table 37).

Acc. No. 4801 and 4804 of *C. incana* showed identical banding profile (1500, 1400, 1100, 1000, 850, 700, 600, 400, 300 and 225 bp). Total nine RAPD bands (1500, 1250, 1050, 950, 700, 600, 400, 300 and 225 bp of which 950 bp was unique) were found in Acc. No. 4790. Ten bands (1700, 1500, 1250, 1100, 1000, 850, 600, 500, 400 and 300 bp) were present in Acc. No. 4809 of which 1700 bp was unique in nature (Fig. 203; Table 37).

In *C. juncea*, ten bands (2000, 1550, 1400, 1050, 900, 650, 500, 480, 450 and 350 bp) were observed of which seven (2000, 1550, 900, 650, 480, 450 and 350 bp) were unique in nature (Fig. 203; Table 37).

There was no common band found with this primer. Therefore, all of 76 bands were polymorphic and the percentage of polymorphism was 100% (Fig. 203; Table 37).

3.8.13 Primer OPG-6

With the primer OPG-6, total 77 RAPD bands were found among ten germplasm.

Among the five germplasm of *C. pallida,* Acc. No. 4250 and 4805 shared identical five bands (850, 750, 650, 500 and 300 bp). Six fragment size of 1850, 850, 750, 650, 500 and

300 bp were common in Acc. No. 4803, 4806 and 4807. Besides these common fragments, few additional bands were observed such as 1200 bp in Acc. No. 4807, fragment size of 1350, 1200, 100 bp in Acc. No. 4806 and 1550, 1350, 1100 bp in Acc. No. 4803. Band at 1550 and 1100 bp of Acc. No. 4803 were unique in nature (Fig. 204; Table 38).

In case of *C. incana*, six bands (1850, 1350, 1000, 700, 500 and 350 bp of which 700 and 350 bp were unique) were found in Acc. No. 4790. Five bands (1600, 1000, 500, 450 and 400 bp) were common in Acc. No. 4801, 4804 and 4809. Besides these bands four (1350, 650, 200 and 150 bp of which 150 bp was unique), five (1450, 1400, 800, 750 and 250 bp of which 1400 and 250 bp were unique) and six (2000, 1450, 1350, 750, 650 and 200 bp of which 2000 bp was unique) more bands were found in Acc. No. 4804, 4809 and 4801, respectively (Fig. 204; Table 38).

In *C. juncea* six bands (1600, 1500, 1350, 1050, 800 and 450 bp) were found of which 1500 and 1050 bp were unique (Fig. 204; Table 38).

With this primer all the 77 polymorphic bands showed 100% polymorphism (Fig. 204; Table 38).

3.8.14 Primer OPG-9

A total of 50 RAPD bands were observed within ten germplasm of *Crotalaria* species with the primer OPG-9.

Among the five germplasm of *C. pallida,* Acc. No. 4250, 4805, 4806 and 4807 showed almost similar banding pattern with three common bands (1400, 1000 and 950 bp). Moreover, one more band (750 bp) was present in Acc. No. 4807 and two additional bands (500 and 600 bp) were found in Acc. No. 4805. A number of different sized bands were observed in Acc. No. 4803 (2500, 1800, 1550, 1400, 1250, 1050, 950, 750, 600, 500, 400 and 350 bp) of which five (2500, 1800, 1250, 400, 350 bp) were unique in nature (Fig. 205; Table 39).

Comparatively few bands were found in *C. incana*. A single band was present in Acc. No. 4804 (350 bp). In contrast, two bands (450 and 300 bp) in Acc. No. 4801, three bands (800, 700 and 450 bp of which 700 bp was unique band) in Acc. No. 4809 and nine bands

(1100, 1000, 800, 750, 600, 500, 450, 300 and 150 bp of which 1100, 150 bp were unique) in Acc. No. 4790 were observed with this primer (Fig. 205; Table 39).

In *C. juncea* eight bands (1550, 1400, 1150, 950, 800, 600, 500 and 50 bp) were found of which two were unique at 1150 and 50 bp (Fig. 205; Table 39).

No common bands were found with this primer. Therefore, all bands were polymorphic and the percentage of polymorphism was 100% among ten *Crotalaria* germplasm (Fig. 205; Table 39).

3.8.15 Genetic distances

The values of pair-wise Nei's (1972) genetic distances analyzed by using computer software "popgene32" among ten germplasm of *Crotalaria* species were computed using combined data for the fourteen RAPD primers ranging from 0.0970 to 0.7139 (Table 41). The highest genetic distance (0.7139) was observed between a germplasm of *C. pallida* (Acc. No. 4803) and *C. incana* (Acc. No. 4801). Among the five germplasm of *C. pallida* the lowest (0.0970) genetic distance was observed between Acc. No. 4806 and Acc. No. 4807. On the other hand, the highest genetic distance (0.3834) was found among Acc. No. 4803 and 4806 of *C. pallida.* In case of four germplasm of *C. incana,* the lowest (0.1714) genetic distance was observed between Acc. No. 4801 and Acc. No. 4804 whereas, the highest genetic distance (0.4950) was found between Acc. No. 4790 and 4809 (Fig. 206; Table 41).

3.9 SSR analysis

Initially, five selected primer pairs were used for SSR analysis of ten *Crotalaria* germplasm. Four primer pairs exhibited good quality banding patterns in germplasm of *Crotalaria* with sufficient variability. Therefore, four primer pairs were used for further analysis. The details of primer pairs used in this study have been given previously in Materials and Methods section in (Table 8). Each primer pair showed different banding profiles. The pair-wise SSR profiles and banding patterns of ten germplasm of *Crotalaria* were as follows:

3.9.1 Primer AL-365892

A total of 63 bands were found among ten *Crotalaria* germplasm with the primer pair AL-365892.

The five germplasm of *C. pallida* showed more or less identical banding patterns and shared five common fragments such as 900, 750, 450, 350 and 230 bp. Moreover, one more band was present in Acc. No. 4250 (300 bp) and Acc. No. 4803 (200 bp) whereas, three more bands were present in Acc. No. 4806 (600, 300 and 200 bp) and 4807 (600, 500 and 200 bp) (Fig. 207; Table 42).

The four germplasm of *C. incana* also showed more or less similar banding pattern with a little difference. DNA fragment size of 950, 700, 500, 250 and 150 bp were common in all germplasm. However, one more band of 400 bp was present in Acc. No. 4801 and 4809, two more bands such as 400 and 200 bp were present in Acc. No. 4804. On the other hand, three more bands (780, 600 and 200 bp) were present in Acc. No. 4790 (Fig. 207; Table 42).

In *C. juncea* three bands (780, 650 and 180 bp) were found of which two at 650 and 180 bp were unique bands (Fig. 207; Table 42).

No common bands were found in this case. Therefore, the percentage of polymorphism was 100% among ten germplasm of *Crotalaria* (Fig. 207; Table 42).

3.9.2 Primer AW-127626

Few bands were found among ten *Crotalaria* germplasm with the primer pair AW-127626. No band was found in any germplasm of *C. pallida* and *C. incana* except Acc. No. 4809. This germplasm of *C. incana* was found to possess four bands (300, 250, 200 and 100 bp) of which three (300, 250, 200 bp) were unique in nature (Fig. 208; Table 43).

In *C. juncea,* three bands (270, 230 and 100 bp) were found of which two of 270 and 230 bp were unique bands (Fig. 208; Table 43).

Common band was absent among the germplasm with this primer pair. Therefore, the percentage of polymorphism was 100% (Fig. 208; Table 43).

3.9.3 Primer AW-584539

The primer pair AW-584539 generated total 21 bands within the ten germplasm of *Crotalaria* species.

Among the five germplasm of *C. pallida*, only Acc. No. 4803 showed six bands (700, 600, 500, 400, 300 and 100 bp) of which four were unique in nature (700, 500, 400 and 300 bp). No band was found in other four germplasm of *C. pallida* (Fig. 209; Table 44).

In *C. incana*, Acc. No. 4790 and 4801 showed no band. Only three bands (900, 800 and 450 bp) were present in Acc. No. 4804 and nine bands (1500, 900, 800, 750, 600, 450, 250, 200 and 100 bp) were observed in Acc. No. 4809 of which four bands of 1500, 750, 250 and 200 bp were unique (Fig. 209; Table 44).

Three bands (600, 270 and 230 bp) were found in *C. juncea* of which two (270, 230 bp) were unique bands (Fig. 209; Table 44).

There was no common band found among these germplasm. Therefore, all the bands were polymorphic and the percentage of polymorphism was found to be 100 % (Fig. 209; Table 44).

3.9.4 Primer MtSSRNFAW-142

Total 16 bands were observed with this primer pair among ten germplasm.

Few bands were found in case of *C. pallida*. No band was found in Acc. No. 4250 and Acc. No. 4807. The Acc. No. 4803, 4805 and 4806 shared a common band (280 bp). In addition, a band at 500 bp was also present in Acc. No. 4805 (Fig. 210; Table 45).

In case of *C. incana*, Acc. No. 4801 and 4804 shared three common bands (800, 600 and 500 bp). Two bands (800 and 550 bp) were found in Acc. No. 4809. The rest one germplasm (Acc. No. 4790) had four bands (1350, 800, 600 and 300 bp) of which two (1350 and 300 bp) were unique in nature (Fig. 210; Table 45).

There was no band found in *C. juncea* with this primer pair (Fig. 210; Table 45).

All the 16 bands were polymorphic bands and the percentage of polymorphism was 100 % (Fig. 210; Table 45).

3.9.5 Genetic distances

The values of pair-wise Nei's (1972) genetic distances analyzed by using computer software "popgene32" among ten germplasm of *Crotalaria* species. were computed from combined data for the four SSR primer pairs ranging from 0.0690 to 1.0986 (Table 47). The highest genetic distance (1.0986) was found between a germplasm of *C. pallida* (Acc. No. 4806) and *C. incana* (Acc. No. 4809). The highest genetic distance was also observed between Acc. No. 4803 of *C. pallida* and Acc. No. 4809 of *C. incana*. Among the five germplasm of *C. pallida* the lowest (0.0690) genetic distance was observed between Acc. No. 4806 and Acc. No. 4807. This lowest distance was also observed in Acc. No. 4250 with 4805 and 4807. In contrast, the highest genetic distance (0.2231) was found between Acc. No. 4250 and 4803. On the other hand, same genetic distance was present in Acc. No. 4803 and 4807 of *C. pallida.* In case of four germplasm of *C. incana,* the lowest (0.0931) genetic distance was observed between Acc. No. 4801 and Acc. No. 4804 but the highest genetic distance (0.6286) was found between Acc. No. 4790 and 4809 (Fig. 211; Table 47).

Table 26. RAPD analysis with primer OPA-4 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	2 (1000,800)	1 (800)	1 (1000)	-	-
	4803	5 (2500,1400,1200, 1000,800)	4 (2500,1400,1200, 800)	1 (1000)	-	-
	4805	7 (1900,1500,1200, 1000, 800,650,500)	6 (1900,1500,1200, 800,650,500)	1 (1000)	-	-
	4806	7 (1900,1500,1200, 1000,800,650,500)	6 (1900,1500,1200, 800,650,500)	1 (1000)	-	-
	4807	10 (3000,2700,1900, 1500,1200,1000, 900,650,500,250)	8 (3000,2700,1900, 1500,900,650,500, 250)	2 (1200,1000)	2 (900, 250)	-
C. incana	4790	15 (3000,2700,2500, 2400,2100,1750, 1500,1300,1200, 1000,850,700, 600,400,300)	9 (3000,2700,2500, 2400,1300,800, 700,600,300)	6 (2100,1750, 1500,1200, 1000,400)	5 (1750, 1300, 850,600, 400)	-
	4801	10 (3500,2700,2400, 1500,1400,1200, 1000,800,650,300)	7 (3500,2700,1500, 1200,800, 650,300)	3 (2400,1400, 1000)	-	-
	4804	8 (3500,2800,2400, 1500,1400,1200, 1000,800)	6 (3500,2800, 1500, 1200,1000,800)	2 (2400, 1400)	1 (2800)	-
	4809	7 (2100,1900,1500, 1200,1000,800,650)	7 (2100,1900,1500, 1200,1000,800, 650)	-	-	-
C. juncea	Local	-	-	-	-	-

Table 27. RAPD analysis with primer OPA-18 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	5 (2000,1200,1100, 1000,450)	5 (2000,1200,1100, 1000,450)	-	-	-
	4803	6 (2000,1400,1100, 1000,600,300)	6 (2000,1400,1100, 1000,600,300)	-	3 (1400,600, 300)	-
	4805	7 (1200,1100,1000, 750, 700,500,450)	7 (1200,1100,1000, 750, 700,500,450)	-	-	-
	4806	7 (1200,1100,1000, 750,700,500,450)	7 (1200,1100,1000, 750,700,500,450)	-	-	-
	4807	7 (1200,1100,1000, 750,700,500,450)	7 (1200,1100,1000, 750,700,500,450)	-	-	-
C. incana	4790	6 (1200,1100,1000, 850,730,500)	6 (1200,1100, 1000,850,730,500)	-	1 (730)	-
	4801	6 (1200,1100,850, 750,550,350)	6 (1200,1100, 850,750,550,350)	-	-	-
	4804	-	-	-	-	-
	4809	8 (2000,1600,1200, 1000,750,550, 350,200)	8 (2000,1600,1200, 1000,750,550, 350,200)	-	1 (200)	-
C. juncea	Local	5 (1600,1200, 750,500,400)	4 (1600,1200, 500,400)	1 (750)	1 (400)	-

Table 28. RAPD analysis with primer OPAB-5 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	3 (850,700,200)	2 (700,200)	1 (850)	-	-
	4803	3 (850,700,200)	2 (700,200)	1 (850)	-	-
	4805	1 (850)	-	1 (850)	-	-
	4806	6 (1200,850,700, 650,450,200)	5 (1200,700, 650,450,200)	1 (850)	-	-
	4807	6 (1200,850,700, 650,450,200)	5 (1200,700, 650,450,200)	1 (850)	-	1 (850)
C. incana	4790	7 (1400,1100, 1000,850,700, 650,400)	6 (1400,1100, 1000,700, 650,400)	1 (850)	-	-
	4801	7 (1300,1100,850, 700,650,400,250)	6 (1300,1100, 700,650, 400,250)	1 (850)	1 (250)	-
	4804	6 (2500,1300, 1100,850, 650,400)	5 (2500,1300, 1100,650,400)	1 (850)	-	-
	4809	7 (2500,1500, 1300,1100, 1000,850,400)	6 (2500,1500, 1300,1100, 1000,400)	1 (850)	1 (1500)	-
C. juncea	Local	7 (1400,1300, 1100,850,650, 550,400)	5 (1400,1300, 1100,650, 400)	2 (850,550)	1 (550)	-

Table 29. RAPD analysis with primer OPAB-6 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	3 (1050,950,600)	3 (1050,950,600)	-	-	-
	4803	4 (1100,1050, 950,600)	4 (1100,1050, 950,600)	-	-	-
	4805	4 (1100,1050, 950,600)	4 (1100,1050, 950,600)	-	-	-
	4806	4 (1100,1050, 950,600)	4 (1100,1050, 950,600)	-	-	-
	4807	4 (1100,1050, 950,600)	4 (1100,1050, 950,600)	-	-	-
C. incana	4790	7 (1050,850,650, 550,500,450,400)	7 (1050,850,650, 550,500,450,400)	-	2 (650,500)	-
	4801	9 (1200,850,700, 550,450,400, 300,280,250)	9 (1200,850,700, 550,450,400, 300,280,250)	-	-	-
	4804	9 (1200,850,700, 550,450,400, 300,280,250)	9 (1200,850,700, 550,450,400, 300,280,250)	-	-	-
	4809	5 (1200,700,550, 450,400)	5 (1200,700,550, 450,400)	-	-	-
C. juncea	Local	-	-	-	-	-

Table 30. RAPD analysis with primer OPC-10 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	5 (2000,1600, 1100,850,600)	5 (2000,1600,1100,850,600)	-	-	-
	4803	17 (2400,2000,1750, 1600,1500,1400, 1100,1050,850, 650,600,500,400, 350,300,270,250)	14 (2400,2000,1600, 1500,1400, 1050, 650,600,500,400, 350,300,270,250)	3 (1750,1100, 850)	2 (1750,270)	-
	4805	17 (3500,1600,1500, 1400,1100,1050, 900,850,750,700, 650,600,500, 400,350,300,250)	14 (3500,1500, 1400,1100,1050, 900,850,750, 700,650,600, 400,300,250)	3 (1600,500, 350,)	2 (3500,900)	-
	4806	6 (1600,1400,550, 500,300,250)	6 (1600,1400,550, 500,300,250)	-	-	-
	4807	9 (2400,2000,1600, 1100,950,850, 600,500,400)	9 (2400,2000,1600, 1100,950,850, 600,500,400)	-	-	-
C. incana	4790	4 (1600,950, 850,700)	4 (1600,950, 850,700)	-	-	-
	4801	7 (2100,2000, 1600,800,550, 400,300)	7 (2100,2000,1600, 800,550,400,300)	-	-	-
	4804	3 (2100,1600,800)	3 (2100,1600,800)	-	-	-
	4809	3 (2100,1600,750)	3 (2100,1600,750)	-	-	-
C. juncea	Local	-	-	-	-	-

Table 31. RAPD analysis with primer OPC-13 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	3 (1600,1200,700)	3 (1600,1200,700)	-	1 (700)	-
	4803	2 (1200,900)	2 (1200,900)	-	1 (900)	-
	4805	2 (1600,1200)	2 (1600,1200)	-	-	-
	4806	1 (1600)	1 (1600)	-	-	-
	4807	1 (1600)	1 (1600)	-	-	-
C. incana	4790	-	-	-	-	-
	4801	1 (1350)	1 (1350)	-	-	-
	4804	1 (1350)	1 (1350)	-	-	-
	4809	1 (1350)	1 (1350)	-	-	-
C. juncea	Local	-	-	-	-	-

Table 32. RAPD analysis with primer OPC-14 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	8 (1700,1500,1350, 1100,900,800, 700,550)	8 (1700,1500,1350, 1100,900,800, 700,550)	-	-	-
	4803	11 (2400,2100,1900, 1700,1350,1100,900, 800,700,550,400)	11 (2400,2100,1900, 1700,1350,1100,900, 800,700,550,400)	-	1 (2100)	-
	4805	11 (2400,1700,1500, 1400,1350,1100,900, 800,700,550,300)	11 (2400,1700,1500, 1400,1350,1100,900, 800,700,550,300)	-	-	-
	4806	11 (2400,1700,1500,14 00,1350,1100,900, 800,700,550,300)	11 (2400,1700,1500, 1400,1350,1100,900, 800,700,550,300)	-	-	2 (1100, 800)
	4807	11 (2400,1700,1500,14 00,1350,1100,900, 800,700,550,300)	11 (2400,1700,1500, 1400,1350,1100,900, 800,700,550,300)	-	-	-
C. incana	4790	10 (1900,1700,1500, 1400,1350,1100, 900,800,700,350)	10 (1900,1700,1500, 1400,1350,1100, 900,800,700,350)	-	-	-
	4801	10 (1700,1500,1400, 1350,1100,900,800, 700,550,350)	10 (1700,1500,1400, 1350,1100,900,800, 700,550,350)	-	-	-
	4804	10 (1700,1500,1400, 1350,1100,900,800, 700,550,350)	10 (1700,1500,1400, 1350,1100,900,800, 700,550,350)	-	-	-
	4809	8 (1800,1700,1500, 1400,1100,900, 800,350)	8 (1800,1700,1400, 1100,900,350)	-	-	-
C. juncea	Local	12 (1900,1800,1500, 1400,1350,1100, 800,700,600, 500,400,350)	12 (1900,1800,1500, 1400,1350,1100, 800,700,600, 500,400,350)	-	1 (600)	-

Table 33. RAPD analysis with primer OPC-16 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	-	-	-	-	-
	4803	-	-	-	-	-
	4805	3 (200,1600,750)	3 (200,1600,750)	-	-	-
	4806	12 (2800,2450,2000, 1600,1450,1300, 1100,1000,900, 750,600,450)	12 (2800,2450,2000, 1600,1450,1300, 1100,1000,900, 750,600,450)	-	1 (2800)	-
	4807	11 (2450,2000,1600, 1450,1300,1100, 1000,900,750, 600,450)	11 (2450,2000,1600, 1450,1300,1100, 1000,900,750, 600,450)	-	-	-
C. incana	4790	11 (2450,1600,1100, 1000,850,700, 550,500,350, 300,200)	11 (2450,1600,1100, 1000,850,700, 550,500,350, 300,200)	-	-	-
	4801	13 (1600,1250,1100, 1000,850,750, 700,550,500,450, 300,250,200)	13 (1600,1250,1100, 1000,850,750, 700,550,500,450, 300,250,200)	-	1 (1250)	-
	4804	-	-	-	-	-
	4809	8 (1600,850,700, 550,400,350, 300,250)	8 (1600,850,700, 550,400,350, 300,250)	-	1 (400)	-
C. juncea	Local	5 (1600,1100,850, 800,750)	5 (1600,1100,850, 800,750)	-	1 (800)	-

Table 34. RAPD analysis with primer OPC-26 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	8 (2150,1900,1450, 950,850,800, 550,400)	8 (2150,1900, 1450,950,850, 800,400)	1 (550)	-	-
	4803	9 (3000,2150,1900, 1650,1350,800, 550,500,400)	8 (3000,2150,1900, 1650,1350,800, 500,400)	1 (550)	3 (1650, 1350, 500)	-
	4805	7 (2150,1900,1450, 950,850,550,400)	6 (2150,1900,1450, 950,850,400)	1 (550)	-	-
	4806	7 (2150,1900,1450, 950,850,550,400)	6 (2150,1900,1450, 950,850,400)	1 (550)	-	-
	4807	7 (2150,1900,1450, 950,850,550,400)	6 (2150,1900,1450, 950,850,400)	1 (550)	-	-
C. incana	4790	9 (2350,2050,1500, 1300,1150,900, 800,700,200)	8 (2350,2050,1500, 1150,900,800, 700,200)	1 (1300)	2 (2350,2050)	-
	4801	13 (3000,2000,1800, 1500,1400,1200, 1000,900,800, 750,650,550,200)	10 (3000,2000,1800, 1500,1000,900, 800,650, 550,200)	3 (1400, 1200, 750)	-	-
	4804	17 (3000,2000,1800, 1500,1400,1300, 1200,1000,900, 800,750,650,550, 450,400,250,200)	17 (3000,2000,1800, 1500,1400,1300, 1200,1000,900,800, 750,650,550,450, 400,250,200)	-	-	-
	4809	13 (3000,2000,1800, 1400,1200,1000, 900,800,750,650, 550,400,250)	9 (3000,2000, 1800, 1000, 900,800,650, 550,250)	4 (1400, 1200, 750, 380)	-	-
C. juncea	Local	19 (2600,1900,1800, 1500,1450,1300, 1150,1100,1000, 950,800,700,600, 550,450,380,300, 230,100)	17 (2600,1900,1800, 1500,1450,1300, 1150,1100,1000, 950,800,700,550, 380,300,230,100)	2 (600, 450)	7 (2600, 1100, 600,380, 300,230, 100)	-

Table 35. RAPD analysis with primer OPC-96 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	5 (1600,900,800, 700,550)	5 (1600,900,800,700, 550)	-	-	-
	4803	14 (2500,2100,1900, 1600,1500,1400, 1200,900,800, 700,600,500, 400,250)	13 (2500,2100,1900, 1500,1400,1200, 900,800,700,600, 500,400, 250)	1 (1600)	6 (2500,2100, 1900,1500, 600,400)	-
	4805	8 (1600,1300,900, 800,700,650, 550,250)	7 (1900,1300,1000, 850,700,550,250)	1 (1600)	-	-
	4806	8 (1600,1300,900, 800,700,650, 550,250)	7 (1900,1300,1000, 850,700,550,250)	1 (1600)	-	-
	4807	3 (1600,800,700)	3 (1600,850,700)	-	-	2 (1600, 700)
C. incana	4790	12 (2000,1600,1400, 1200,950,900, 800,700,500, 450,300,250)	12 (2000,1600,1400, 1200,950,900, 800,700,500, 450,300,250)	-	3 (900,450, 300)	-
	4801	9 (2000,1600,1300, 1100,800,700, 500,350,250)	9 (2000,1600,1300, 1100,800,700, 500,350,250)	-	-	-
	4804	9 (2000,1600,1300, 1100,800,700, 500,350,250)	9 (2000,1600,1300, 1100,800,700, 500,350,250)	-	-	-
	4809	9 (2000,1600,1300, 1100,800,700, 500,350,250)	9 (2000,1600,1300, 1100,800,700, 500,350,250)	-	-	-
C. juncea	Local	8 (2400,1600,1250, 1100,750,700, 650,350)	8 (2400,1600,1250, 1100,750,700, 650,350)	-	3 (2400, 1250,750)	-

Table 36. RAPD analysis with primer OPF-22 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	1 (750)	1 (750)	-	-	-
	4803	4 (1950,750, 600,350)	4 (1950,750, 600,350)	-	-	-
	4805	4 (850,750, 600,400)	4 (850,750, 600,400)	-	-	-
	4806	3 (850,750,600)	3 (850,750,600)	-	-	-
	4807	3 (750,600,350)	3 (750,600,350)	-	-	-
C. incana	4790	6 (1600,1400, 1250,950, 700,500)	5 (1600,1250, 950,700,500)	1 (1400)	-	-
	4801	10 (1950,1600, 1450,1050,950, 750,700,550, 400,250)	10 (1950,1600,1450, 1050,950,750, 700,550, 400,250)	-	-	-
	4804	9 (1950,1400,1250, 1050,950,700, 550,350,250)	9 (1950,1400,1250, 1050,950,700, 550,350,250)	-	-	-
	4809	8 (1600,1250,1050, 950,700,550, 400,250)	7 (1600,1250,1050, 950,700, 550,400)	1 (250)	-	-
C. juncea	Local	8 (2200,1450, 1400,950,750, 600,500,300)	8 (2200,1450, 1400,950,750, 600,500,300)	-	1 (2200)	-

Table 37. RAPD analysis with primer OPG-3 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	-	-	-	-	-
	4803	6 (1500,1100,750, 600,500,400)	6 (1500,1100,750,600, 500,400)	-	-	-
	4805	6 (1500,1100,750, 600,500,400)	6 (1500,1100,750, 600,500,400)	-	-	-
	4806	8 (1500,1100,750, 600,500,400, 300,200)	8 (1500,1100,750,600, 500,400,300,200)	-	1 (200)	-
	4807	7 (1500,1100, 750,600,500, 400,300)	7 (1500,1100,750, 600,500, 400,300)	-	-	-
C. incana	4790	9 (1500,1250,1050, 950,700,600, 400,300,225)	8 (1500,1250,1050, 950,700, 400, 300,225)	1 (600)	1 (950)	-
	4801	10 (1500,1400,1100, 1000,850,700, 600,400, 300,225)	10 (1500,1400,1100, 1000,850,700, 600,400, 300,225)	-	-	-
	4804	10 (1500,1400,1100, 1000,850,700, 600,400, 300,225)	10 (1500,1400,1100, 1000,850,700, 600,400, 300,225)	-	-	-
	4809	10 (1700,1500,1250, 1100,1000,850, 600,500, 400,300)	10 (1700,1500,1250, 1100,1000, 850,600,500, 400,300)	-	1 (1700)	-
C. juncea	Local	10 (2000,1550,1400, 1050,900,650, 500,480, 450,350)	8 (2000,1550,1400, 1050,900,650, 500,350)	2 (480,450)	7 (2000, 1550,900, 650,480, 450,350)	-

Table 38. RAPD analysis with primer OPG-6 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	5 (850,750,650, 500,300)	4 (850,750, 650,300)	1 (500)	-	-
	4803	9 (1850,1550, 1350,1100,850, 750,650, 500,300)	8 (1850,1550,1350, 1100,850,750, 650,300)	1 (500)	2 (1550, 1100)	-
	4805	5 (850,750,650, 500,300)	4 (850,750, 650,300)	1 (500)	-	-
	4806	9 (1850,1350,1200, 1000,850,750, 650,500,300)	8 (1850,1350,1200, 1000,850,750, 650,300)	1 (500)	-	-
	4807	7 (1850,1200,850, 750,650, 500,300)	6 (1850,1200,850, 750,650,300)	1 (500)	-	-
C. incana	4790	6 (1850,1350,1000, 700,500,350)	5 (1850,1350,1000, 700,500)	1 (350)	2 (700, 350)	-
	4801	11 (2000,1600,1450, 1350,1000,750, 650,500,450, 400,200)	10 (2000,1600,1450, 1350,1000,750, 650, 450, 400,200)	1 (500)	1 (2000)	-
	4804	9 (1600,1350,1000, 650,500,450, 400,200,150)	9 (1600,1350,1000, 650,500,450, 400,200,150)	-	1 (150)	-
	4809	10 (1600,1450,1400,1 000,800,750, 500,450, 400,250)	9 (1600,1450,1400,10 00,800,750, 450,400,250)	1 (500)	2 (1400, 250)	-
C. juncea	Local	6 (1600,1500,1350, 1050,800,450)	5 (1600,1500,1350, 1050,800)	1 (450)	2 (1500, 1050)	-

Table 39. RAPD analysis with primer OPG-9 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	3 (1400,1000,950)	3 (1400,1000,950)	-	-	-
	4803	12 (2500,1800,1550, 1400,1250,1050, 950,750,600, 500,400,350)	12 (2500,1800,1550, 1400,1250,1050, 950,750,600, 500,400,350)	-	5 (2500, 1800, 1250, 400,350)	-
	4805	5 (1400,1000,950, 600,500)	5 (1400,1000, 950,600,500)	-	-	-
	4806	3 (1400,1000,950)	3 (1400,1000,950)	-	-	-
	4807	4 (1400,1000, 950,750)	4 (1400,1000, 950,750)	-	-	-
C. incana	4790	9 (1100,1000,800, 750,600,500, 450,300,150)	9 (1100,1000,800, 750,600,500, 450,300,150)	-	2 (1100, 150)	-
	4801	2 (450,300)	2 (450,300)	-	-	-
	4804	1 (350)	1 (350)	-	-	-
	4809	3 (800,700,450)	3 (800,700,450)	-	1 (700)	-
C. juncea	Local	8 (1550,1400, 1150,950,800, 600,500,50)	8 (1550,1400, 1150,950,800, 600,500,50)	-	2 (1150,50)	-

Table 40. Compilation of RAPD analysis in ten germplasm of *Crotalaria* species.

Primer codes	Total bands	Size ranges (bp)	Number of Polymorphic bands	Number of Common bands	Number of unique bands	Polymorphisms (%)
OPA-4	71	250-3500	71	-	8	100
OPA-18	57	200-2000	57	-	6	100
OPAB-5	53	200-2500	43	10	3	81.13
OPAB-6	49	250-1200	49	-	2	100
OPC-10	71	250-3500	71	-	4	100
OPC-13	12	700-1600	12	-	2	100
OPC-14	102	300-2400	82	20	2	80.39
OPC-16	63	200-2800	63	-	4	100
OPC-26	109	100-3000	109	-	12	100
OPC-96	85	250-2500	65	20	12	76.47
OPF-22	56	250-2200	56	-	1	100
OPG-3	76	200-2000	76	-	10	100
OPG-6	77	150-200	77	-	10	100
OPG-9	50	50-2500	50	-	10	100
	931	**50-3500**	**881**	**50**	**86**	**95.57**

Table 41. Summary of Nei's (1972) genetic distances of ten germplasm of *Crotalaria* species by RAPD analysis.

Species	Acc. No./ Identity	*C. pallida*					*C. incana*				*C. juncea*
		4250	4803	4805	4806	4807	4790	4801	4804	4809	Local
C. pallida	4250	****									
	4803	0.2786	****								
	4805	0.1837	0.2923	****							
	4806	0.2129	0.3834	0.1434	****						
	4807	0.2003	0.3586	0.1796	0.0970	****					
C. incana	4790	0.5006	0.6661	0.6016	0.5708	0.5410	****				
	4801	0.5293	0.7139	0.5953	0.5410	0.5708	0.4564	****			
	4804	0.4297	0.5953	0.5469	0.5293	0.5588	0.4673	0.1714	****		
	4809	0.4783	0.7069	0.5293	0.5588	0.5892	0.4950	0.2607	0.2343	****	
C. juncea	Local	0.4350	0.7069	0.5410	0.5588	0.5769	0.6016	0.6205	0.5351	0.6016	****

Table 42. SSR analysis with primer AL-365892 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	6 (900,750,450, 350,300,230)	6 (900,750,450, 350,300,230)	-	-	-
	4803	6 (900,750,450, 350,230,200)	6 (900,750,450, 350,230,200)	1 (450)	-	-
	4805	5 (900,750,450, 350,230)	5 (900,750,450, 350,230)	-	-	-
	4806	8 (900,750,600, 450,350,300, 230,200)	8 (900,750,600, 450,350,300, 230,200)	-	-	-
	4807	8 (900,750,600, 500,450,350, 230,200)	8 (900,750,600, 500,450,350, 230,200)	-	-	-
C. incana	4790	8 (950,780,700, 600,500,250, 200,150)	8 (950,780,700, 600,500,250, 200,150)	-	-	-
	4801	6 (950,700,500,400, 250,150)	6 (950,700,500,400, 250,150)	-	-	-
	4804	7 (950,700,500, 400,250, 200,150)	7 (950,700,500, 400,250, 200,150)	-	-	-
	4809	6 (950,700,500, 400,250,150)	6 (950,700,500, 400,250,150)	-	-	-
C. juncea	Local	3 (780,650,180)	2 (780,650)	1 (180)	2 (650,180)	-

Table 43. SSR analysis with primer AW-127626 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	-	-	-	-	-
	4803	-	-	-	-	-
	4805	-	-	-	-	-
	4806	-	-	-	-	-
	4807	-	-	-	-	-
C. incana	4790	-	-	-	-	-
	4801	-	-	-	-	-
	4804	-	-	-	-	-
	4809	4 (300,250, 200,100)	4 (300,250, 200,100)	-	3 (300,250, 200)	-
C. juncea	Local	3 (270,230, 100)	3 (270,230, 100)	-	2 (270,230)	-

Table 44. SSR analysis with primer AW-584539 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	-	-	-	-	-
	4803	6 (700,600,500, 400,300,100)	6 (700,600,500, 400,300,100)	-	4 (700,500, 400,300)	-
	4805	-	-	-	-	-
	4806	-	-	-	-	-
	4807	-	-	-	-	-
C. incana	4790	-	-	-	-	-
	4801	-	-	-	-	-
	4804	3 (900,800,450)	3 (900,800,450)	-	-	-
	4809	9 (1500,900,800, 750,600,450, 250,200,100)	9 (1500,900,800, 750,600,450, 250,200,100)	-	4 (1500,750, 250,200)	-
C. juncea	Local	3 (600,270,230)	3 (600,270,230)	-	2 (270,230)	-

Table 45. SSR analysis with primer MtSSRNFAW-142 of ten germplasm of *Crotalaria* species.

Species	Acc. No./ Identity	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. pallida	4250	-	-	-	-	-
	4803	1 (280)	1 (280)	-	-	-
	4805	2 (500,280)	2 (500,280)	-	-	-
	4806	1 (280)	1 (280)	-	-	-
	4807	-	-	-	-	-
C. incana	4790	4 (1350,800, 600,300)	4 (1350,800, 600,300)	-	2 (1350, 300)	-
	4801	3 (800,600,500)	3 (800,600,500)	-	-	-
	4804	3 (800,600,500)	3 (800,600,500)	-	-	-
	4809	2 (800,550)	2 (800,550)	-	-	-
C. juncea	Local	-	-	-	-	-

Table 46. Compilation of SSR analysis in ten germplasm of *Crotalaria* species.

Primer pairs	Total bands	Size ranges (bp)	No. of Polymorphic bands	No. of unique bands	Polymorphisms (%)
AL-365892	63	150-950	63	2	100
AW-127626	7	100-300	7	5	100
AW-584539	21	100-1500	21	10	100
MtSSRNFAW-42	16	300-1350	16	2	100
	107	**100-1350**	**107**	**19**	**100**

Table 47. Summary of Nei's (1972) genetic distances of ten germplasm of *Crotalaria* species by SSR analysis.

Species	Acc. No./ Identity	*C. pallida*					*C. incana*				*C. juncea*
		4250	4803	4805	4806	4807	4790	4801	4804	4809	Local
C. pallida	4250	****									
	4803	0.2231	****								
	4805	0.0690	0.1957	****							
	4806	0.0690	0.1957	0.0931	****						
	4807	0.0931	0.2231	0.1178	0.0690	****					
C. incana	4790	0.5108	0.7156	0.5486	0.4745	0.3727	****				
	4801	0.4055	0.6712	0.3727	0.5108	0.4055	0.1691	****			
	4804	0.5486	0.7621	0.5108	0.5878	0.4745	0.2231	0.0931	****		
	4809	0.9163	1.0986	0.9734	1.0986	0.9163	0.6286	0.4394	0.3727	****	
C. juncea	Local	0.4055	0.5878	0.4394	0.5108	0.4745	0.5486	0.5108	0.6712	0.8622	****

Results

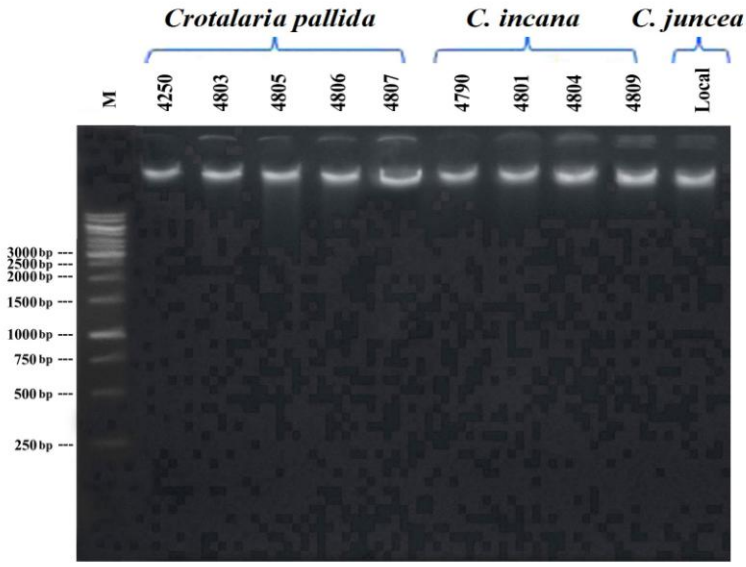


Fig. 191. Electrophoretogram of ethidium bromide stained genomic DNA samples of ten germplasm of *Crotalaria* species. Lane M:1 kb DNA ladder.

Results

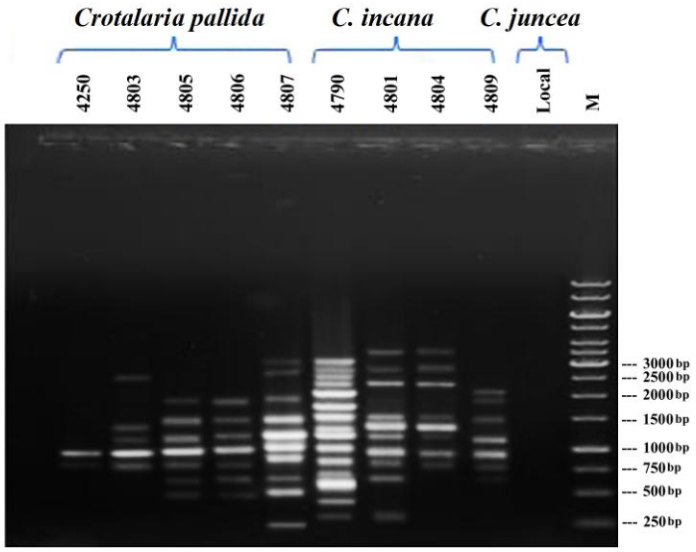


Fig. 192. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPA-4 primer. Lane M:1 kb DNA ladder.

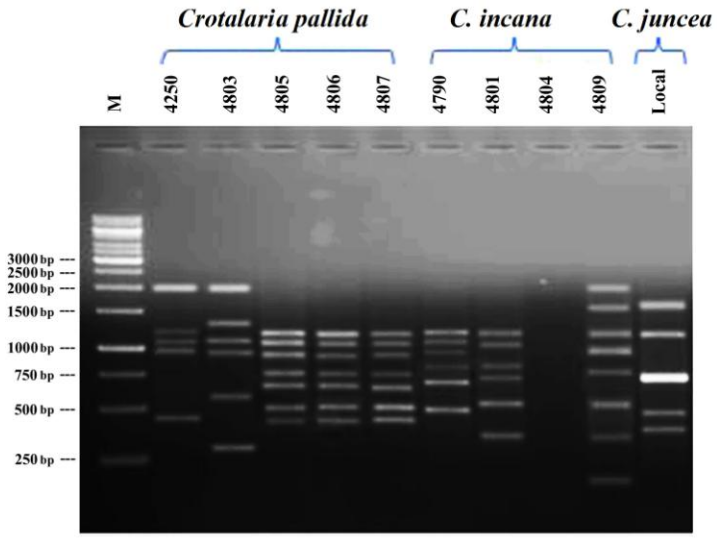


Fig. 193. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPA-18 primer. Lane M:1 kb DNA ladder.

Results

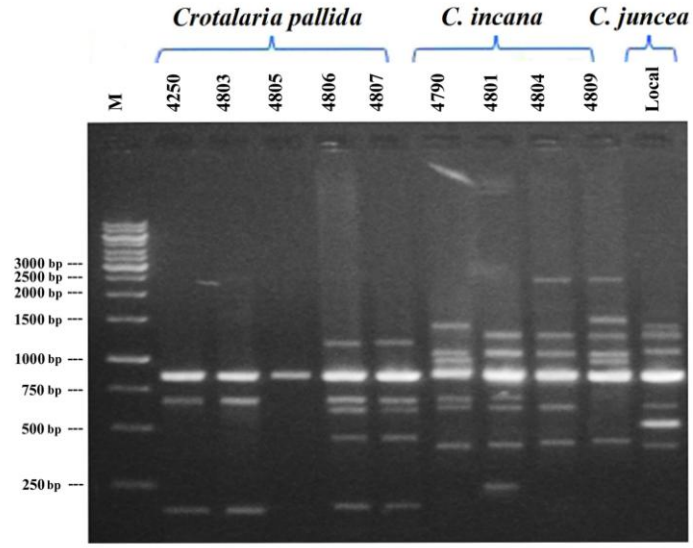


Fig. 194. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPAB-5 primer. Lane M:1 kb DNA ladder.

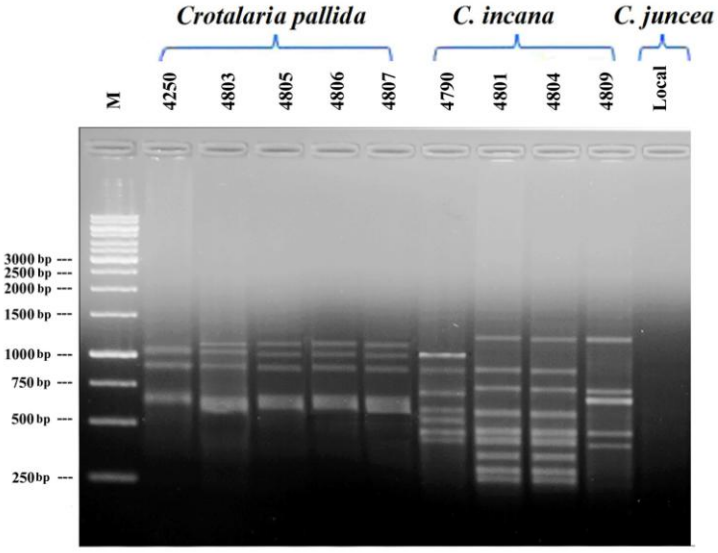


Fig. 195. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPAB-6 primer. Lane M:1 kb DNA ladder.

Results

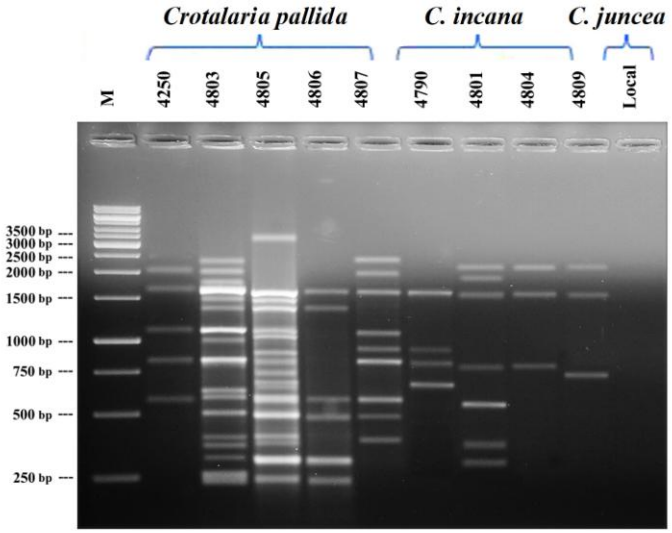


Fig. 196. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPC-10 primer. Lane M:1 kb DNA ladder.

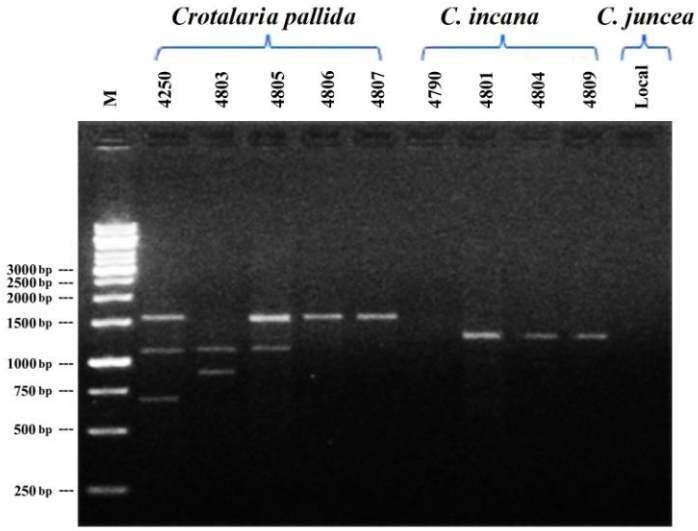


Fig. 197. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPC-13 primer. Lane M:1 kb DNA ladder.

Results

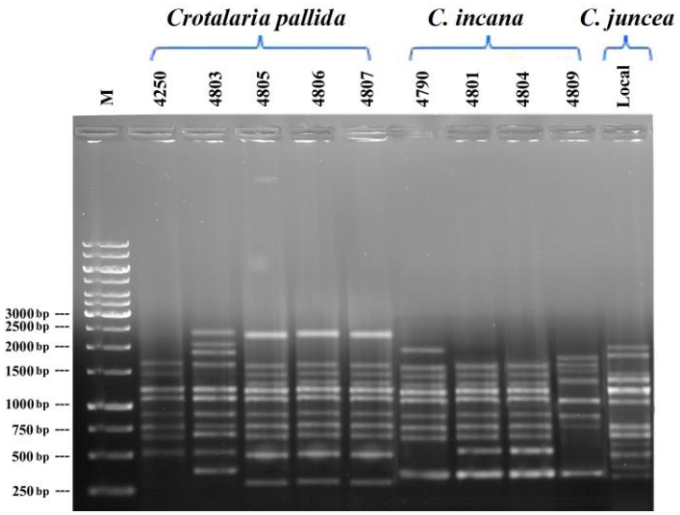


Fig. 198. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPC-14 primer. Lane M:1 kb DNA ladder.

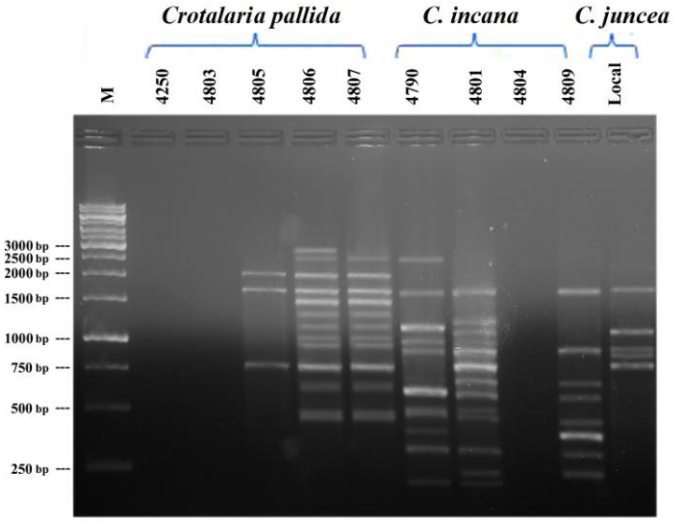


Fig. 199. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPC-16 primer. Lane M:1 kb DNA ladder.

Results

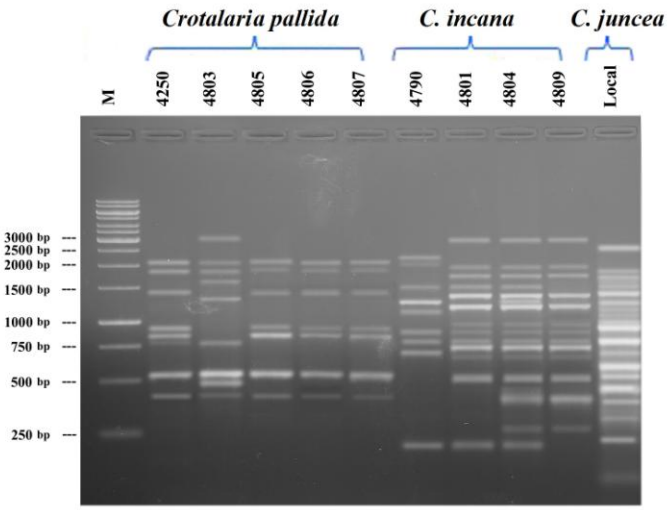


Fig. 200. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPC-26 primer. Lane M:1 kb DNA ladder.

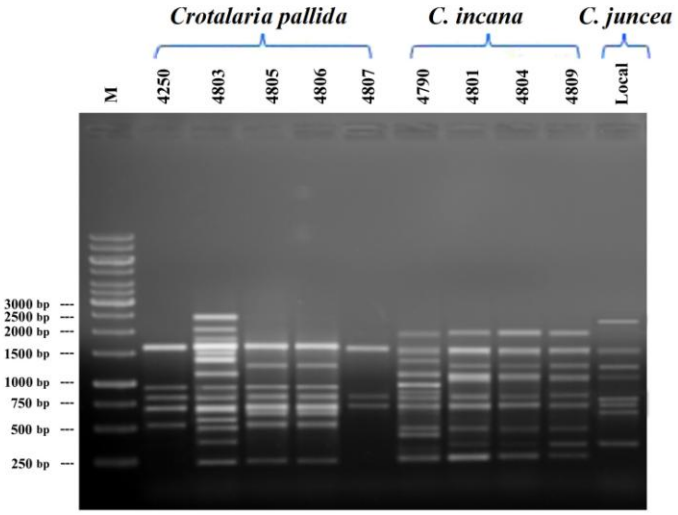


Fig. 201. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPC-96 primer. Lane M:1 kb DNA ladder.

Results

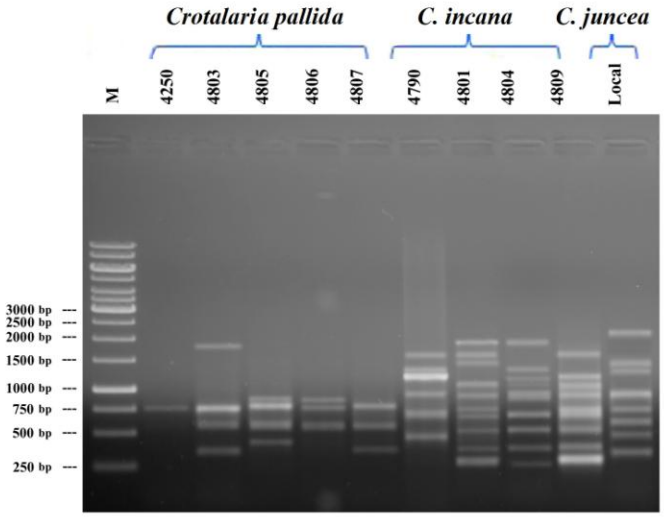


Fig. 202. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPF-22 primer. Lane M:1 kb DNA ladder.

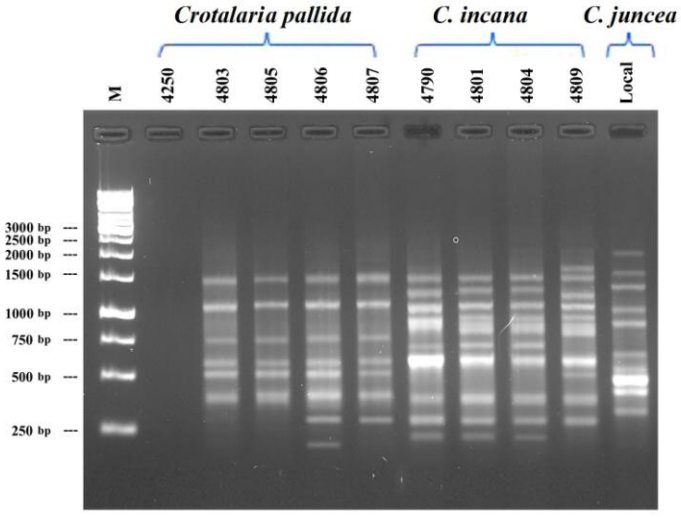


Fig. 203. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPG-3 primer. Lane M:1 kb DNA ladder.

Results

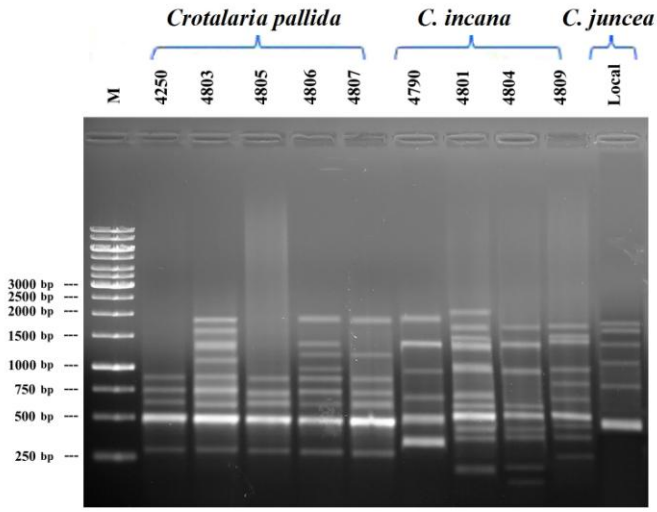


Fig. 204. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPG-6 primer. Lane M:1 kb DNA ladder.

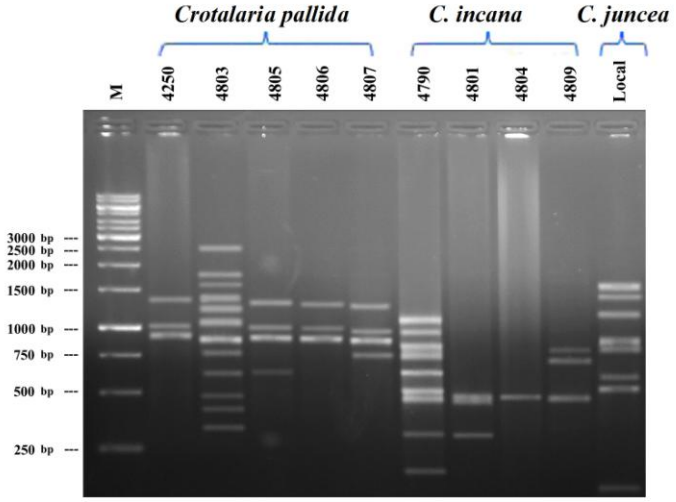


Fig. 205. RAPD profiles of ten germplasm of *Crotalaria* species showing amplification of bands with OPG-9 primer. Lane M:1 kb DNA ladder.

Results

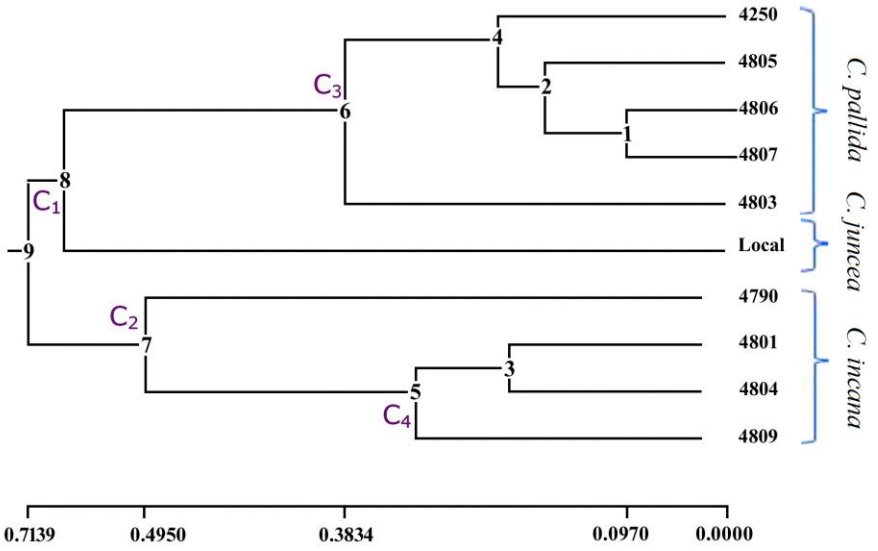


Fig. 206. UPGMA dendrogram contructed based on Nei's (1972) genetic distance summarizing the data on differentiation among ten germplasm of *Crotalaria* species by RAPD analysis.

Results

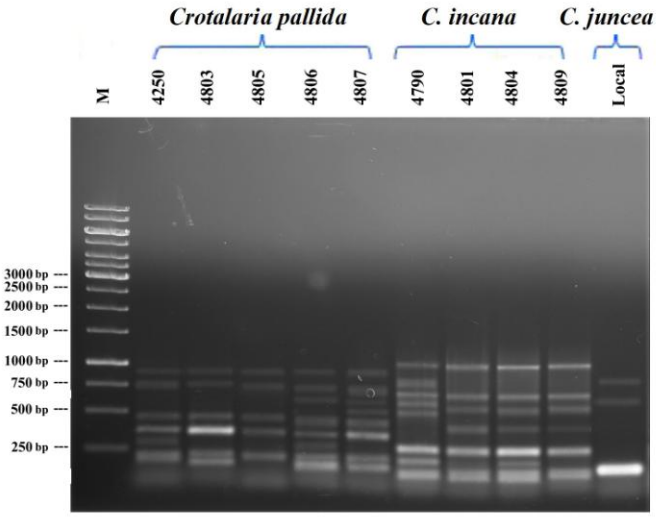


Fig. 207. SSR profiles of ten germplasm of *Crotalaria* species showing amplification of bands with primer pair AL-365892. Lane M:1 kb DNA ladder.

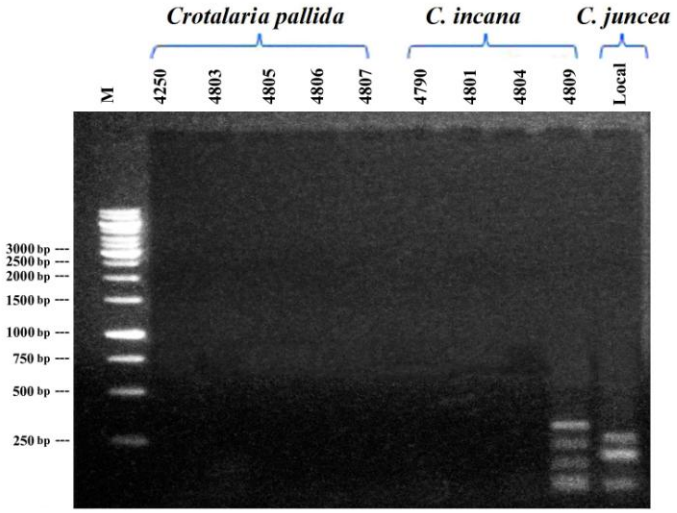


Fig. 208. SSR profiles of ten germplasm of *Crotalaria* species showing amplification of bands with primer pair AW-127626. Lane M:1 kb DNA ladder.

Results

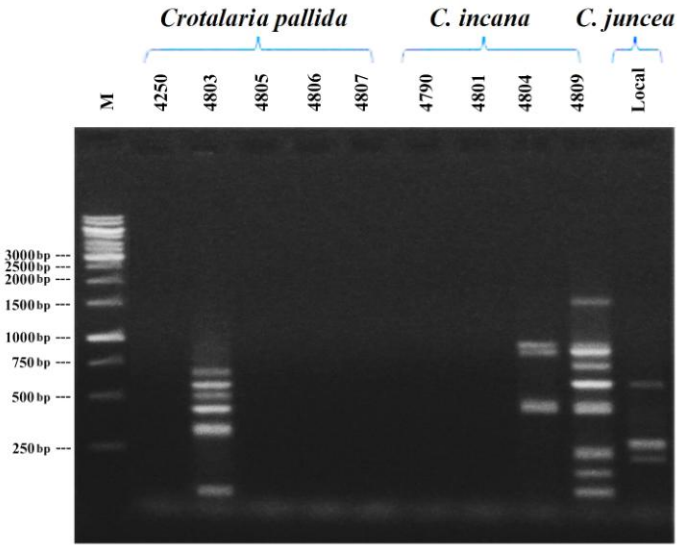


Fig. 209. SSR profiles of ten germplasm of *Crotalaria* species showing amplification of bands with primer pair AW-584539. Lane M:1 kb DNA ladder.

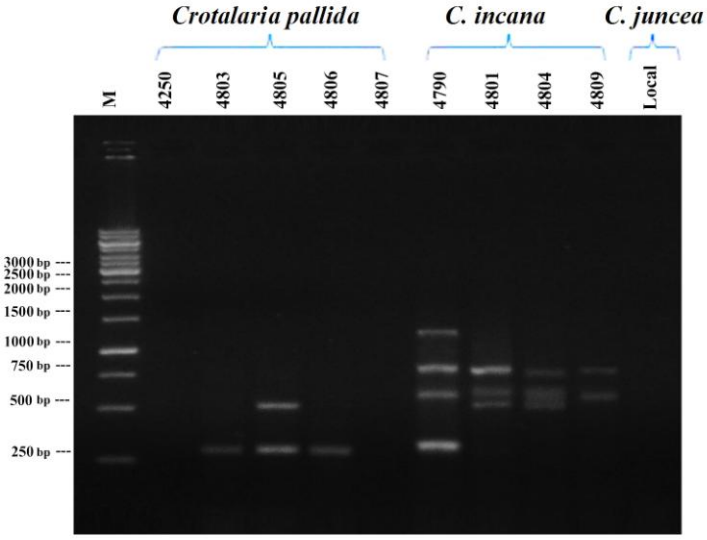


Fig. 210. SSR profiles of ten germplasm of *Crotalaria* species showing amplification of bands with primer pair MtSSRNFAW-142. Lane M:1 kb DNA ladder.

Results

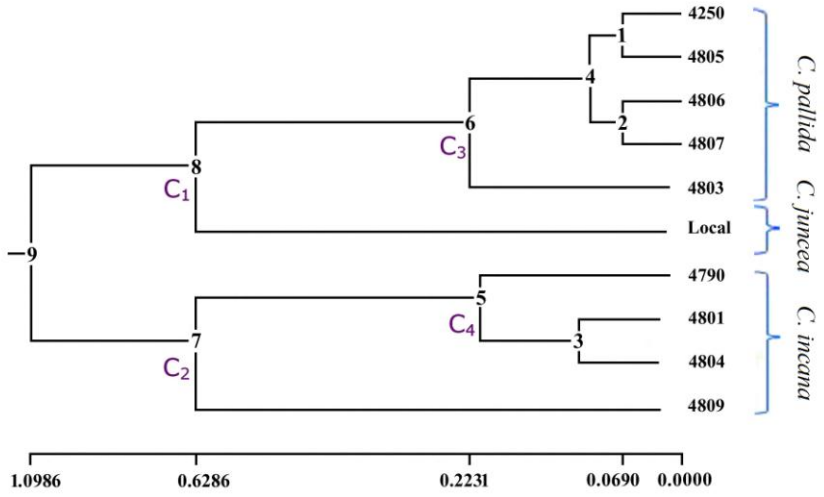


Fig. 211 UPGMA dendrogram contructed based on Nei's (1972) genetic distance summarizing the data on differentiation among ten germplasm of *Crotalaria* species by SSR analysis.

4. DISCUSSION

4. DISCUSSION

Ten germplasm of *Crotalaria* species *viz. C. pallida* (Acc. No. 4250, 4803, 4805, 4806 and 4807), *C. incana* (Acc. No. 4790, 4801, 4804 and 4809) and *C. juncea* (Local) were characterized cytogenetically following orcein, CMA- and DAPI-staining. The nature of differential staining property of interphase nuclei and prophase chromosomes were accomplished to get additional information towards characterization of the *Crotalaria* germplasm. Apart from this cytogenetical investigation, experiments were conducted to study the genetic relationship among the *Crotalaria* germplasm with the help of RAPD and SSR markers.

4.1 Orcein-staining properties of interphase nuclei and prophase chromosomes

The staining properties of interphase nuclei and prophase chromosomes usually provide karyomorphological features that help to characterize different germplasm. Tanaka (1971) was the pioneer of proposing these criteria for karyomorphological investigation. He found that the nature of staining of heterochromatins present in the interphase nuclei and prophase chromosomes were different in different species. On the basis of the staining property he classified interphase nuclei and prophase chromosomes in five different categories. Later different investigators applied these criteria in characterizing different plant materials of diverge nature (Alam *et al.* 1993, Begum and Alam 2004, Hossain *et al.* 2016, Sultana and Alam 2016a, Bonna *et al*. 2018).

In this study, interphase nuclei of ten *Crotalaria* germplasm were grouped into three categories such as “Diffuse type”, “Simple chromocenter type” and “Complex chromocenter type” on the basis of nature of staining properties of heterochromatin after orcein staining.

Interphase nuclei of Acc. No. 4250 and 4807 of *C. pallida,* Acc. No. 4804 of *C. incana* were homogenously stained with orcein (Figs 41, 45 and 48; Table 48). According to Tanaka (1971), these could be regarded as “Diffuse Type”. In contrast, several number of small heteochromatic regions were scatterdly distributed around the nucleus of Acc. No. 4803 and 4806 in *C. pallida* which could be considered as “Simple chromocenter type” according to Tanaka 1971 (Figs 42 and 44; Table 48). On the other hand, few large hererochromatic regions were observed in the interphase nuclei of Acc. No. 4805 in *C.*

pallida, Acc. No. 4790, 4801, 4809 in *C. incana* and local germplasm of *C. juncea* (Figs 43, 46, 47 and 49; Table 48). These types of interphase nuclei are considered as "Complex chromocenter type" according to Tanaka 1971.

The prophase chromosome of Acc. No. 4250, 4805 in *C. pallida* and local germplasm of *C. juncea* were darkly stained at one end and gradually become faint towards another end (Figs 71, 73 and 80; Table 48). According to Tanaka 1971, this type of prophase chromosome could be regarded as "Gradient type". However, "Continuous type" of prophase chromosomes were found in Acc. No. 4803, 4806, 4807 in *C. pallida* and 4790, 4801, 4804, 4809 in *C. incana* where prophase chromosomes were uniformly stained along the length with orcein (Figs 72, 74, 75, 76, 77, 78 and 79; Table 48).

Usually, germplasm with "Diffuse type" of interphase nuclei showed "Continuous type" of prophase chromosome and "Simple or Complex chromocenter type" of interphase nuclei showed "Gradient type" of prophase chromosomes. In this study, Acc. No. 4805, 4807 in *C. pallida*; 4804 in *C. incana* and local germplasm of *C. juncea* followed the general rule. However, the other six germplasm belonging to three *Crotalaria* species did not follow the usual regulation of heterochromatin. Presence of facultative heterochromatin might be one of the reasons for this type of observation. Whatever the reason is, the ten germplasm could be characterized on the basis of these characters of interphase nuclei and prophase chromosomes.

Table 48. Type of Interphase nuclei and prophase chromosomes of *Crotalaria* species after staining with orcein.

Species	Acc. No./ Identity	Type of Interphase Nuclei	Type of Prophase Chromosomes
C. pallida L.	4250	Diffuse type	Gradient type
	4803	Simple chromocenter type	Continuous type
	4805	Complex chromocenter type	Gradient type
	4806	Simple chromocenter type	Continuous type
	4807	Diffuse type	Continuous type
C. incana L.	4790	Complex chromocenter type	Continuous type
	4801	Complex chromocenter type	Continuous type
	4804	Diffuse type	Continuous type
	4809	Complex chromocenter type	Continuous type
C. juncea L.	Local	Complex chromocenter type	Gradient type

4.2 Nucleolus

A nucleolus was observed in interphase and prophase stages in Acc. No. 4803 and 4806 of *C. pallida* (Figs 42, 44, 72 and 74). In addition, although nucleolus was not found in interphase nuclei but was appeared in prophase stage in Acc. No. 4807of *C. pallida* (Fig. 75). In *C. incana*, a nucleolus was found in interphase nuclei of all germplasm except Acc. No. 4804 (Figs 46, 47 and 49). However, a nucleolus was found in prophase chromosome in all germplasm studied (Figs 76, 77, 78 and 79). In Acc. No. 4806 of *C. pallida*, this nucleolus was observed in metaphase stage also (Figs 104a-104c). Usually the nucleolus disappears at late prophase of mitosis. There is considerable evidence that it is not unusual for plant nucleoli to persist in mitotic metaphase or later. Persistent nucleolar materials were of frequent occurrence at prometaphase, metaphase, anaphase and even sometimes in telophase. In the majority of cases, nucleoli appeared as clear entities, usually almost round in shape. They varied in size from small, hardly detectable structures to large conspicuous ones. These observations suggested the late transcription of rDNA to rRNA and late transportation of rRNA from the nucleus to the cytoplasm. Persistent nucleolus was observed in few species such as *Spartocera fusca* (Cattani and Papeschi 2004), *Zea mays* (Zirkle 1928), telophase stage in *Oryza sativa* (Ramanujam 1938), *Ceiba pentandra* (Tijo 1948), 16 species of *Cassia* (D'Amato Avanzi 1953), 10 species of *Reseda* (Gori 1956), 11 species of *Gossypium* (Sultana and Alam 2016a) and 45 species of the family Gramineae (Walter and Emery 1957). However, no available report on persistent nucleolus was found in any species of *Crotalaria.* Therefore, the persistent nature of nucleolus is considered as a salient feature of *Crotalaria* species.

4.3 Orcein-karyotype

For genetic characterization karyotype analysis with orcein-staining has been reported as a stable and reliable method which provides basic information about genetic makeup of a particular material.

4.3.1 2n chromosome number

In this investigation, three species of *Crotalaria* L. *viz. C. pallida, C. incana* and *C. juncea* were found to possess different somatic chromosome numbers. In *C. pallida* 2n = 16 (Acc. No. 4803, 4805 and 4807) and 2n = 18 (Acc. No. 4250 and 4806) chromosomes were observed. According to the previous chromosome number records, 2n = 8, 14 and

16 chromosomes were reported for *C. pallida* (Table 1). The results of the present investigation only correlates with the previous reports of 2n = 16 (Gupta and Gupta1978a, Gupta and Gupta1978b, Raina and Verma 1979, Verma and Raina1983, Li 1988, Kumari and Bir 1990, Mangotra and Koul 1991) . Therefore, 2n = 18 chromosomes observed in this study is the first report for *C. pallida* (Figs 101a-101c and 104a-104c; Tables 13 and 16). *Crotalaria incana* were found to possess 2n = 16 (Acc. No. 4801); 2n = 17 (Acc. No. 4790 and 4804) and 2n = 18 (Acc. No. 4809) chromosomes. Previously 2n = 14 and 2n = 16 chromosomes were reported in *C. incana* of which 2n = 16 was similar to the present finding (Mangotra and Koul 1991). Therefore, 2n = 17 and 18 chromosomes found in *C. incana* was the first report for this species (Figs 106a-106c, 108a-108c and 109a-109c; Tables 18, 20 and 21). 2n = 16 somatic chromosome number was observed in *C. juncea* (Figs 110a-110c; Table 22). Previously different chromosome number was recorded for *C. juncea* such as 2n = 8, 14 and 16 (Table 1). However, 2n chromosome number 8 and 14 was not found in the present observation.

The presence of different chromosome number in different germplasm of *Crotalaria* has created a confusion regarding the basic chromosome number. According to previous reports, *Crotalaria* could be considered as a genus with multiple basic chromosome number *viz.* x = 7 and x = 8 (Subramaniam and Pandey 2013). Therefore, the Acc. No. 4803, 4805 and 4807 of *C. pallida*; Acc. No. 4801 of *C. incana* and local germplasm of *C. juncea* with 2n = 16 represents basic chromosome number x = 8. On the other hand, 2n = 18 of Acc. No. 4250 and 4806 of *C. pallida*; Acc. No. 4809 of *C. incana* represents basic chromosome number x = 9. However, 2n = 8 and 2n = 14 was not found in this investigation. 2n = 17 in Acc. No. 4790 and 4804 of *C. incana* might be originated either by intra-specific hybridization between 2n = 18 and 2n = 16 germplasm or by aneuploid origin that correlates with their phenotypic features (seedless pod production). On the other hand, somatic chromosome number 2n = 8 (Verma and Raina 1978) for the species of *C. pallida* and *C. juncea* might be originated from hybridization followed by uniparental chromosome elimination, reductional mitosis or hypo-euploidy. In contrast, 2n = 14 (Patil and Chennaveeraiah 1975, Gupta and Gupta 1978, Patil 1983, Verma and Raina 1991, Palomino and Ricardo 1991, Olivera and Aguiar-Perecin 1999, Almada 2006 and Flores 2006) might represent basic chromosome number x = 7. Previous studies indicated almost 87% of species studied in the genus *Crotalaria* had basic chromosome number x = 8 (Bhandari *et al.* 2016). It could be conceptualized that the species with the

basic chromosome number x = 7 and x = 9 might be evolved from those with basic chromosome number x=8. Whatever the reason is, the basic chromosome number x=9 (2n = 18) is a new report for the genus *Crotalaria*.

The above observation indicated that aneuploidy and hybridization played important role in the evolution of a series of new basic chromosome numbers, accompanied with the diversification of species within the genus *Crotalaria*.

4.3.2 Centromeric formulae

In five germplasm of *C. pallida*, the range of chromosomal length was almost negligible *i.e.* distance between small and large chromosomes was about 1 µm (Table 23). Out of five germplasm, all metacentric chromosomes were found in Acc. No. 4805. Presence of all metacentric chromosomes is a feature of symmetric karyotype (Stebbins 1971). In contrast, few submetacentric chromosomes were observed in rest four germplasm of *C. pallida* (Table 23). This feature indicated relatively asymmetric nature of their karyotype. (Figs 101-105 and 131-135; Table 23).

In *C. incana*, Acc. No. 4790 and 4801 were found to possess all metacentric chromosomes which indicated their symmetric nature of karyotype (Figs 106, 107, 136 and 137, Table 23). In contrast, Acc. No. 4804 and 4809 showed two and fourteen submetacentric chromosomes, respectively (Figs 108, 109, 138 and 139, Table 23). On the basis of this karyotypic feature, these two germplasm could be regarded as moderately asymmetric in nature (Table 23). In these four germplasm, the range of chromosomal length was almost negligible *i.e.* distance between small and large chromosomes was about 1 µm (Table 23). As a result, lesser difference in chromosomal length was observed in their karyotypes (Table 23). Therefore, a combination of symmetric and relatively asymmetric karyotype were found in different germplasm of *C. incana*. Stebbins (1971) mentioned that the symmetric karyotypes were primitive character. Among the four germplasm of *C. incana*, Acc. No. 4809 was comparatively advanced than the other three germplasm.

Crotalaria juncea were found to possess all metacentric chromosomes representing the homogenous karyotype. According to Stebbins (1971) this karyotype was primitive in nature.

4.3.3 Probable reasons for the origin of submetacentric chromosomes

The submetacentric chromosomes might be originated from metacentric chromosomes by some chromosomal aberration *viz.* terminal deletion, pericentric inversion, non reciprocal or unequal translocation between fragments of chromosomes within *Crotalaria* species.

4.4 Fluorescent-Banding

Chromomycin A_3 (CMA) and 4´-6-Diamidino-2-Phenylindole (DAPI) are two fluorochromes specific to GC- and AT-rich base specific segments, respectively (Schweizer 1976). Fluorescent banding gives decisive analysis of karyotype, even chromosome having similar morphology and other conventional karyotypic features. In this study, these two fluorochromes were used for critical analysis of karyotype of ten *Crotalaria* germplasm.

Characterization of *Crotalaria* germplasm by CMA and DAPI was the pioneer attempt in Bangladesh.

4.4.1 CMA-banding pattern

CMA-banding technique provides information regarding GC-rich repeats in the genome. In addition, chromosomal aberrations could also be detected by this method.

4.4.1.1 Nature of CMA-band

After CMA-banding, ten *Crotalaria* germplasm generated 31 centromeric and 34 terminal bands which indicated a tendency of accumulating GC-rich sequences at centromeric regions or chromosomal ends (Zaman and Alam 2009). In addition, 12 chromosomes were entirely fluoresced with CMA. In this entirely fluoresced chromosomes, GC-rich repeats were not confined to the terminal or centrometic region rather distributed along the chromosomes. The possible reason for these entirely fluoresced chromosomes was tandem duplication of GC-rich repeats (Schweizer 1976, Hiron *et al.* 2006, Mahbub *et al.* 2007, Sultana and Alam 2007, Khatun and Alam 2010, Sultana and Alam 2016b).

The karyotypes of ten *Crotalaria* germplasm were compared critically after CMA-staining. The germplasm used in this study have distinct CMA-banding pattern. The number, location, distribution and intensities of CMA-bands varied in different

germplasm. The number of CMA-bands varied from 3 (Acc. No. 4804 and 4809 of *C. incana*) to 16 (*C. juncea,* and Acc. No. 4803 of *C. pallida*) (Figs 141-150 and 171-180; Table 24). Total length of CMA-positive banded region ranging from 0.60 ± 0.05µm (Acc. No. 4809 of *C. pallida*) to 7.21 ± 0.05µm (Acc. No. 4807 of *C. pallida*). The percentage of GC-rich repeats was lowest (2.53µm) in Acc. No. 4809 of *C. incana* and highest (31.57 µm) in Acc. No. 4807 of *C. pallida* (Table 24).

4.4.1.2 Heteromorphic CMA-band

In case of *C. pallida*, heteromorphicity was found in chromosome pair IV, V and IX of Acc. No. 4250. One member of pair IV and V, showed centromeric and terminal band, respectively while no band was observed in their homologue suggesting probable deletion of the banded region from the respective chromosomes. In pair IX, one chromosome fluoresced entirely while its homologue member had terminal CMA-positive band on short arm. This feature of heteromorphicity was also found in same pair of Acc. No. 4806. In this case, GC-rich repeats of one member may tandemly duplicated along the length of the respective chromosomes (Figs 141 and 144). In Acc. No. 4803, heteromorphicity was observed in chromosome pair VIII. In this germplasm, a terminal band was found in a member of that pair, whereas the other member had an interstitial band. These banding features indicated a probable inversion either from terminal to interstitial or interstitial to terminal region (Fig. 142). Heteromorphicity was observed in chromosome pair V and VIII in Acc. No. 4805. One member of these pairs had terminal band while their homologue member did not show band. Similar kind of heteromorphicity was observed in pair III of Acc. No. 4807. The reasons for this heteromorphicity may be deletion of the banded region from the respective chromosomes (Figs 143 and 145).

In case of *C. incana*, heteromorphicity was found in all germplasm after CMA-staining. In Acc. No. 4790 and 4804, heteromorphicity was observed in chromosome pair I. One member of pair I in Acc. No. 4790 had centromeric band while no band was observed in its homologue. Similarly an interstitial band was observed in one member of pair I in Acc. No. 43804 without any band in its homologue. The above feature suggested probable deletion of the banded region from the respective chromosomes (Figs 146 and 148). In case of Acc. No. 4801 and 4809, heteromorphicity was found in chromosome pair VI and VIII, respectively. In these pairs one member had a terminal band while no

band was present in the other member. The reasons for this heteromorphicity may be deletion of the banded region from the respective chromosomes (Figs 147 and 149).

However, comparative karyotype analysis after CMA-banding revealed the occurrence of structural aberration such as deletion, tandem duplication and inversion in the ten *Crotalaria* germplasm.

4.4.2 DAPI-banding pattern

Fluorescent banding technique with DAPI fluorochromes helps to provided information regarding AT-rich repeats in the genome. In addition, different type of chromosomal aberration such as deletion, duplication, inversion, etc. could also be detected by this method.

4.4.2.1 Nature of DAPI-bands

The number of DAPI-bands was less than that of the CMA-band found in different germplasm of *Crotalaria*. After DAPI-banding ten germplasm of *Crotalaria* generated a total of 16 terminal bands. The presence of terminal DAPI-bands indicated a tendency of accumulating AT-rich repetitive sequences at the chromosomal ends (Sultana and Alam 2007, Sultana and Alam 2016b). Few DAPI-bands were found at centromeric regions of the respective chromosomes revealing the presence of AT repeats in those regions. In addition to terminal and centromeric bands, few chromosomes were entirely fluoresced with DAPI. In these entirely fluoresced chromosomes, AT-rich repeats were not confined to the terminal or centromeric region rather distributed along the chromosomes. The probable reason for entirely florescence was i) either these chromosomes were completely AT-rich by nature or ii) due to successive duplication of AT-rich repeats (Hiron *et al.* 2006, Mahbub *et al.* 2007, Sultana and Alam 2016b).

The karyotypes of ten *Crotalaria* germplasm were compared critically after DAPI-staining. The germplasm used in this study have distinct DAPI-banding pattern. The number, location, distribution and intensities of DAPI-bands varied in different germplasm. The number of DAPI-bands varied from 2 (Acc. No. 4809 of *C. pallida*) to 9 (Acc. No. 4804 of *C. incana*) among ten germplasm of *Crotalaria* (Figs 151-160 and 181-190; Table 25). Total length of DAPI-positive banded region ranging from 0.58 ± 0.05µm (Acc. No. 4809 of *C. pallida*) to 5.35 ± 0.04µm (Acc. No. 4804 of *C. incana*).

The percentage of GC-rich repeats was lowest (2.45µm) in Acc. No. 4809 of *C. incana* and highest (24.49µm) in Acc. No. 4804 of *C. incana* (Table 25). No DAPI-band was found in two germplasm of *C. pallida* (Acc. No. 4805 and 4807) and *C. juncea* (Table 25).

4.4.2.2 Heteromorphic DAPI-band

In case of *C. pallida*, after DAPI-staining, heteromorphicity was found in chromosome pair IV and VII of Acc. No. 4250. In pair IV, one chromosome fluoresced entirely while its homologue member had DAPI -positive bands along with long arm. In this case, AT-rich repeats of one member may be tandemly duplicated along the length of the respective chromosomes. A terminal band was found in a member of pair VII while the other member had no band. The reasons for this heteromorphicity may be deletion of the banded region from the respective chromosomes (Figs 151 and181). In Acc. No. 4803, heteromorphicity was observed in chromosome pair V. In this pair, one chromosome fluoresced entirely except centromere while its homologue member had no DAPI-positive band. In this case, AT-rich repeats of one member may be tandemly duplicated along the length except centromere of the respective chromosomes (Figs 152 and 182). Heteromorphicity was observed in chromosome pair IX in Acc. No. 4806. In this pair, one chromosome fluoresced entirely while its homologue member had terminal DAPI-positive band on its short arm. In this case, AT-rich repeats of one member may be tandemly duplicated along the length of the respective chromosomes (Figs 154 and 184).

In case of *C. incana*, heteromorphicity was found in three germplasm after DAPI-staining. In Acc. No. 4790, heteromorphicity was observed in chromosome pair VII. One chromosome fluoresced entirely while its homologue member had no DAPI-positive band in this pair. This type of heteromorphicity was also found in pair IV of Acc. No. 4804. In this case, AT-rich repeats of one member may be tandemly duplicated along the length of the respective chromosomes. On the other hand, in pair VI of Acc. No. 4790, a chromosome had terminal band and no band was observed in its homologue suggesting probable deletion of the banded region from the respective chromosome. Similar kind of heteromorphicity was found in pair IV of Acc. No. 4801. Besides this, heteromorphicity was also found in pair VIII of Acc. No. 4801. A chromosome of this pair had band on whole short arm while no band was observed in its homologue suggesting probable

deletion of the banded region from the respective chromosome (Figs 156-158 and 186-188).

However, comparative karyotype analysis after DAPI-banding revealed the occurrence of structural aberration such as deletion and tandem duplication in the ten *Crotalaria* germplasm.

4.4.2.3 Marker chromosomes

In Acc. No. 4803 of *C. pallida*, a member of chromosome pair V fluoresced entirely with DAPI except centromere (Figs 152 and 182). In Acc. No. 4804 of *C. incana*, both members of chromosome pair I had DAPI-positive bands at centromere and also at terminal region (Figs 158 and 188). These chromosomes are unique since absent in the rest germplasm and thus could be used as a marker for these germplasm.

4.5 DNA fingerprinting

The second phase of this investigation was conducted to study the genetic relationship among the ten *Crotalaria* germplasm with the help of RAPD and SSR method technology. For this purpose fourteen oligonucleotide primers and four microsatellite primer pairs for RAPD and SSR were utilized, respectively. The data obtained following RAPD and SSR techniques were analyzed using "popgene32" computer package. The data were used to generate dissimilarity matrix for both RAPD and SSR bands obtained through gel electrophoresis. The distance matrix between each pair of germplasm was used to construct two separate dendograms using unweighted pair group method of arithmetical means (UPGMA). Ten *Crotalaria* germplasm represented a broad spectrum of variation in both RAPD and SSR banding pattern.

4.5.1 DNA fingerprinting by Random Amplified of Polymorphic DNA (RAPD)

RAPD is a PCR based marker technique that has been used for estimation of genetic diversity of populations and for studying the genetic relationships among different genotypes (Esmail *et al.* 2008).

4.5.1.1. Primer selection

A total of eighteen primers were used initially in this study those differed greatly in their efficiency for revealing polymorphism. Four of them failed to amplify DNA. Since all of the reaction parameters were identical for all primers, differences in the clarity of the banding patterns are likely due to the specific requirements (such as PCR reagents or temperature profile) of a primer. Weeden *et al.* (1992) also reported variation in the efficiency of primer amplification. Fritsch *et al.* (1993) demonstrated the importance of the G+C content of primers on the PCR yield of detectable on amplified products. However, no correlation between G+C content and the clarity of the banding pattern was noted in the present study.

On the other hand, the remaining fourteen primers generated multiband fingerprinting, which easily scorable and reproducible. This suggested that fourteen primers could be able to bind with suitable priming sites of the genomic DNA. Moreover, this revealed the proper adjustment of PCR parameters (reagents and thermocycler parameters) and thus optimized the amplification (Devos and Gale 1992). Therefore, these fourteen primers have been selected for subsequent analysis.

4.5.1.2 Polymorphism as detected by RAPD analysis

The primer sequence, band size and banding pattern of ten *Crotalaria* germplasm were shown in Tables 7 and 26-40. The fourteen primers generated 931 distinct bands of which 881 were considered as polymorphic. However, these ten *Crotalaria* germplasm shared 50 common DNA fragments (4.43%) after RAPD analysis with fourteen primers. Band size ranging from 50-3500 bp of PCR amplification products scored for all primers. Light and bright bands were produced in the RAPD reactions. Light bands produced from low homology between the primer and the pairing site on the DNA strand (Thormann *et al.* 1994).

A diverse level of polymorphism in different crops have been reported earlier such as Chickpea 98.14% (Rasool 2013), 87.00% (Datta *et al*. 2010), 14.56% (Sant *et al*. 1999) and 25.5% (Sonnante *et al*. 1997); *Brassica* 98.03% (Ghosh *et al.* 2009); Eggplant 57.89% (Biswas *et al*. 2009); Chilli 90% (Paran *et al.* 1998); Cotton 84.95% (Esmail *et al.* 2008), 90.96% (Maleia *et al.* 2010), 69.37% (Saravanan *et al.* 2006), 63.20% (Hussain *et al.* 2007) and 100% (Sultana and Alam 2016c); peanut 96% (Lang and Hang 2007), 42.7% (Raina *et al.* 2001) and 21% (He and Prakash 1997); Groundnut 6.68%

(Subramanian *et al.* 2000); *Crotalaria* 48% (Wang *et al.* 2006). The results of the present investigation showed 95.57% polymorphism among ten germplasm. The broad range of polymorphism revealed wide diversity in *Crotalaria* germplasm.

Therefore, the ten germplasm used in this study were highly diversed from each other. The diversification would be very useful for improved breeding programme of *Crotalaria*.

4.5.1.3 Unique RAPD markers

In addition to polymorphism, 86 unique RAPD sequences were identified in ten *Crotalaria* germplasm using fourteen different primer combinations. The term unique sequence means that the sequence found in a germplasm with a certain primer was absent in other germplasm (Figs 192-205, Tables 26-40). In the earlier literature, there was no information about unique band (Wang *et al.* 2006). The unique bands were stable and specific for the respective germplasm and thus could be used as a tool for characterization.

4.5.1.4 Genetic relationships among ten *Crotalaria* germplasm

The values of pair-wise Nei's (1972) genetic distances analyzed by using computer software "popgene32" among ten germplasm of *Crotalaria* species were computed from combined data for the fourteen RAPD primers ranging from 0.0970 0.7139 (Table 41). The highest genetic distance (0.7139) was observed between a germplasm of *C. pallida* (Acc. No. 4803) and *C. incana* (Acc. No. 4801). Among the five germplasm of *C. pallida* the lowest (0.0970) genetic distance was observed between Acc. No. 4806 and Acc. No. 4807. On the other hand, the highest genetic distance (0.3834) was found among Acc. No. 4803 and 4806 of *C. pallida*. In case of four germplasm of *C. incana*, the lowest (0.1714) genetic distance was observed between Acc. No. 4801 and Acc. No. 4804 whereas, the highest genetic distance (0.4950) was found between Acc. No. 4790 and 4809 (Fig. 206; Table 41).Wide genetic diversity in different crops have been reported earlier (Molla *et al.* 2010, Sultana and Alam 2016c)

The difference between the highest and the lowest value of genetic distance revealed the wide range of variability persisting among the ten *Crotalaria* germplasm. High genetic distance values between germplasm pairs were found due to difference in genetic

constituent. The germplasm of lowest genetic distance can be used as parental source for breeding line to improve *Crotalaria* germplasm.

4.5.1.5 Cluster analysis (Tree Diagram)

A cluster analysis on the basis of DNA fingerprinting by RAPD was carried out. Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) segregates ten germplasm of *Crotalaria* into two major clusters *viz.* cluster -1 (C_1) and cluster -2 (C_2). Cluster -1 placed *C. juncea* alone with a high genetic distance of 0.7139. In contrast, the five germplasm of *C. pallida* formed cluster -3 (C_3) under cluster -1. According to cluster -3, Acc. No. 4806 and 4807 are closely related with narrow genetic distance 0.0970 while Acc. No. 4803 is distantly related from other four germplasm of *C. pallida* with large genetic distance (0.3834). Acc. No. 4790 of *C. incana* was placed alone under cluster -2 whereas other three germplasm grouped into cluster -4 (C_4). Within cluster -4, Acc. No. 4801 and 4804 was closely related with narrow genetic distance of 0.1714 (Fig. 206).

4.5.2. DNA fingerprinting by Simple Sequence Repeats (SSRs)

Five SSR primer pairs were used in this study of which four generated well-defined and reproducible polymorphic bands. The primer sequence, band size and banding pattern of ten *Crotalaria* germplasm were given in Tables 8, 43-47 and in Figs 207-210.

4.5.2.1. Polymorphism as detected by SSR analysis

The four primer pairs generated 107 distinct bands of which all were considered as polymorphic and thus showed 100% polymorphisms which indicated the high level of polymorphisms (Table 47). The size of band was ranging from 100 to 1350 bp. Out of four, the primer pair AL-365892 produced highest number (63) of polymorphic bands (Fig. 207; Table 47). Lowest number (7) of polymorphic bands was produced by the primer pair AW-127626 (Fig. 208; Table 47). In contrast, the primer pairs did not generate any common band. The common band indicated the sharing of similar DNA fragments among ten *Crotalaria* germplasm. Thus absence of common band resulting the 100% polymorphism in ten germplasm of *Crotalaria.* The present study supported the observations of several scientists on the potential utility of SSR markers in

characterization of different plant species (Satyavathi *et al.* 2006, Sethy *et al.* 2006, Tang *et al.* 2006, Shoba *et al.* 2010, Bharadwaj *et al.* 2010, Sultana and Alam 2016c).

Therefore, ten germplasm used in this study were highly diverged from each other. The diversification would be useful for improved breeding programme of *Crotalaria*.

4.5.2.2 Unique SSR markers

In addition to polymorphism, 19 unique SSR sequences were identified in ten *Crotalaria* germplasm using four different primer pair combinations. Primer pair AL-365892 produced two unique bands of 650 and 180 bp in *C. juncea* (Tables 42 and 46). With primer pair AW-127626, Acc. No. 4809 of *C. incana* showed three unique bands of 300, 250, 200 bp and *C. juncea* showed two unique band of 270 and 230 bp (Tables 43 and 46). Primer pair AW-584539 produced four unique bands of 700, 500, 400 and 300 bp in Acc. No. 4803 of *C. pallida*. Moreover, unique band were observed in Acc. No. 4809 of *C. incana* in 1500, 750, 250 and 200 bp. Besides, two unique bands were present in *C. juncea* (Tables 44 and 46). With primer pair MtSSRNFAW-142, Acc. No. 4790 of *C. incana* showed two unique bands of 1350 and 300 bp (Tables 45 and 46). The unique bands were stable and specific for the respective germplasm and thus could be used as marker. Except polymorphic band no reports of unique SSR band was available for *Crotalaria* species (Raj *et al.* 2011, Roux *et al.* 2013). Therefore, this is first time report about unique SSR band for *Crotalaria* germplasm.

4.5.2.3 Genetic distances

The values of pair-wise Nei's (1972) genetic distances analyzed by using computer software "popgene32" among ten germplasm of *Crotalaria* species. were computed from combined data for the four SSR primer pairs ranging from 0.0690 to 1.0986 (Table 47). The highest genetic distance (1.0986) was found between a germplasm of *C. pallida* (Acc. No. 4806) and *C. incana* (Acc. No. 4809). This highest genetic distance was also observed between Acc. No. 4803 of *C. pallida* and Acc. No. 4809 of *C. incana*. Among the five germplasm of *C. pallida* the lowest (0.0690) genetic distance was observed between Acc. No. 4806 and Acc. No. 4807. This lowest distance was also observed in Acc. No. 4250 with 4805 and 4807. In contrast, the highest genetic distance (0.2231) was found between Acc. No. 4250 and 4803. On the other hand, same genetic distance was present in Acc. No. 4803 and 4807 of *C. pallida.* In case of four germplasm of *C. incana,*

the lowest (0.0931) genetic distance was observed between Acc. No. 4801 and Acc. No. 4804 but the highest genetic distance (0.6286) was found between Acc. No. 4790 and 4809 (Fig. 211; Table 47). Wide genetic diversity in different crops have been reported earlier (Molla *et al.* 2010, Sultana and Alam 2016c)

The difference between the highest and the lowest value of genetic distance revealed the wide range of variability persisting among the ten *Crotalaria* germplasm. High genetic distance values between germplasm pairs were found due to difference in genetic constituent. The germplasm of lowest genetic distance can be used as parental source for breeding line to improve *Crotalaria* germplasm.

4.5.2.4 Cluster analysis (Tree Diagram)

A cluster analysis on the basis of DNA fingerprinting by SSR was carried out. Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) segregates ten germplasm of *Crotalaria* into two major clusters *viz.* cluster -1 (C_1) and cluster -2 (C_2). Cluster -1 placed *C. juncea* alone with a high genetic distance of 0.8622. In contrast, the five germplasm of *C. pallida* formed cluster -3 (C_3) under cluster -1. According to cluster-3, Acc. No. 4806 and 4807 are closely related with narrow genetic distance (0.0690). On the other hand, Acc. No. 4250 are also closely related with Acc. No. 4805 and 4806 with narrow genetic distance while Acc. No. 4803 is distantly related from other four germplasm of *C. pallida* with large genetic distance (0.2231). Acc. No. 4809 of *C. incana* was placed alone under cluster -2 whereas other three germplasm grouped into cluster -4 (C_4). Within cluster -4, Acc. No. 4801 and 4804 was closely related with narrow genetic distance of 0.0931 (Table 47).

4.6 Acc. No. 4803 of *C. incana* is distinct from the rest of the germplasm

The germplasm Acc. No. 4803 of *C. pallida* is different from the rest nine germplasm in various morphological**,** cytogenetical and molecular aspects (Tables 24, 25, 41 and 47). This germplasm is different than others on the basis of their morphology. Such as yellow coloured seed, stem hairy and size of pod is comparatively smaller than the other germplasm (Figs 22 and 32).

Acc. No. 4803 was also distinct from the rest in respect of CMA-banding pattern. Highest number of CMA-band was found among other germplasm of *C. pallida*. (Figs 142 and 172; Table 24). In case of DAPI- banding pattern, a member of chromosome pair V had fluoresced entirely DAPI-positive bands except centromere (Figs 152 and 182). This chromosome is unique since absent in the rest germplasms and thus could be used as a marker for this germplasm. This germplasm is different in respect of RAPD and SSR finger printing. Several unique sequences were found with various primers (Tables 27, 30, 31, 32, 34, 35, 38 and 39). The RAPD and SSR dendrogram placed this germplasm alone in a separate cluster (Figs 206 and 211).

The above data made Acc. No. 4803 of *C. pallida* distinct from the rest nine germplasm of *Crotalaria.*

4.7 Presence of trisomy in two germplasm of *Crotalaria incana*

In Acc. No. 4790 and 4804 of *C. incana*, 2n = 17 chromosomes were observed after orcein, CMA and DAPI-staining. In addition about 70 per cent pods of these two germplasm were seedless. The above features indicated probable aneuploid nature of these germplasm. This aneuploidy might be trisomy (2n+1) with basic chromosome number 8 (2n + 1 = 16 + 1 = 17) or monosomy with basic chromosome number 9 (2n – 1 = 18 – 1 = 17). Moreover Acc. No. 4790 was placed alone with high genetic distance from other four germplasm of *C. incana* after cluster analysis through RAPD. Therefore, these two germplasm should be avoided for further breeding programme.

4.8 Conclusions

In the available literatures and internet sources, report on the nature of mitotic interphase and prophase chromosomes, CMA and DAPI banding pattern, unique RAPD and SSR bands and combined cytogenetical and molecular analysis of *Crotalaria* was not found. Therefore, in this study, characterization of ten germplasm of *Crotalaria* by the above parameters was the pioneer attempt.

Alterations of chromosomal segments were found in ten *Crotalaria* germplasm. Different basic chromosome number, different 2n chromosome number, diversification and reshuffling of CMA- and DAPI-positive banded regions were observed in these ten germplasm of *Crotalaria* studied. The number, location and distributions of GC- and AT-

rich repeats were specific for each germplasm. Therefore, each germplasm has its characteristic CMA- and DAPI-banding pattern. The ten germplasm showed unique RAPD and SSR DNA fingerprinting useful for authentic characterization.

Therefore, the ten germplasm of *Crotalaria* available in Bangladesh could be characterized authentically by combined cytogenetical and molecular analysis.

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* Original not seen.