III. MATERIAL AND METHODS

Material for the present investigation was collected extensively from different parts of South-Western India and from other parts like Aurangabad, Baroda, Cuttack and Delhi. Material was also obtained from other countries like Africa, China, U.S.A. and U.S.S.R. For each collection, voucher number was given. Whenever possible, young plants were collected and transferred to the experimental garden for further study. Otherwise seeds were collected. Field notes were maintained for all the collections. Herbarium sheets were prepared with usual procedure by selecting healthy twigs for each collection.

Suitable flower buds were fixed in Carnoy's fixative for 2-3 hours to 24 hours and later transferred to 70% alcohol for further storage. The stored buds were suitable for meiotic preparations at least for a month. This procedure was followed only at the time of collection tours. For material from the experimental garden, the meiotic preparations were made after 2 hours of fixation. Species investigated in the present work are given below with their collection numbers and localities. The map facing the page shows the places of collections (Fig. 1).

Species	Coll. No.	Place of collection or source
C. alata Hamilt.	163	Delhi
	096	Kodaikanal
C. albida Heyne.	003	Mahabaleshwar
C. barbae Scheinf.	138	Kew Gardens
C. brevidens Benth.	141	Delhi
	105	N. Rhodesia
	106	E. Africa
	142	U.S.A.
C. browani Bert. ex DC.	090	Australia
	099	Australia
	080	Cuttack
	155	Delhi
	178	Panji
C. calycina Schrank	[illegible]	
	066	Kodaikanal
	049	Nandi Hills
C. ferruginea var.	017	Baroda
β pilosissima Benth.	018	Baroda
	020	Baroda
	069	Kodaikanal
C. filipes Benth.	004	Mahabaleshwar
	004	Panchagani
C. Grahamiana W. and A.	061	Kodaikanal
	071	Kodaikanal
C. grantiana Harms.	102	Brasil
	101	S. Africa

Species	Coll. No.	Place of collection or source
C. impressa Roxb.	135	Delhi
	162	Delhi
C. incana Linn.	193	Ceylon
	147	Delhi
	104	Spain
	092	Sweden
	139	U.S.A.
C. juncea Linn.	091	Brazil
	108	Brazil
	005	Dharwar
	006	Dharwar
	024	Dharwar
	025	Dharwar
	107	Japan
	007	Hansur
	015	Sankeshwar
	143	U.S.A.
	149	U.S.S.R.
C. laburnifolia Linn.	072	Kodaikanal
	038	Mercara
	009	Ootacmund
C. lanceolata R. Hey.	110	China
	102	S. Africa
C. Leschenaultii DC.	064	Kodaikanal
	047	Nandi Hills

Species	Coll. No.	Place of collection or source
C. mediogaines var. luxuriens Bak.	083	Aurangabad
	073	Tiruchchirapalli
C. muoronata Desv.	060	Curtallum
	057	Ernakullam
	058	Ernakulam
	059	Ernakulam
	170	Tiruchchirapalli
C. mysorensis Roth.	161	Delhi
	013	Kesrangundi
C. nana Burm.	065	Dharwar
	068	Kodaikanal
C. nitens H.B.K.	023	Sweden
C. orixensis Rottl.ex Wild.	134	Delhi
	152	Delhi
	026	Dharwar
C. pauli@nn Linn.	117	Brazil
	118	Mexico
C. prostrata Roxb.	011	Dharwar
	037	Londa
	040	Londa
	035	Supa
	043	Supa

Species	Coll. No.	Place of collection or source
C. retusa Linn.	078	Anmod
	918	Baroda
	079	Cuttack
	132	Delhi
	153	Delhi
	008	Dharwar
	001	Mahabaleshwar
	074	Mahabaleshwar
	048	Nandi Hills
C. saltiana Andr.	123	China
	088	Thailand
	122	Thailand
C. sericea Retz.	080	Australia
	124	Australia
	131	Delhi
	158	Delhi
	165	Delhi
C. shevaroyensis Gamb.	010	Yercaud
C. spectabilis Roth.	114	U.S.A.
	125	T. Africa
C. stipularia Desv.	094	Sweden
C. striata DC.	078	Bangalore
	045	Hemmerugatta
	154	Delhi
	039	Londa
	044	Londa

Species	Coll. No.	Place of collection or source
C. striata var. acutifolia Trim.	014	Kemsangundi
C. triquetra Dalz.	034	Dandeli
	012	Dharwar
	038	Londa
	042	Supa
C. verrucosa Linn.	081	Cuttack
	053	Dharwar
	082	Ranebennur
	130	S. Africa
	129	Spain
	145	U.S.A.
C. Wightiana Greh.	062	Kodaikanal
	065	Kodaikanal
	067	Kodaikanal
C. zanzibarica Benth.	127	Australia
	087	China
	157	Delhi
	128	Italy

Due to the presence of hard seed coat there was trouble in seed germination. Therefore the seeds were treated with Con. H_2SO_4 for 20-25 minutes, then were thoroughly washed in tap water. Afterwards they were placed in a petridish on a moist blotting paper. There used to be 100 percent germination. The young seedlings were then transferred to the pots. Scraping of the seed coat was also tried. No doubt there was 100 percent germination, but the radicle used to come out first, usually through the scrapped region. While doing so it used to detach itself from the cotyledons. Thus further germination of the seedling used to be of no use. Therefore the acid treatment is the best for the hard nature of the seed coat.

The mitotic study was made following Fjic and Levan's (1950) oxyquinoline aceto-orcein squash method. This proved to be satisfactory, since constrictions were exaggerated and chromosome arms were suitably shortened.

Earlier workers followed different processes. Srivastava (1958) pretreated the root tips in 0.00175 M. solution of 8-hydroxyquinoline for 1½ hours at 14°-20°C and followed the usual procedure for hydrolysis and staining. Magoon et al. (1963) pretreated the root tips with saturated aqueous paradichlorobenzene for 1½ hours at 16°-20°C, washed thoroughly with water, then hydrolysed and stained as usual. Datta and Biswas (1963) and Datta

and Choudhary (1966) followed the elaborated process. They pretreated the root tips in saturated aqueous solution of aesculine for 1-2 hours at 6°-8°C. Then the root tips were fixed in acetic-alcohol (1:2) for an hour. Later they were hydrolysed and kept for staining for about 30 minutes in 2% acetic-orcein. Patta and Ganguly (1967) pretreated the root tips with 0.002 M. to 0.001 M. solution of 8-hydroxyquinoline for 2-3 hours at 8°-10°C. After this pretreatment, the root tips were fixed in 45% propionic acid-alcohol (1:2) for an hour. Then each root tip was hydrolysed and kept for staining in 2% propionic-orcein for about 30 minutes to get well stained and spread karyotypes. It is observed that in all the above cases the preparations were not good, especially the morphology of the chromosomes and spreading of the chromosomes in a plate was not proper. Most of the workers have not been able to notice the secondary constriction and interstitial satellite, the latter being very characteristic of the genus.

For the present work healthy root tips were taken from seedling and adult plants growing in the pots for mitotic study. Two hours before obtaining the root tips the plants were sufficiently watered to ensure full turgidity of the root tip cells. Excised root tips were cleaned and treated with 0.002 mol. 8-hydroxyquinoline for 2-2½ hours at 10°-12°C. Then the root tips were hydrolysed in 2% aceto-orcein (B.Merck) and 1N HCl (8:2) and squashed

in 2% aceto-orcein. The slides were sealed with gum-mastic and they remained good for study upto a period of a fortnight. For karyotypic study of each species, a number of preparations were made from different root tips. The divisions in root tips were found to be maximum between 9.00 A.M. to 10.30 A.M. and it mostly depended on the diurnal changes. A comparative karyotypic study was carried out to ascertain particularly the chromosome numbers and detailed morphology of the chromosomes. Only the best plate among the freshly prepared slides were selected for drawing and photography. Microphotographs were taken after a day. All the drawings were made at table level using Carl-Zeiss drawing apparatus with builtin polarised filters, using 25x eyepiece and 1.32 apochromatic 100 oil immersion objective at a magnification of 3680x. Photo-micrographs for mitosis and meiosis were taken with 35 mm. Kodak retina reflex Camera using Zeiss periplanatic eyepiece 10x and 1.32 apochromatic 100 oil immersion objective and later enlarged to a suitable size.

The chromosome measurements were made from the drawn figure. For curved chromosomes, they were marked into smaller sections and then measured. First measurements were made in m.m. and then converted into microns by comparing with the drawn scale of the stage micrometer under the same magnification.

Long arm, short arm and satellites were measured separately and tabulated. The gap caused by constriction whether primary or secondary was not taken into consideration because they were affected by pretreatment. All the measurements of chromosomes in a complement were tabulated, then homologous chromosomes were paired. The mean values for long arm, short arm and satellites of the pair were taken. The mean of the total length of the chromosomes were also calculated. Then the chromosome pairs were arranged in a serial order of decreasing length. All these measurements were then converted into microns. The mean total of all the chromosome pairs in the complement gives the total length of the chromosomes of the haploid complement in micron. The karyotype preparations involve the pretreatment and other factors. Chromosome measurements made might vary to a little extent and this should however, be minimised by considering 'Relative length' expressed as the percentage length of the chromosome to the total length of all the chromosomes in a complement. The relative length is constant for each species.

Idiograms were first drawn on the graph paper in decreasing order of length from left to right. The ends of long arm were directed downwards lying on the same abscissa. Then the drawings were transferred to the drawing paper. The width of the chromosomes and gap of the primary or secondary constrictions were maintained uniformly throughout the work, which do not add to the length of

the chromosomes. The scale is represented in microns by the side of the idiogram. Then chromosomes of the idiograms were numbered left to right. The karyotype formula is also given.

Most of the meiotic preparations were done by fixing suitable flower buds in Carnoy's fixative (6 parts of alcohol: 3 parts of chloroform: 1 part acetic acid) for about 2-3 hours. The buds were fixed from the experimental garden and it was never stored. The fixation time was between 11.00 A.M. to 1.00 P.M. The anthers were dissected from the buds and were squashed in a drop of 1% acetocarmine on a clean slide. After removing the debris the cover slip was placed on it and excess of stain was removed by placing between the folds of the blotting paper and applying little pressure on cover slip to get uniform spreading of the pollen mother cells. A gentle heating over a spirit flame intensified the staining. Then the slide was sealed with paraffin and kept for 8 hours to overnight for the stain to intensify. Then the slide was made permanent by removing the sealed paraffin ring with the help of a blade and passing through acetic acid and n-butyl alcohol series (improved McClintock's method (1929)). Using n-butyl alcohol in place of absolute

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alcohol and Xylol.) After removing the paraffin ring the slide was kept topsyturvy and slanting in a petridish containing 1:1 acetic acid and n-butyl alcohol till the cover-slip and slide separated. The slide and cover-slip were then transferred to second and third petridish containing n-butyl alcohol only. In each grade they were left for 1-2 minutes. It was then the slide and the cover-slip were recombined using euparol as mounting medium. There was no shrinkage of pollen mother cells in this method. After perfect drying of the slide, they were studied and observations were recorded. Only desired stages were photographed. It was found that Carnoy's fixative is good to get perfect spreading of chromosomes at metaphase and anaphase.

In hybridisation experiments, even though the buds were quite big and handy in some species they were delicately attached to the plants. Emasculation was done very carefully without much disturbing the buds. Only the necessary parts like keel petals and all the ten anthers were removed in the buds which were likely to bloom next day. Other flowers and buds in the racemes were removed. The emasculated buds were protected from pollination by extraneous pollen by covering the entire racemes with butter paper bags. The label was tied to them denoting the female parent, the number of buds emasculated and date. Pollination was affected the next day (7.30 A.M. to 9.00 A.M.) by carefully removing the

butter paper bags. For pollination just dehisced anthers were used. On the label the Coll. No. of the male parent, number of the flowers pollinated and date were noted. The bag was replaced. This procedure was repeated in the afternoon to ensure perfect pollination and it is known that other set of five small round anthers dehisce in the afternoon (3.00 P.M. to 4.00 P.M.) with full blooming of the flower. It was found that at the interval of 2-4 days, flowers used to wither and fall. Therefore, it was felt necessary to use the growth harmones like Indole butyric acid of 0.01%. Before pollination the stigmatic surface of the emasculated flowers was applied with I.B.A. of 0.01% and then pollinated with desired male parent. Around the ovary moist cotton dipped in I.B.A. of 0.01% was kept, so that it can prevent the withering of the flowers. Only in few cases the pod development has been noticed with few shrivelled seeds.

For comparison, herbarium plants were photographed under Leitz reprovit using Leica camera and later enlarged to a suitable size.

In the present investigation the taxa from different localities have been studied morphologically. Short descriptions of the taxa based on actual collections and observations have been given. The morphological data based on actual specimens were used in drawing the polygraphs adopting Hutchinson's technique (1933, Love and Nadeau 1961).

For plotting the polygraph twelve morphological characters were selected and five index values were used (the number of index values were kept constant for all the characters) for the morphological variations observed (Table 1).

Table 1

Characters selected for Polygraphs

	Characters	Variations	Index value(IV)
1.	Nature of the plant	Prostrate	1
		Spreading	2
		Dwarf (less than 30 c.m.)	3
		Medium (30 to 90 c.m.)	4
		Tall (90 c.m. and above)	5
2.	Leaves	Simple sub-sessile	1
		Simple stalked	2
		Three foliate	3
		Five foliate	4
		Seven foliate	5
3.	Leaf or leaflet size	Small	1
		Medium	3
		Large	5

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	Characters	Variations	Index value (I?)
4.	Leaf or leaflet shape	Elliptic	1
		Ovate or obovate	2
		Lanceolate or oblanceolate	3
		Oblong	4
		Linear	5
5.	Leaf or leaflet apex	Rounded	1
		Obtuse	2
		Acute	3
		Setuse	4
		Mucronate	5
6.	Stipules	Absent	1
		Small	2
		Lanceolate	3
		Foliaceous	4
		Adnate	5
7.	Calyx size	Small (less than 1 c.m.)	1
		Medium (1.0 – 2.5 c.m.)	3
		Big (2.5 and above)	5
8.	Standard petal	Coloured veins on both sides	1
		Coloured veins outside	3
		Coloured veins inside	5
9.	Pod's length	Short (less than 2.5 c.m.)	1
		Medium (2.5 to 5.0 c.m.)	3
		Long (5.0 c.m. and above)	5

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Characters	Variations	Index value(IV)
10. Pod's nature	Glabrous	1
	Deflexed	3
	Hairy	5
11. Seed colour	Bluish black or black	1
	Light brown or brown	2
	Yellowish white or Pink	3
	Green mosaic or Bottle green	4
	Grey mosaic	5
12. Number of seeds in a pod	Less (less than 10)	1
	Average (10 to 20)	3
	More (20 and above)	5

To construct a polygraph a 'key' is made in the shape of a wheel with radiating spokes (Figs. 2a). The centre of the wheel is represented by a point. Each spoke denotes one morphological character and the distance along the spoke represents the variation of that particular character. Only twelve important characters were selected to represent twelve spokes to avoid the crowding on the polygraph figure. Out of twelve characters four could be estimated objectively and the rest eight had to be estimated subjectively. In plotting the polygraph, graph papers were used, which enabled easily to point out the

particular position of a character with variation on the spoke. These points were later joined to form polygonal figure or polygraph.

Suppose there is a plant with following characters:

	Characters	Variations	Index value
1.	Nature of the plants	Tall	5
2.	Leaves	Simple stalked	2
3.	Leaf size	Large	5
4.	Leaf shape	Obovate, Ovate, oblong	2,4
5.	Leaf apex	Acute	3
6.	Stipules	Foliacious and adnate	4-5
7.	Calyx size	Medium	3
8.	Standard petal	Coloured veins inside	5
9.	Pod's length	Medium	3
10.	Pod's nature	Glabrous and deflexed	1-3
11.	Seed colour	Bluish-black	1
12.	Number of seeds per pod	Average	3

Then the polygraph will be as in figure 8b .