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Study of morphological features and karyomorphology of East Indian Lemongrass a.k.a Cochin grass or Malabar grass {*Cymbopogon flexuosus* (Nees ex Steud.) W. Watson}

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Abstract
Cymbopogon flexuosus, also known as Malabar grass or Cochin grass is abundantly found in the South Indian states of Karnataka, Kerala and Tamil Nadu. It has been an important cash crop along with *Cymbopogon citratus* for manufacture of various essential oils. Though the grass has been subject of many studies, our study focuses on the morphological and karyomorphological features of the wild species of the *Cymbopogon flexuosus* (Nees ex Steud.)

Keywords: *Cymbopogon flexuosus*, morphology, karyomorphology, wild species, medicinal plant

1. Introduction
The genus *Cymbopogon* is well known for the production of the secondary metabolites which are used in various industries such as pharmaceuticals, perfumery & fragrances, food industry and others. The genus *Cymbopogon* belongs to the tribe andropogoneae of the family Poaceae (Gramineae). *Cymbopogon flexuosus* is an elite member of this genus and is distributed widely in the Southern States of India (S. Singh *et al*. 2000). The plants of this genus has reputation in traditional and folk medicine as abortifacient, aphrodisiac and analgesic properties. In nature the plant occurs in fields, often on limestone and on slopes and ridges at 100-2200m altitude, in mixed deciduous, dipterocarps and teak forest (M. Singh, Ganesha Rao, and Ramesh 1997) [10]. Understanding the genetic resources and diversity is very important for the breeding programs and improvement of several economically important plant species including *Cymbopogon* sp. Karyotype studies can become one important resource for devising breeding and germplasm conservation for *Cymbopogon* sp., Karyotype study holds great importance as it enables scientists to view the layout of chromosomes in a particular species. By mapping out specific chromosome pairs, we can readily identify the sex of a particular species. Also, it enable us to detect certain abnormalities that may exist due to abnormality in chromosome number (i.e. extra chromosome, lacking a chromosome, or having a chromosome that is not fully developed).

2. Materials and Methods
Wild C. *flexuosus* were collected from hilly regions of South India i.e. Himavad Gopala hills from Karnataka, Shevaroy hills from Tamil Nadu, Travancore hills from Kerala and Tirumala hills from Andhra Pradesh. The plant collections were considered as four different populations and were maintained in the departmental garden of Microbiology and Biotechnology, Bangalore University, Bangalore under uniform conditions for further studies.

1. **Morphological studies:** The selection of the plant for study was based on the habit of the plant (morphology) and its aroma value. The morphological characters of the plant such as plant appearance, plant height, leaf size, leaf colour, stem colour and inflorescence were recorded.
2. **Karyomorphological studies:** The different populations of C. *flexuosus* were subjected to cytological studies to know the ploidy nature of the plant.

2.1 Mitosis

2.1.1 Pre-treatment, fixation and storage of tissues: Healthy root tips from different populations of C. *flexuosus* were collected between 1200 Hrs to 1400 Hrs and studies for mitotic activity, as the cell division is found to be high during this period.

The root tips were washed with tap water followed by pre-treatment with 0.002M 8-hydroxyquinoline (8HQ) for 3 hours at 4 °C. The root tips were washed with water and fixed in 1:3 acetic acid ethanol or propionic ethanol for 24 hours before squashing. The materials were stored in the fixative at 4 °C till the squashes were made. After fixing the sample, squash preparations were made within one or two weeks which gave good staining and spreading of chromosomes.

2.1.2 Squash preparation: Root tip squash were prepared by treating the fixed root tips with a mordant of 4% iron-alum in 45% propionic acid for 15-30 minutes. They were treated with 4% haematoxylin stain in 45% propionic acid for 30-60 minute followed by squashing in 45% propionic acid. Temporary slides were prepared and observed under apochromatic lens system of Carl Zeiss microscope using oil immersion and photographed for measuring the chromosomes.

2.1.3 Karyotype analysis: The karyotype measurements were made using digital callipers and the readings were expressed in microns. The karyotypes recognized were compared based on absolute size, relative size, position of centromere, basic chromosome number, number and position of satellite in chromosomes.

The degree and distribution of heterochromatin region in the chromosomes were also studied at mitotic prophase and prometaphase. Based on the arm ration the chromosomes were classified into specific groups. The nomenclature proposed by Leven *et al*., (1964) [8] (Levan, Fredga, and Sandberg 1964) [8] was adopted for classification and accordingly six regions were recognized based on the centromere position in the chromosome i.e. M (middle), T (ends), m (median), sm (sub-median), t (terminal) and st (sub-terminal) region. The m, sm, t and st represent half the length of the chromosome from the centromere position.

The chromosomes in the karyotype had two types of secondary constrictions, the first type was long and fibre like consisting of very small satellite, the constriction being situated on the short arm and considered as true satellite chromosome. In the second type, the secondary constriction was short and represented by small gap in the satellite chromosome with a big satellite. Such chromosomes are referred to as small secondary constrictions or simply secondary constriction chromosomes. The relative length of chromosomes was expressed as percentage of the total sum of the length of the chromosomes of the complement. Based on their centromeric position the chromosomes were grouped into M, m and sm type and within each group the chromosomes were arranged in decreasing order of their length.

2.1.4 Determination of karyotype asymmetry: To analyse the karyotype asymmetry, the system proposed by Stebbins (1971) [3] (Stebbins, 1971) [3] was followed in which chromosome asymmetry was categorized based on three degrees of difference between the largest and smallest chromosome of the complement and four degrees with respect to the proportion of chromosome between acro- or telocentric chromosomes. Accordingly twelve categories of chromosome asymmetry were recognized viz., 1A, 2A, 3A, 4A, 1B, 2B, 3B, 4B, 1C, 2C, 3C and 4C.

2.2 Meiosis

2.2.1 Pre-treatment, fixation and storage of tissues: The spikelet of appropriate developmental stage were selected and fixed in Carnoy's fluid i.e., in 6:3:1ratio of absolute alcohol : chloroform : acetic acid between 0900 Hrs and 1030 Hrs. Propionic acid gave good spreading and staining in place of acetic acid. Before smear preparation the material was fixed for 24 hours and stored in refrigerator at 4 °C. The fixed spikelet were used within two weeks to obtain good spreading and staining of the chromosome.

2.2.2 Smear preparation: The smears of the pollen mother cells (PMCs) were made using propiono-iron haematoxylin stain (1%) which was diluted to half strength (0.5%) using 45% propionic acid. Generally 1ml of the diluted stain was mixed with 1 drop of mordant which can be varied accordingly to required intensity of staining. The cytological observations were made from the temporary slides and later slides were made permanent using butanol-acetic acid and butanol series.

3. Results

3.1 Morphological studies: The C. *flexuosus* collected from different locations and its morphological characters are shown in Fig. 1 and 2. Morphological features of wild C. *flexuosus* studied are as follows:

C. *flexuosus* is a tall plant having short and thick rhizome, the culm grows up to 3 m tall with a nodding inflorescence. The stem is erect, stout, smooth, polished, solid and globous at nodes.

The leaf blades are linear acuminate, tapering at both ends, attenuate upwards to long filiform tip, glaucous green, suffused with purple or reddish tinge, minutely scabrid on both surfaces. The leaf is coarsely hairy on the upper surface, shortly hairy on the lower surface near the junction with the sheath over 1, on length and up to 1.5cm in width. The sheaths ate very loose, long, and persistent and become leathery, curled, falling away from the culms. The ligule is cretaceous to coriaceous when old and 2-3mm long.

The inflorescence is large, loose with many long flexuous branches (Fig. 6). It branches, rebranches and ends in spatheole subtending a pair of racemes seated upon peduncle up to 60cm long and 30cm broad. The axis of the inflorescence is 12 noded, joints are smooth and polished, slightly expanded at the tip, flattened, smooth and glabrous but ciliate or villous towards the top on the edges, usually villous just at the node. The branches and branchlets are similar. The spatheole is 18-20mm long, narrowly elliptic-acumimate when flattened, many nervered, hyaline on the margins, greyish green and turns pale red or brownish red. The peduncle is 3-3.5mm long, wiry smooth and glabrous but shortly haired near the tip. The racemes are 15-20mm long, one sub sessile, the other shortly pedicelled, very hairy in the fork; joints of the raceme are 2.5mm long, compressed, linear, expanded at the tip, densely ciliate along the margins. The pedicle is similar but shorter. The lowest pair of spikelet in the sub sessile raceme is homologous male; pedicle of the pedicelled spikelet not swollen but very short, all remaining pairs in both racemes is heterogamous. The hermaphrodite spikelets are 4.5-5mm long with short bearded callus, oblong-acute or wide above the middle.

The lower glume size and shape of the spikelet is 2-keeled, smooth, and glabrous but scabrid on the margins of the keels, flat on the back, shallowly concave below, obscurely or clearly 1-3 nerved between the carnials. The upper glume of the spikelet is boat shaped, rounded on the back, keeled and

winged, smooth and glabrous, scabrid on the keel. The lower floret is empty, lemma lanceolate acute with hyaline scale, 2-keeled, ciliate on the flaps, palea absent. The upper floret is bisexual, lemma narrow, hyaline, cleft to the middle, awned in the sinus, palea absent, awn 12mm long; column 5mm twisted. The styles are two in number and stigma is plumose and purple. The stamens are three in number, anthers 1.75-2mm long, lodicules truncate-cuneate, pedicelled spikelets ale or neuter, elliptic-acuminate in shape, 3.5mm long. The lower glume is many nerved, smooth, glabrous and upper glume is 3-nervered, boat shaped, smooth and glabrous. The florets are reduced to lanceolate-acute hyaline scale 3mm long with stamens 3 and anthers 2mm long (Fig. 7).

The different populations of C. *flexuosus* were studied for its appearance, height, leaf colour and size and colour of the stem and inflorence (Table 1). The plant collected from Himavad Gopala Hills were tall, bushy with broad leaves of bluish green colour. The culm of the plant was dark red in colour possessing maroon inflorescence. C. *flexuosus* collected from Shevaroy hills and Travancore hills showed dispersed appearance, the culm was red in colour with narrow leaves and brownish inflorescence. The plant from Shevaroy hills were of medium height with pale bluish green colour leaves, the plants from Travancore hills were tall with dark green leaves.

3.2 Karyomorphological studies: Cytogenetic analysis of different varieties of C. *flexuosus* were performed to determine the ploidy level and stability of the chromosomes. Both mitotic and meiotic studies were performed:

Mitosis: Plants collected from Travancore hills were identified as diploid race (2n-=20) based on the karyotype analysis. The karyotype comprised of 1 pair of M, 4 pairs of m and 5 pairs of sm chromosomes. The total chromatin length of haploid set was 22.56µm. The length of the longest chromosome was 2.77µm and the shortest chromosome was 1.96µm. A pair of true satellite is universally present but in majority of metaphase plates only one of the satellite of that pair expressed well. In addition, the third pair of chromosome had small secondary constrictions on the long arm. The karyotype asymmetry was 2A type (Table 2 and Fig. 3).

The plant collected from Himvad Gopala Hills were identified as tetraploid race of C. *flexuosus*. The karyotype comprised of 6 pairs of M, 11 pairs of m and 2 pairs of sm chromosomes. The total chromatin length of the complement was 42.72µm. The longest and the shortest chromosomes measured were 2.76µm and 1.66µm respectively. Two pairs of satellite chromosomes and a pair of secondary constrictions were characteristic features of the karyotype. The karyotype asymmetry was found to be 1A type (Table 3 and Fig. 4).

The karyotype analysis of hexaploid race of C. *flexuosus* comprised of 8 pairs of M, 18 pairs of m and 4 pairs of sm chromosomes. The total chromatin length of the haploid set was found to be 60.32µm. The length of the longest chromosome measured was 3.2µm and the shortest chromosome was 1.26µm. The longest and shortest chromosomes were either M or m recognised based on the centromere position. Two pairs of true satellite chromosome and a pair with secondary constrictions were characteristics of the karyotype. The karyotype showed highest ratios in the longest and shortest chromosomes of the complement in the genus. The karyotype asymmetry was 2B type which is the highest in the genus (Table 3 & Fig. 5). Comparison of karyomorphological data in different races of C. *flexuosus* is given in Table 4. The karyomorphological analysis in wild population of C. *flexuosus* indicated its polyploidy nature. C. *flexuosus* collected from H.G. hills showed chromosome number of 2n=40 (Tetraploid). The plant collected from Shevaroy hills showed chromosome number of 2n=6- (Hexaploid) and those collected from Travancore hills showed chromosome number of 2n=20 (Diploid).

Meiosis: The meiosis observed in pollen mother cells of diploid race was normal forming 10 bivalents during prophase I and showed 10-10 segregation at anaphase I (Fig. 8). The meiotic analysis of the tetraploid race is given in table 12. The meiotic preparations from the pollen mother cells revealed the 40 autosomes (As) which formed 20 bivalents and 6B chromosomes (Fig. 9). B chromosomes showed the formation of 2 bivalents and 2 univalent in 80% of the metaphase I cells. Rarely the B chromosomes formed all bivalents (20II+3II). The plant showed secondary association without the formation of trivalent, tetravalent or hexavalent chromosomes. Majority of the cells showed normal 23-33 segregation during Anaphase I, including the B chromosome. About 90% of the cells in telophase I were normal, while the remaining were abnormal with 1 or 2 lagging chromosomes. The second divisions were also normal. The six extra chromosomes in pollen mother cells were regarded as supernumerary of B chromosomes based on their behaviour. There was presence of six extra chromosomes in addition to 20 bivalents (2n=40+6). In meiosis, the pollen mother cells exhibited normal behaviour similar to diploids with the formation of 30 bivalents during the course of first meiotic division. In majority of cells at diakinesis and metaphase I stages, the bivalent showed characteristics lateral association in twos and threes, indicating secondary association. Formation of secondary association between the bivalents and interconnections among chromosomes during second meiotic division in pollen mother cells were the characteristic feature of hexaploid race of C. *flexuosus*. The normal segregation of 30-30 chromosomes was noted at anaphase I (Fig. 10). Interconnections between and among segregation chromosomes were also observed.

4. Discussion

In the present research, wild species of C. *flexuosus* were screened and selected for morphological and karyomorphological study. A great variability is seen in morphology, chemotype and genotype of C. *flexuosus* (Lavania 1987) [7]. The karyomorphological analysis revealed the polyploid nature of wild population of C. *flexuosus* (diploid, tetraploid and hexaploid races). Variants of *Cymbopogon* species with different ploidy levels and chemotaxonomic complexities are wide spread in nature. Polyploidization offer the opportunity to enhance the DNA methylation and production of secondary metabolites in plants. Karyotype asymmetry in *Cymbopogon* indicate the advance nature of the genus which indicates the culmination in the line of evolution as it represents the highest ploidy level in the genus and has more advance type of karyotype (Narayan, 1966) [1].

The presence of additional chromosomes may take part in the formation of nucleolus which indicates the multiple origins of the chromosomes involved in the synthesis of nucleolus. The association of chromosomes in polyploidy is due to the closely related duplicated genomes in their complement. Secondary associations between the bivalents have been observed in hexaploid race of C. *flexuosus* which has been interpreted as genetically related chromosomes by many authors (Christopher 1978; Gupta 1965; Raghunath 1967; Tateoka 1965) [5, 6, 9, 12]. The present cytological investigation reveal the allopolyploid nature of the plant due to formation of typical

bivalents. The interconnections among bivalents was reported earlier in the same genus by Subrahmanya and Jagadish chandra (Subrahmanya, 1980) [4]. The interconnection indicate the uncoiling or incomplete coiling of the chromatin formed during repair mechanism of chromosome following the recombination process during first or second meiotic divisions. The ploidy associated changes in morphology are strongly associated with epigenetic changes (Subrahmanya, 1980) [4]. Polyploids are more frequently found in higher altitudes than diploids which are usually distributed at lower altitudes (Sreenath, 1988) [2].

The karyomorphological studies confirm the polyploid nature viz., diploid, tetraploid and hexaploid races in wild population of C. *flexuosus*.

Tables and Figures

Tables

Table 1: Morphological characters in different population of C. *flexuosus*.

Parameter	Himavad Gopala hills, Karnataka	Shevaroy hills, Tamil Nadu	Trvancore hills, Tamil Nadu
Plant appearance	Bushy	Spread	Spread
Plant height	Tall	Medium	Tall
Leaf colour	Bluish green	Pale bluish green	Deep green
Leaf size	Broad	Narrow	Narrow
Stem colour	Dark red	Red	Red
Inflorescence colour	Maroon	Brownish	Brownish

Table 2: Karyomorphological data of diploid race (2n=20) of C. *flexuosus*.

Chromosome pair	Short arm (sa) (µm)	Long arm (la) (µm)	Ratio (la/sa)	Total chromosome length (µm)	Relative length	Chromosome type
1	1.065	1.065	1.0	2.13	9.44	M
2	1.3	1.47	1.13	2.77	12.27	m
3	0.86	1.0+0.43(sat)	1.66	2.30	10.19	m
4	0.93	1.15	1.23	2.08	9.21	m
5	0.83	1.13	1.36	1.96	8.68	m
6	0.86	1.75	2.01	2.60	11.52	sm
7	0.83	1.53	1.84	2.36	10.46	sm
8	0.76+0.2(sat)	1.33	1.75	2.30	10.19	sm
9	0.75	1.31	1.75	2.06	9.13	sm
10	0.73	1.26	1.73	2.00	5.58	sm

Total chromatin length: 22.56 µm
Karyotype formula: 1M+4m+5sm
Karyotype asymmetry: 2A

Table 3: Karyomorphological data of tetraploid race (2n=40) of C. *flexuosus*.

Chromosome pair	Short arm (sa) (µm)	Long arm (la) (µm)	Ratio (la/sa)	Total chromosome length (µm)	Relative length	Chromosome type
1	1.38	1.38	1.0	2.76	6.46	M
2	1.10	1.10	1.0	2.20	5.15	M
3	1.03	1.03	1.0	2.06	4.82	M
4	0.95	0.95	1.0	1.90	4.45	M
5	0.91	0.91	1.0	1.83	4.28	M
6	0.83+0.15(sat)	0.83	1.0	1.81	4.24	M
7	1.2	1.56	1.30	2.76	6.46	m
8	0.98	1.51	1.54	2.50	5.85	m
9	1.0	1.43	1.43	2.43	5.69	m
10	1.03	1.30	1.26	2.33	5.45	m
11	0.86	1.33	1.55	2.20	5.15	m
12	0.86	1.25	1.45	2.11	4.94	m
13	0.86	1.16	1.35	2.03	4.75	m
14	0.78	1.21	1.55	2.00	4.68	m
15	0.83	0.5+0.5(sat)	1.20	1.83	4.28	m
16	0.73	1.1	1.50	1.83	4.28	m
17	0.66	1.0	1.51	1.66	3.89	m
18	0.8	1.4	1.75	2.20	5.15	sm
19	0.76+0.1(sat)	1.33	1.75	2.20	5.15	sm
20	0.76	1.31	1.73	2.08	4.87	sm

Total chromatin length: 42.72 µm
Karyotype formula: 6M+11m+3sm
Karyotype asymmetry: 1A

Table 3: Karyomorphological data of hexaploid race (2n=60) of C. *flexuosus*.

Chromosome pair	Short arm (sa) (µm)	Long arm (la) (µm)	Ratio (la/sa)	Total chromosome length (µm)	Relative length	Chromosome type
1	1.53	1.53	1.0	3.06	5.07	M
2	1.40	1.40	1.0	2.80	4.64	M
3	1.25	1.25	1.0	2.50	4.14	M
4	1.10	1.10	1.0	2.20	3.64	M
5	0.86	0.86	1.0	1.73	2.87	M
6	0.80	0.80	1.0	1.60	2.65	M
7	0.66	0.66	1.0	1.33	2.20	M
8	0.63	0.63	1.0	1.26	2.08	M
9	1.43	1.0+0.76(sat)	1.23	3.20	5.31	m
10	1.16	1.56	1.35	2.73	4.53	m
11	1.0	1.66	1.66	2.66	4.41	m
12	0.93+0.16(sat)	1.33	1.43	2.42	4.01	m
13	1.13	1.26	1.12	2.40	3.98	m
14	0.93	1.33	1.43	2.26	3.75	m
15	0.96	1.20	1.25	2.16	3.58	m
16	0.86	1.16	1.35	2.03	3.37	m
17	0.76	1.23	1.62	2.00	3.32	m
18	0.73	1.13	1.55	1.86	3.08	m
19	0.73	1.06	1.46	1.80	2.98	m
20	0.53+0.36(sat)	0.76	1.44	1.66	2.75	m
21	0.73	0.93	1.27	1.66	2.75	m
22	0.66	1.0	1.51	1.66	2.75	m
23	0.66	1.0	1.51	1.66	2.75	m
24	0.73	0.90	1.23	1.63	2.70	m
25	0.70	0.80	1.14	1.50	2.49	m
26	0.56	0.90	1.60	1.46	2.42	m
27	0.80	1.43	1.79	2.23	3.69	sm
28	0.60	1.30	2.16	1.90	3.15	sm
29	0.56	0.96	1.72	1.53	2.54	sm
30	0.48	0.95	1.97	1.43	2.37	sm

Total chromatin length: 60.32 µm
Karyotype formula: 8M+18m+4sm
Karyotype asymmetry: 2B

Table 4: Comparison of karyomorphological data in different races of C. *flexuosus*.

Sl. No	Race	Karyotype formula	Total chromatin length (µm)	Longest chromosome length and type	Shortest chromosome length and type	Ratio of longest and shortest chromosome	Satellite chromosome and type	Karyotype assymetry
1	2n=20	1M+4m+5sm	22.56	2.77 µm (m)	1.96 µm (m)	1.41	2 pairs (1m+1sm)	2A
2	2n=40	6M+1m+3sm	42.72	2.76 µm (M,m)	1.66 µm (m)	1.66	3 pairs (1M+ 1m+1sm)	1A
3	2n=60	8M+18m+4sm	60.32	3.2 µm (m)	1.26 µm (M)	2.53	3 pairs(3m)	2B

Figures

a b

Fig 1: Collections of C. *flexuosus* from different locations; a. Himavada Gopala hills, b. Shevaroy hills, c. Travancore hills and d. Tirumala hills

Fig 2: Morphological characters of C. *flexuosus*. a. Whole plant, b. leaves, c. stem, d. inflorescence.

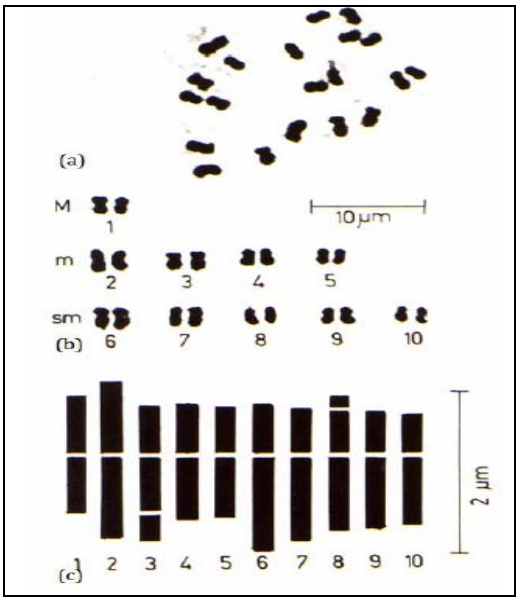

Fig 3: Karyomorphology of diploid race of C. *flexousus*. a. Mitotic metaphase, b. Karyotype (showing 1M, 4m & 5sm), c. I diogram

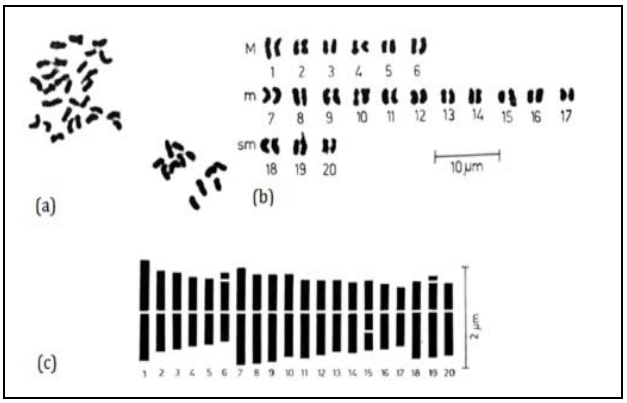

Fig 4: Karyomorphology of tetraploid race (2n=40) of C. *flexuosus*. a. Mitotic metaphase, b. Karyotype (showing 6M, 11m & 3sm), c. I diogram

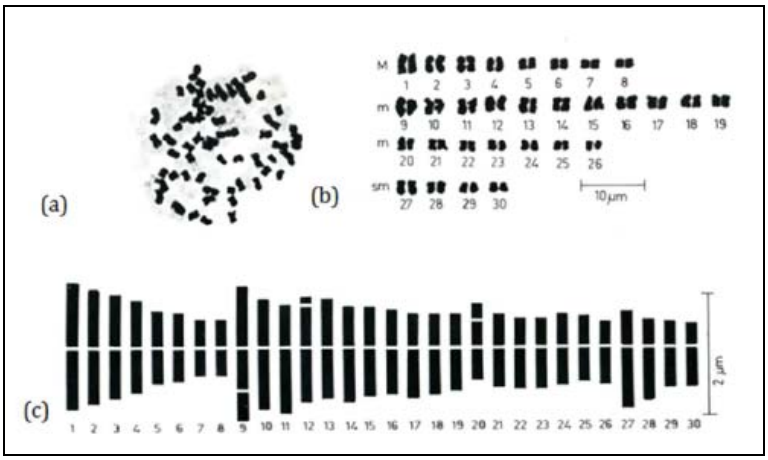

Fig 5: Karyomorphology of hexaploid race (2n=60) C. *flexuosus*. a. Mitotic metaphase, b. Karyotype (showing 1M, 4m, 5sm), c. I diogram

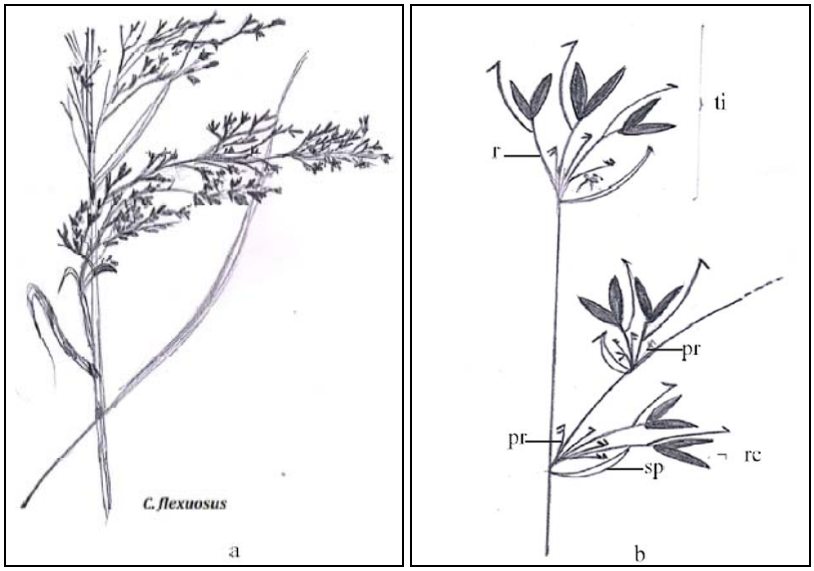

Fig 6: Diagrammatic representation of inflorescence: a- whole inflorescence, b- part of inflorescence, pr- prophyllum, sp- spathe, ti- tier, r- ray, re- raceme

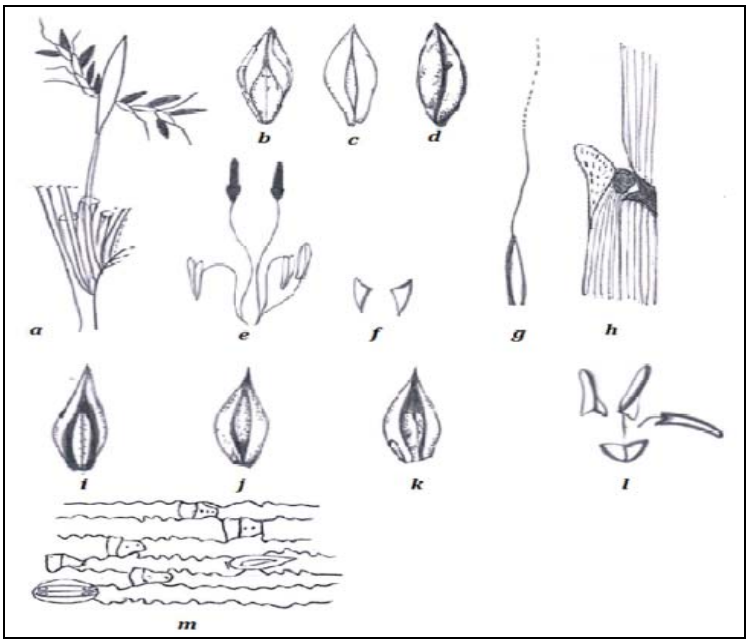

Fig 7: Diagrammatic representation of different parts of inflorescence and stomata: a- sessile perfect spikelet, b- lower glume, c- upper glume, d- lemma, e- stamens and pistil, f- lodicules, g- lemma and awn, h- ligule or pedicelled spikelet, i- lower glume, j- upper glume, k-lemma, l- stamens and lodicules, m- leaf section showing stomata

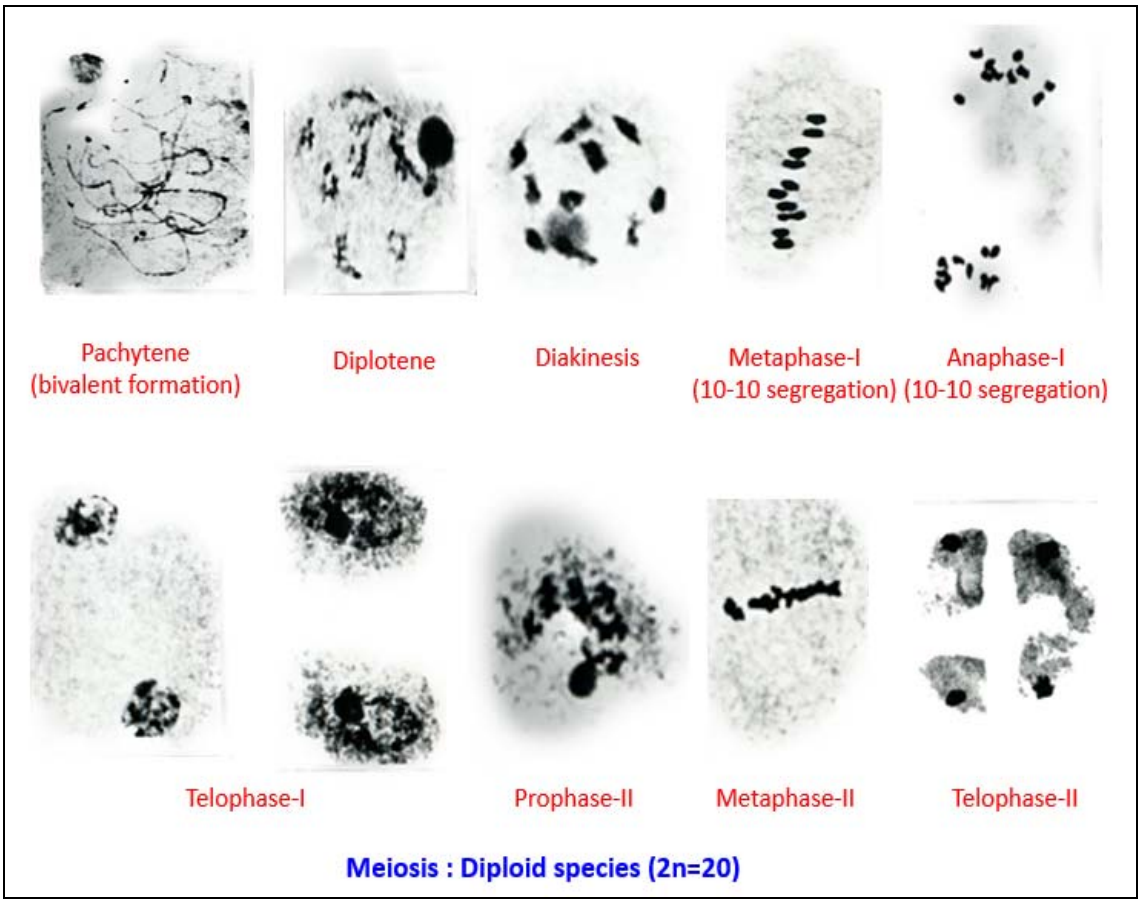

Fig 8: Meiosis in pollen mother cells of diploid race of C. *flexuosus (2n=20)*

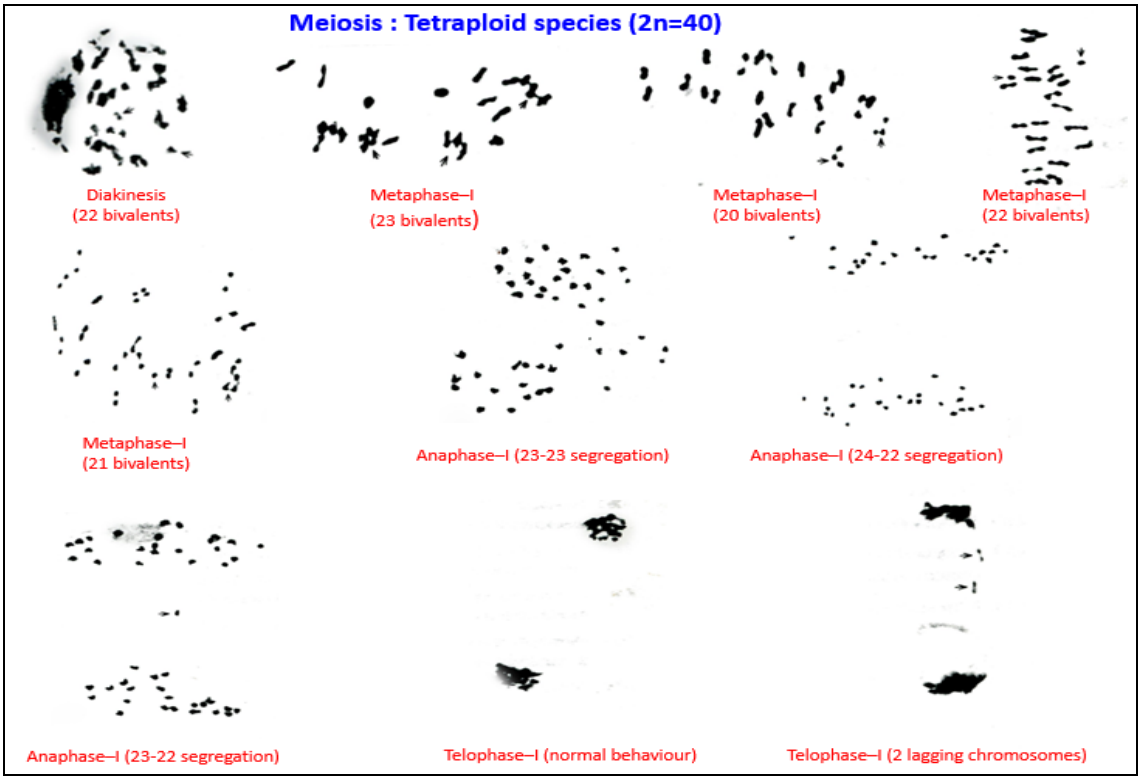

Fig 9: Meiosis in pollen mother cells of diploid race of C. *flexuosus (2n=40)*

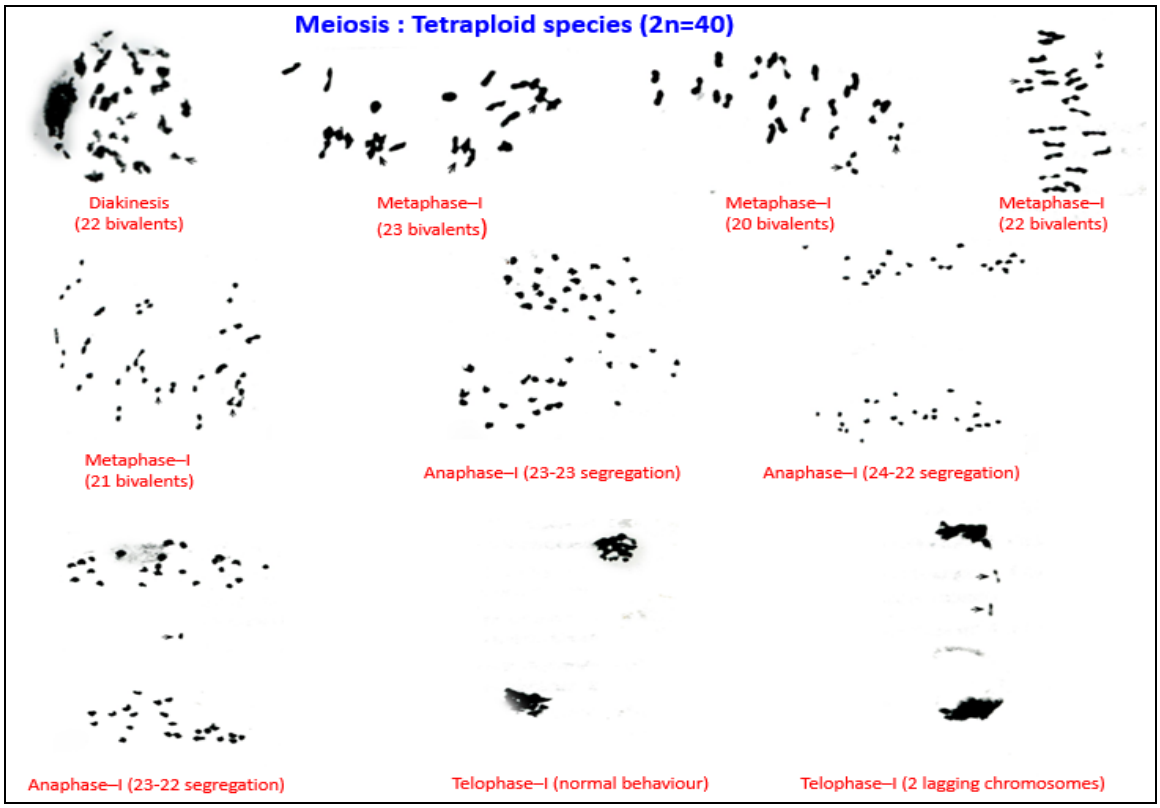

Fig 10: Meiosis in pollen mother cells of diploid race of C. *flexuosus (2n=60)*

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