Differences in Karyotype and Fluorochrome Banding Patterns among Variations of *Trichosanthes cucumerina* with Different Fruit Size

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Summary Snake gourd is an agriculturally important cucurbit vegetable recognized presently as the cultivar ‘Anguina’ of *Trichosanthes cucumerina* ssp. *cucumerina*. The wild type plant occurs naturally that produces small fruits (TCSF) and thus can be distinguished from the cultivar with large elongated fruits (TCLF). Presently, chromosomal features were revealed by modern cytogenetic methods to characterize the two types of plants. Chromosome preparations were standardized by an enzymatic maceration and air-drying method (EMA). The cultivars had considerably different karyotypes than the TCSF plants in spite of the same chromosome numbers ($2n=22$). EMA-DAPI based meiotic cell preparations reconfirmed gametic chromosome number ($n=11$) and showed regular chromosome behavior in both plant types. Karyomorphometric study with 14 inter- and intra-chromosomal symmetry/asymmetry indices advocated higher asymmetry in the karyotype of TCLF. The fluorochrome banding pattern of somatic chromosomes revealed the differential distribution of CMA and DAPI bands in the types of plants. TCSF plants were characterized by the presence of CMA bands in two pairs of chromosomes with secondary constrictions while DAPI bands were found in all chromosomes of the complements. On the contrary, DAPI bands were completely absent in TCLF while distal CMA bands were scored in two pairs of chromosomes with secondary constrictions and one pair of other chromosomes. CMA-DAPI banding patterns and karyomorphometric parameters had a significant impact on the evaluation of karyotype relations. The plants of TCSF and TCLF formed distinct groups and clusters in PCA plot and UPGMA phenogram, respectively. The variation in karyoevolutionary status and fluorochrome banding pattern between TCSF and TCLF plants could be utilized for further cytotaxonomic and phylogenetic revisions.

Key words EMA-Giemsa, CMA, DAPI, Snake gourd, *Trichosanthes cucumerina* L., Karyotype asymmetry, Meiosis.

The family Cucurbitaceae has been esteemed since ancient times for being the world’s second large vegetable producing family extensively cultivated in tropical countries. Like many other cucurbit crops, snake gourd is one of the widely consumed vegetables in India which was known as a variety of *Trichosanthes cucumerina* L. [viz. *T. cucumerina* L. var. *anguina* (L.) Haines] for a long time (Haines 1922). *T. cucumerina* is a native species of *Trichosanthes* in India (Renner and Pandey 2013) belonging to the tribe Sicyoeae of Cucurbitaceae (Schaefer and Renner 2011). *T. cucumerina* produces small ovoid fruits while the snake gourds are elongated and sometimes twisted fruits used as a vegetable when unripe. The distinct difference in fruit morphology was the reason why *T. cucumerina* and *T. anguina* were origi-

nally entitled as different species (Linnaeus 1753) before the inclusion of snake gourd as a variety of *T. cucumerina* (Haines 1922). Chakravarty (1959) re-established the rank of two distinct species based on fruit character. Five species of *Trichosanthes* were later assembled into *T. cucumerina* group (Jeffrey 1980a, b, Chakravarty 1982) including *T. cucumerina* and *T. anguina* owing to variable and overlapping morphological characters. Molecular phylogenetic studies supported the assignment of the vegetable snake gourd as a variant form of *T. cucumerina* (Ali and Al-Hemaid 2010, Cooper and de Boer 2011). Of late, *T. cucumerina* group has been worked out in detail and includes infraspecific ranks based on reliable morphological characters (Pradheep *et al.* 2015). *T. cucumerina* has three subspecies of which *T. cucumerina* ssp. *cucumerina* is the one distinguished from its cultivar again by one attribute *i.e.*, elongated fruits (the snake gourds). Hence snake gourd has been regarded as the cultivar *T. cucumerina* ssp. *cucumerina* Anguina. (L.) K. Pradheep, D.R. Pani & K.C. Bhatt, cult. nov. of the wild *T. cucumerina* ssp. *cucumerina* (Pradheep *et al.*

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This paper is dedicated to the memory of our teacher and renowned Plant Cytogeneticist Professor Arun Kumar Sharma (31st December 1924–6th July 2017), who worked tirelessly to improve plant science teaching and research in the country.

2015) and not a variant form or variety.

Genomic or cytogenetic analysis of the species is missing although snake gourd ('*Anguina*') cultivar and its wild type plant have huge distribution and rich germplasm in India. The recent taxonomic revision is not substantiated by the genomic or chromosomal study. A cytogenetic investigation also has a major role in breeding programs for many important crops like wheat or maize (Albert *et al.* 2010, Icsó *et al.* 2014). The dearth of cytogenetic information encouraged us to study the basic chromosomal features of *T. cucumerina*. So far, chromosome numbers in *T. cucumerina* ($2n=22$, $n=11$) were reported (McKay 1931, Nakajima 1936, Sen and Datta 1975, Singh and Roy 1975, Datta and Basu 1978, Sarker *et al.* 1987, Beevy and Kuriachan 1996) while conventional chromosome staining was followed to construct karyotypes in *T. cucumerina* and *T. anguina* (Sarker *et al.* 1987, Alam *et al.* 2012, 2018). Cucurbit species have small chromosomes and dense cytoplasmic content (Bhowmick and Jha 2015, Ghosh *et al.* 2018) that might obstruct thorough analysis *via* conventional methods. The EMA (Fukui 1996) helps in the elimination of cell wall and cytoplasm required for the scattering of chromosomes (Bhowmick *et al.* 2012). We have taken up this method for mitotic and meiotic chromosome study in *T. cucumerina*. This was accompanied by the analysis of fluorochrome banding pattern in the chromosomes using chromomycin A₃ (CMA) and 4',6-diamidino-2-phenylindole (DAPI). We discuss how cytogenetic information can be used to understand the karyoevoutionary status and possible variation between wild plants of *T. cucumerina* and the snake gourd cultivar which is a prerequisite for further cytotaxonomical analyses.

Materials and methods

Plant materials

For the convenience of interpretation, *T. cucumerina* ssp. *cucumerina* was designated as the small fruit producing plant (TCSF, fruit size 4–5 cm, Fig. 1a) and *Trichosanthes cucumerina* ssp. *cucumerina* '*Anguina*' (L.) was designated as large fruit producing cultivar *i.e.*, snake gourd (TCLF, fruit elongated, 15–30 cm long or even more, Fig. 1b, c). TCSF plants grow naturally in various regions of India. Entire TCSF plants were collected from Ballygunge, Kolkata, and University of Agricultural Sciences, Bangalore and seeds were collected from NBPGR, Thrissur (Table 1). Seeds of TCLF were collected from Bongaon, West Bengal, nurseries of Bhagalpur, Bihar and Adiganahalli, Bengaluru (Table 1). Seedlings were grown and plants were maintained in the Experimental Garden of University of Calcutta.

Chromosome preparation

Somatic chromosomes from root tips were derived from germinating seedlings and underground rhizomes.

Fig. 1. TCSF plant with enlarged flower and fruit in insets (a). TCLF plant (cultivar) with an enlarged flower in the inset (b) and the fruit of TCLF (snake gourd) cultivar (c). Scale bar=5 cm.

Table 1. Collection details of *Trichosanthes cucumerina*.

Type of fruits	Population	Abbreviation of population
TCSF	Ballygunge, Kolkata, West Bengal	KOL
	NBPGR, Thrissur, Kerala	NBP
	University of Agricultural Sciences GKV, Bengaluru, Karnataka	BEN
TCLF	Bongaon, West Bengal	BON
	Bhagalpur, Sabour Diara, Bihar	BHA
	Adiganahalli, North Bengaluru, Karnataka	ADI

Healthy roots (0.5–1 cm in length) were pretreated in 2 mM hydroxyquinoline at 15°C for 4 h and fixed in freshly prepared aceto-methanol (1:3). Root tips were subjected to EMA (Fukui 1996) with useful modifications. Root tips were digested in a cocktail of 1% cellulase Onuzuka RS (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.15% pectolyase Y-23 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.75% macerozyme R-10 (Kyowa Chemical Products Co., Ltd., Japan) and 1 mM EDTA (pH 4.2) for 42 min at 37°C followed by maceration on clean glass slides in a drop of the fixative. The air-dried slides were stained with 2% Giemsa [Giemsa solution (Merck): 1/15 M phosphate buffer: distilled water=2:50:48] for 20 min. Metaphase plates were observed under a Leitz GMBH microscope attached with a ProgRes CT5 Jenoptik D07739 camera and a ProgRes CapturePro ver 2.8.8 software was used for capturing photographs and taking measurements [long arm length (l), short arm length (s), chromosome length (CL), total diploid chromosome length (TCL)]. For karyotype

analysis, chromosomes were classified on the basis of centromeric indices [$Ci=(s/CL)\times 100$] according to Levan *et al.* (1964) and designated as metacentric (M) ($Ci>47.5-50.0$), nearly metacentric (m) ($Ci>37.5-47.5$), submetacentric (sm) ($Ci>25.0-37.5$) and subtelocentric (st) ($Ci>12.5-25.0$).

Analysis of karyomorphometric parameters

At least five metaphase plates from five individual plants of TCSF and those of TCLF from each population had been selected for karyotype preparation and estimation of the degree of symmetry/asymmetry by 14 different parameters. The chromosomes were arranged in decreasing order of length for presenting the karyotype. Inter- and intra-chromosomal asymmetry/symmetry in the karyotypes were evaluated after calculating values of indices *viz.* total form percentage (TF%) (Huziwar 1962), intrachromosomal asymmetry index (AI) and interchromosomal asymmetry index (A2) (Zarco 1986), coefficients of variation of chromosome length (CV_{CL}) and arm ratio (CV_r) (Paszko 2006), index of karyotype symmetry (Syi) (Greilhuber and Speta 1976), asymmetry index of karyotype (AsK%) (Arano 1963), degree of karyotype asymmetry (A) (Watanabe *et al.* 1999), mean centromeric index (X_{Ci}) (Seijo and Fernández 2003), coefficient of variation of centromeric index (CV_{Ci}) (Paszko 2006), symmetry index (S%) (Liang and Chen 2015), dispersion index (DI) (Lavanina and Srivastava 1992), asymmetry index (AI) (Paszko 2006) and mean centromeric asymmetry (M_{CA}) (Peruzzi and Eroğlu 2013).

Fluorochrome staining of somatic metaphase chromosomes

The Giemsa stained metaphase chromosome slides were marked under a fluorescent microscope Zeiss Axioscop 2 followed by destaining in 70% methanol for 45 min and air drying for sequential DAPI and CMA staining according to the protocol (Bhowmick *et al.* 2012). Slides were incubated in McIlvaine buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 , pH 7.0) for 15 min and stained with $20\mu g mL^{-1}$ DAPI for 10 min in the dark phase, washed in the buffer, blow-dried, mounted in non-fluorescent glycerol and observed under the fluorescence microscope with a UV filter cassette. After observation with DAPI, the slides were de-stained in the fixative for 30 min, air-dried and incubated in the buffer with 5 mM $MgSO_4$ for 10 min. Next, the slides were stained with $0.1 mg mL^{-1}$ CMA for 10–15 min in the dark phase, washed with the buffer with 5 mM $MgSO_4$, blow-dried and finally mounted in glycerol. Observations were taken under a BV filter cassette in the fluorescence microscope. Images were captured with the camera ProgRes MF scan Jenoptik D07739 attached to the fluorescence microscope using the software ProgRes CapturePro 2.8.8.

Preparation of meiotic chromosomes by EMA-DAPI technique

Dense cytoplasmic content in the PMCs was eliminated by EMA-DAPI staining (Bhowmick and Jha 2015) with minor modifications. The male flower buds of 0.5 cm length were collected at around 10 am and macerated in the enzyme mixture for 3–4 min at 37°C. Macerated anthers were pipetted out in a clean glass slide and PMCs were spread in the fixative and air-dried for 1–3 h. The slides were kept in the buffer for 12 min. Slides were then stained with $20\mu g mL^{-1}$ DAPI solution for 20 min, washed in the buffer and finally mounted in glycerol. The observation was taken under the UV filter in the fluorescence microscope and images were captured using the camera.

Evaluation of chromosomal relationships by statistical analyses

One way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was conducted for chromosome morphometric data and karyotype study considering triplicate values of the TCSF and TCLF plants using IBM SPSS Statistics 16.0. Quantitative parameters of chromosome measurements and karyomorphometric values were used to assess significant differences ($p\leq 0.05$ at 95% confidence level) between TCSF and TCLF if any. A cluster analysis with nine numerical karyotype parameters (CV_{CL} , CV_r , AsK%, CV_{Ci} , M_{CA} , DI, AI, number of chromosomes with DAPI and CMA signals) was conducted to normalize data matrix and produce an unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on average Euclidean distance using Infostat 2017/d software. Apart from this, we also focused on the principal component analysis (PCA) with the above mentioned karyomorphometric parameters using the same software since PCA is a useful statistical measure for multivariate analysis (McVean 2009, Marghali *et al.* 2014).

Results

Karyotype features based on EMA-Giemsa staining

Standardization of pretreatment conditions combined with EMA-Giemsa method resulted in scattered metaphase plates with clear chromosomes against a cytoplasm free background suitable for karyotype analysis in the plants (Fig. 2). The number of chromosomes in somatic cells was $2n=22$ in both TCSF (Fig. 2a–c) and TCLF (Fig. 2f–h). Chromosome length in a complement ranged from 2.26–4.99 μm in TCSF (Table 2). The karyotype was composed of 18 nearly metacentric and four nucleolar chromosomes. One of the nucleolar pairs was submetacentric with a secondary constriction on the short arm and the other pair was nearly metacentric with a secondary constriction on the short arm (Fig. 2a–c). Plants of TCSF were found to have consistency in the

Fig. 2. EMA-Giemsa preparations of somatic metaphase chromosomes ($2n=22$) of TCSF plants collected from populations KOL (a), NBP (b) and BEN (c). d-e Karyotype and corresponding ideogram of TCSF. f-h EMA-Giemsa preparations of somatic metaphase chromosomes ($2n=22$) of TCLF plants collected from populations BON (f), BHA (g) and ADI (h). i-j Karyotype and corresponding ideogram of TCLF. Chromosomes with nucleolar constrictions are indicated by arrows. For abbreviation of populations, see Table 1. Scale bar = $5\mu\text{m}$.

Table 2. Chromosome number, karyotype data and CMA-DAPI banding pattern in *T. cucurmerina*.

Type of fruits	Population	Chromosome number		TCL* (μm)	CSR** (μm)	Karyotype	Chromosomes with CMA bands	Chromosomes with DAPI bands	Fluorochrome banding pattern
		2n	n						
TCSF	KOL	22	11	76.95 ± 1.22	2.26–4.99	$18m+2sm^{st}+2m^{st}$	4	22	12A+6B+4C
	NBP	22	11	74.21 ± 1.05	2.29–4.78	$18m+2sm^{st}+2m^{st}$	4	22	12A+6B+4C
	BEN	22	11	75.97 ± 2.41	2.32–4.68	$18m+2sm^{st}+2m^{st}$	4	22	12A+6B+4C
TCLF	BON	22	11	75.49 ± 2.85	2.77–5.01	$12m+4M+2sm+4sm^{st}$	6	0	2D+2E+2F+6G
	BHA	22	11	76.11 ± 0.57	2.80–4.76	$12m+4M+2sm+4sm^{st}$	6	0	2D+2E+2F+6G
	ADI	22	11	75.20 ± 1.88	2.0–4.98	$12m+4M+2sm+4sm^{st}$	6	0	2D+2E+2F+6G

*TCL: Total diploid chromosome length, **CSR: chromosome size range

morphology of the chromosomes. The nucleolar pairs of chromosomes were the longest in the complement. One of the nearly metacentric pair of chromosomes had a slightly longer length than the other nearly metacentric chromosomes in all TCSF individuals (Fig. 2d). The length of this pair ranged from 4.72 to 4.67 μm in the plants. The total diploid chromosome length of TCSF

was $75.71 \pm 1.38\mu\text{m}$ (Table 2) and modal karyotype was determined as $18m+2sm^{st}+2m^{st}$ (Fig. 2d, e, Table 2).

The TCLF plants had six nearly metacentric pairs, two metacentric pairs, one submetacentric pair and two nucleolar pairs of chromosomes (Fig. 2f–j). The pairs of nucleolar chromosomes were the longest and had secondary constrictions at the submedian and subter-

minimal regions in all plants (Fig. 2i–j). Chromosome size ranged from 2.77–5.01 μm in the TCLF plants (Table 2). Among the chromosomes without secondary constriction, one nearly metacentric pair of chromosomes was larger than the others in TCLF plants. The length of this pair was varying between 4.92 to 4.80 μm . The total diploid chromosome length in TCLF was $76.26 \pm 0.46 \mu\text{m}$ (Table 2) and modal karyotype was found to be $12\text{m}+4\text{M}+2\text{sm}+4\text{sm}^{\text{st}}$ (Fig. 2i, j, Table 2).

Comparative karyomorphometric study in TCSF and TCLF plants

Among the 14 parameters taken for the present analysis, the increase in values of A2, CV_{CL} , and CV_r in TCLF plants provided support for higher inter-chromosomal asymmetry in the karyotype (Table 3). Again, a higher level of asymmetry in TCLF compared to TCSF was supported by an increase in the intra-chromosomal asymmetry indices like Ask% and CV_{CI} (Table 3). Remarkably, the intra-chromosomal asymmetry indices showed greater asymmetry in TCLF (Table 3) than those of TCSF. Some of the intra-chromosomal parameters are inversely related to asymmetry and they showed a distinctly lower value in TCLF (A1, TF%, Table 3). Also, AI value suggested a predisposition of asymmetry in the karyotype of TCLF plants. Cumulatively, 10 out of 14 karyomorphometric parameters depicted an asymmetric nature in the karyotypes of TCLF compared to TCSF plants.

Fluorochrome banding pattern in somatic chromosomes

Differential distribution of CMA and DAPI bands was clear in the two types of plants under study. In TCSF plants, chromosomal DAPI and CMA bands were observed (Fig. 3a–f) and DAPI bands were found in all somatic chromosomes (Fig. 3b, e). A clear examination of DAPI banding revealed six pairs of nearly metacentric chromosomes with DAPI bands in the distal parts of both the arms and three other pairs with distal DAPI bands in the long arm (Fig. 3a, b). CMA staining revealed positive bands in the distal parts of four nucleolar chromosomes (Fig. 3c, d, f). CMA bands were found to be DAPI negative while distal part of the long arm of the same chromosomes was found to have DAPI bands (Fig. 3a–c, e–f).

In the TCLF plants, there were six distinct CMA bands in three pairs of chromosomes (Fig. 3j–m). One of the nucleolar chromosome pair showed distal CMA bands and two CMA spots just below the secondary constriction (Fig. 3i–m). The other chromosome pair displayed a single distal CMA band in the short arm (Fig. 3i–j, k–m). Another distal CMA band was detected in a nearly metacentric pair of chromosomes (Fig. 3i–m). Chromosomes displayed uniform staining with DAPI indicating an absence of any DAPI bands in TCLF plants (Fig. 3n–o).

Table 3. Karyomorphometric configuration of *Trichosanthes cucurmerina*.

Type of fruits	Population	Karyomorphometric parameters*													
		Inter-chromosomal asymmetry parameters						Intra-chromosomal symmetry/asymmetry parameters						Inter- and intra-(combined) chromosomal symmetry/asymmetry parameters	
		A2	CV _{CL}	CV _r	Sy _i	TF%	Ask%	A1	A	X _{CI}	CV _{CI}	M _{CA}	S%		DI
TCSF	KOL	0.19±0.02	19.89±0.21	66.14±0.10	66.52±0.40	44.19±1.08	55.80±0.54	0.64±0.10	0.14±0.01	43.52±0.87	31.60±0.06	14.54±0.30	47.01±0.55	10.45±0.10	6.28±0.20
	NBP	0.18±0.00	21.47±0.19	66.01±0.06	61.27±0.12	46.88±1.21	56.53±0.09	0.64±0.03	0.18±0.02	43.58±0.35	29.68±0.14	18.29±0.20	44.12±0.39	10.60±0.02	6.13±0.00
	BEN	0.19±0.00	21.20±0.13	65.03±0.28	65.39±0.33	44.30±1.00	55.44±0.53	0.64±0.07	0.15±0.00	43.66±0.77	29.54±0.02	18.63±0.11	46.80±0.01	10.31±0.09	6.33±0.04
	Average values	0.186	20.85	65.73	64.39	45.12	55.92	0.64	0.16	43.58	30.27	17.15	45.97	10.45	6.25
TCLF	BON	0.21±0.01	27.86±0.11	70.01±0.23	69.77±0.08	44.71±0.87	66.53±0.13	0.32±0.10	0.14±0.01	44.07±0.08	35.82±0.26	14.10±0.10	44.40±0.90	9.48±0.14	6.85±0.14
	BHA	0.21±0.00	27.94±0.25	67.68±1.06	66.32±0.22	43.88±0.24	65.54±0.33	0.32±0.00	0.10±0.00	44.87±0.11	35.00±0.00	10.80±0.12	44.07±0.08	9.45±0.23	6.33±0.11
	ADI	0.20±0.03	27.66±0.20	69.21±0.31	67.53±0.32	43.57±0.65	69.08±0.41	0.32±0.02	0.13±0.02	45.30±0.42	35.70±0.09	13.60±0.02	45.70±0.12	9.48±0.22	7.83±0.08
	Average values	0.206	27.82	68.96	67.87	44.05	67.05	0.32	0.12	44.74	35.50	12.83	44.72	9.47	7.00

Fig. 3. a–f Somatic metaphase chromosomes of TCSF plants stained with Giemsa (a) DAPI (b and e) and CMA (c, d, and f): a–c KOL, d NBP; e–f BEN. g–h Haploid ideogram (g) and karyotype (h) showing CMA-DAPI banding pattern in the chromosomes of TCSF plants. i–n Somatic metaphase chromosomes of TCLF plants stained with Giemsa (i) CMA (j–m) and DAPI (n): i–j BON, k–l BHA; m–n ADI. o–p Haploid ideogram (o) and karyotype (p) showing CMA banding pattern and lack of DAPI bands in the chromosomes of TCLF plants. Black arrows indicate nucleolar constrictions and white arrows indicate CMA bands. q–t EMA based DAPI stained PMCs showing meiotic chromosomes at metaphase I (q and s) and metaphase II (r and t) stages: q–r TCSF, s–t TCLF. For abbreviation of populations, see Table 1. Scale bar = 5 μ m.

On the basis of occurrence of DAPI and CMA bands, chromosomes were classified into three distinct types in the TCSF plants such as type A where two distal DAPI bands appeared in the opposite arms of a single chromosome, type B chromosomes having one distal DAPI band and type C chromosomes with one distal CMA band in the shortest arm and one distal DAPI band in the longest arm. This prototype was found to be consistent in all TCSF plants and hence the DAPI and CMA banding pattern could be determined as 12A+6B+4C (Fig. 3g–h). Compared to TCSF, plants of TCLF had chromosomes with different fluorochrome banding patterns namely type D with one distal CMA band, type E with one distal CMA band in the nucleolar chromosomes, type F with distal CMA band followed by two CMA spots immediately below the second constriction and type G with no fluorochrome bands. Hence the CMA banding pattern in TCLF plants was determined as 2D+2E+2F+16G (Fig. 3o–p).

Meiotic behavior of the plants studied by EMA-DAPI staining technique

Regular chromosome pairing in early prophase I and presence of 11 bivalents in metaphase I confirmed the gametic chromosome number as $n=11$ in TCSF and TCLF plants (Fig. 3q, s). Observation of 11 chromosomes in metaphase II further confirmed regular segregation in both TCSF and TCLF plants (Fig. 3r, t). A chromosomal anomaly in meiotic divisions was not detected.

Status of karyotype evolution determined by PCA and UPGMA analyses

In the principal component analysis, plants of TCLF and TCSF were located at a considerable distance in the component plot. The plot accounted for 96.3% of the total chromosomal differences between the two types of plants. PC1 accounted for 88.5% of the total variation whereas PC2 accounted for 7.8% of the variation

Fig. 4. a Component plot derived after principal component analysis of TCSF (●) and TCLF (■) plants of six populations using karyomorphometric and fluorochrome banding variables, 1: KOL, 2: NBP, 3: BEN, 4: BON, 5: BHA, 6: ADI. b UPGMA dendrogram worked out from average Euclidean distance on the basis of karyomorphometric indices and fluorochrome banding data of TCSF and TCLF plants of six populations, 1: KOL, 2: NBP, 3: BEN, 4: BON, 5: BHA, 6: ADI. For abbreviation of populations, see Table 1.

in the two-dimensional plot (Fig. 4a). PC1 highlighted the variations due to differences in CV_{CL} , CV_{CI} , $AsK\%$, DI , M_{CA} and number of chromosomes with DAPI bands while PC2 emphasized chromosomal differences owing to variation in CV_r , AI and number of chromosomes with CMA bands. TCSF and TCLF occupied two spatially distinct groups in the component plot (Fig. 4a). Cluster analysis with quantitative parameters along with CMA and DAPI banding pattern resulted in the formation of two distinct clusters of TCSF and TCLF in the UPGMA dendrogram with a cophenetic correlation of 0.984 which was a good fit between the average linkage matrix and cophenetic value matrix (Fig. 4b).

Discussion

T. cucumerina was known to have a taxonomic controversy regarding the inclusion of snake gourd as variety since a long time. The naturally growing TCSF was considered recently as the wild type while TCLF (snake gourd) was regarded as the cultivar (Pradheep *et al.* 2015). We collected populations of both TCSF and TCLF from different regions to see if there is any significant difference in karyotype with respect to geographical variations. The EMA-Giemsa based method of chromosome analysis enabled clarification of chromosome morphology and karyotype analysis in *T. cucumerina*. Meiotic revision of this species by EMA-DAPI staining was found useful to visualize chromosomes and reconfirm constancy in gametic chromosome number and regular chromosome segregation in both types of plants. Considering the early reports, our study corroborates to the number of chromosomes reported ($2n=22$, $n=11$) (McKay 1931, Nakajima 1936, Sen and Datta 1975, Singh and Roy 1975, Datta and Basu 1978, Sarker *et al.* 1987, Beevy and Kuriachan 1996). TCSF plants of three populations had the same chromosome numbers, similar karyotypes, and banding patterns but had minor differences in a chromosome size range and karyotype asymmetry/symmetry indices. Also, the cultivars had an overall constancy in karyotype attributes among the

populations and little difference in chromosome size, pointing towards genomic stability. The difference in karyotypes was mainly pronounced between the TCSF and TCLF in terms of chromosome lengths and centromeric positions which were further reflected in the statistical analysis with karyotype symmetry/asymmetry values. However, the occurrence of three nucleolar chromosome pairs in *T. cucumerina* and *T. anguina* was documented in old illustrations (Sarker *et al.* 1987), while some cultivars of snake gourd were reported to contain variation in chromosome numbers, karyotypes, and a number of satellites chromosomes (Alam *et al.* 2012, 2018). Presently, four chromosomes with the secondary constriction were clearly observed in both TCSF and TCLF plants, having differences in the centromeric position.

The tendency of having symmetric or asymmetric nature in the karyotype of a species has been appreciated as a standard for cytotaxonomic evaluation (Paszko 2006, Peruzzi and Eroğlu 2013). Inter-chromosomal asymmetry resulting from heterogeneity in chromosome sizes (Levitsky 1931) was prominently acquired in TCLF cultivar. Variations in the relative position of centromeres lead to intra-chromosomal asymmetry (Levitsky 1931) which is also higher in TCLF. Some parameters are inversely related to intra-chromosomal symmetry/asymmetry which had lower values (AI and $TF\%$) in TCLF, supporting higher asymmetry in its karyotype. Parameters like AI (Paszko 2006) combines the impact of inter- and intra-chromosomal variations and were also indicative of higher asymmetry in TCLF. The data are based on five TCSF and five TCLF plants from each population and hence the overall asymmetric inclination of karyotype in TCLF is statistically significant.

CMA and DAPI stainings accentuated dissimilarities in chromosome architectures. The presence of DAPI bands in all chromosomes of TCSF (types A, B, and C) demarcated it from TCLF which lacked such bands altogether. The occurrence of DAPI bands in all chromosomes was previously documented in *T. dioica*

(Bhowmick and Jha 2015). There might be a tendency for acquiring DAPI bands in the genus which can be confirmed after study in other species of *Trichosanthes*. The presence of four distal CMA bands in TCSF plants differentiated them from TCLF plants with six distal CMA bands. Previously, CMA bands have been suggested to be GC-rich sequences corresponding to NORs (Schweizer 1976, Guerra 2000) which in this case was corroborating with four nucleolar chromosomes of both TCSF (type C) and TCLF (types E and F) with distal CMA bands. Interestingly, the type F nucleolar chromosome pair of TCLF had an additional interstitial (immediately below the secondary constriction) band. Again, the type D non-nucleolar chromosomes with distal CMA bands were another remarkable feature in TCLF plants. However, the particular nature of these non-nucleolar or interstitial CMA bands awaits the application of *in situ* hybridization with rDNA probe.

Fluorochrome banding patterns and quantitative karyomorphometry parameters directly related to karyotype asymmetry were utilized for assessing the statistical difference between the two types of plants. Inversely related parameters (TF% and Syi) and qualitative indices like Stebbins category were ruled out. The UPGMA analysis and PCA yielded distinct grouping of TCSF plants apart from TCLF and emphasized delineation of the two. The evolution of cultivars might come about as a consequence of artificial selection in agricultural practice. The mechanism of whether and how these processes may lead to certain chromosomal changes is not known in this case. The cultivar 'Anguina' share same chromosome numbers with TCSF unlike many cultivated cucurbits with higher ploidy (Waminal and Kim 2015). A detailed investigation of chromosome morphology and fluorochrome banding pattern clarified the difference between the *T. cucumerina* and its cultivar 'Anguina.' In accordance with the morphological basis of delineation, karyotype and fluorochrome banding pattern analysis in our study provides an archetype to complement further cytotaxonomic revision of *T. cucumerina*. These two types of plants have many similarities in morphological features other than fruit because of which snake gourd was regarded as the cultivar and not a distinct species or subspecies (Pradheep *et al.* 2015). Since TCSF and TCLF plants have the same ploidy but the difference in karyotype attributes with respect to the difference in fruit morphology, we are far from making any conclusion to resolve the taxonomic status of these plants. Karyotype differences were previously documented between the varieties of bitter gourd (Ghosh *et al.* 2018) and varieties and cultivars of cucumber (Zhao *et al.* 2011, Zhang *et al.* 2012). It would be interesting to see the extent of phylogenetic divergence between TCSF and TCLF in future genome analysis. Results of our cytogenetic study lay a foundation for such investigations in *T. cucumerina* that might also facilitate

selective breeding among plants with desirable agricultural and medicinal qualities.

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