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A comparative karyo-morphometric analysis of Indian landraces of *Sesamum indicum* using EMA-giemsa and fluorochrome banding

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Abstract. *Sesamum indicum* commonly known as 'Sesame', 'Til' or 'Gingli' is an age-old high valued oil crop. With distinct seed and floral diversity and no detailed chromosomal analysis is available on Indian landraces of *S. indicum* (2n= 26). The present study demonstrates standardization of enzymatic maceration and air drying (EMA) method of chromosome preparation and comparative karyometric analysis in four Indian landraces of *S indicum.* All the landraces were characterized by very small chromosomes, length ranging from 1.24 ± 0.02 to 2.87 ± 0.09 µm. The EMA- Giemsa based karyotype analysis revealed nine pairs of chromosomes with nearly median primary constriction, three pairs were submedian and a single satellite pair in each of the studied landrace. The CMA staining of *Sesamum* chromosomes revealed the presence of distinct CMA positive (CMA^{+ve}) signals in all the studied landraces. The Black seeded til (BT) and White seeded til (WT) were characterized by six chromosomes with distal CMA^{+ve} signal on short arm, while the Dark brown seeded til (DBT) showed ten chromosomes with distal CMA^{+ve} signal on short arm. The Light brown seeded til (LBT) was characterized by eight chromosomes with distal CMA^{+ve} signal on short arm. The results obtained from the scatter plot of A1 versus A2 and PCA analysis provide a strong relationship with that of the fluorochrome banding analysis. The present research offers an explicit karyo-morphometric characterization of four Indian landraces of *S. indicum* for the first time.

Keywords: fluorochrome banding, karyotype, sesame, *Sesamum indicum*, small chromosomes, til.

INTRODUCTION

Sesamum indicum L. commonly known as 'Sesame', 'Til' or 'Gingli' is an age-old high valued oil crop. As per the Index Kewensis the genus belongs to the family Pedaliaceae and comprises 36 species. However, *S. indicum* is the only cultivated species of this genus (Nayar and Mehra 1970). Sesame seeds are also known as the 'Queen of the oil seeds' and the first oil known to be

consumed by human (Bedigian and Harlan 1986). Beneficial effects exhibited by sesame as antioxidant, antimicrobial, anti-inflammatory, antidiabetic, anticancer on human health has recently renewed the interest in this crop (Amoo *et al.* 2017, Zhang *et al.* 2013). The species was domesticated in India long back (Bedigian 2003; 2010) and now ranked first in production and export of this crop (IOPEPC Kharif 2017). Cultivated *S. indicum* has highly variable genotypes and distinct differences have been noted in floral and seed colour morphology within the cultivated landraces (Raghavan *et al.* 2010). Chromosome analysis has played an important role in genetics and plant breeding for conservation of genetic diversity and improvement of crops. It is felt that chromosome analysis still provides foundational pieces of genomic information (Soltis 2014) and considered "the quickest, cheapest, and easiest way to get any substantial information about the genome of a species which is not possible by any other methods" (Guerra 2008). Chromosome analysis in this cultivated species (2n=26) was reported long back by Morinaga *et al.* (1929), Raghavan and Krishnamurthy (1947) and Kobayashi (1949). Raghavan and Krishnamurthy (1947) reported that all 13 small chromosome pairs have terminal constrictions, while Mukherjee (1959) reported presence of five types of somatic chromosomes. However, Kobayashi (1949; 1991) in his analyses noted five pairs of median and eight pairs of sub median chromosomes. Zhang *et al.* (2013) reported three pairs median, eight pairs sub median and two pairs sub-terminal chromosomes in *S. indicum* cv. Yuzhi 11. It appears from the earlier reports that karyometric analysis of Indian sesame deserves priority as detailed chromosomal analysis is not available on *S. indicum* along with their important landraces. Thus, the present communication for the first time details the standardization of enzymatic maceration and air drying (EMA) method of chromosome preparation and comparative karyometric analysis using non-fluorescent Giemsa and fluorescent DAPI and CMA stains in four distinct Indian landraces of *S indicum*.

MATERIALS AND METHODS

Plant materials

The present study included four Indian landraces of *S. indicum* namely Black seeded till (BT), Dark brown seeded till (DBT), Light brown seeded till (LBT) and White seeded till (WT). Among these four landraces, seeds of BT, WT and LBT were collected from different parts of West Bengal and DBT was collected from Mangalore, Karnataka. All the collected seeds were germinated, grown in earthen pots and maintained under natural environment. Voucher specimens were prepared for all the collected samples.

Somatic chromosome preparation and karyo-morphometric analysis

Nearly 20- 25 seeds from each landrace were imbibed in water for overnight and germinated in dark on moist filter papers to harvest their root tips. A minimum of ten healthy root tips of each sample were pre-treated separately in saturated solution of *p*-dichlorobenzene (PDB) at 14- 16°C for 4- 5 hrs, fixed overnight in glacial acetic acid: methanol (1:3) and finally stored therein at - 20 °C. Enzymatic maceration and air-drying (EMA) method was carried out following our earlier published protocol (Jha and Yamamoto 2012; Jha *et al.* 2015; Jha and Saha 2017) with required modifications of enzyme digestion time (55 min-90 min). Completely air-dried slides were stained with 2% Giemsa solution (Merck; Germany) in 1/15th phosphate buffer solution (pH 6.8) for 10-30 min at room temperature. After 4- 5 times washing with ddH_2O, the slides were air dried, mounted with xylene and observed (a minimum of 20 well scattered metaphase plates for each landrace). They were examined and photographed under Carl Zeiss, Axio. Lab. A1 microscope fitted with CCD Camera using Axiovision L. E4 software.

For karyo-morphometric analysis, different karyological parameters viz. length of long arm (*l*) and short arm (s), absolute chromosome length (CL), relative chromosome length (RL) and total diploid chromatin length (TCL) were used. Five somatic metaphase plates were used for karyometric analysis as well as to prepare ideogram. The centromeric index (CI) was used to classify the chromosomes according to Levan *et al.* (1964) [metacentric (m) (1.00–1.70), submetacentric (Sm) (1.70–3.00), subtelocentric (St) (3.00–7.00) and telocentric (t) (7.00–α)]. The karyotype asymmetry was estimated using intra-chromosomal asymmetry index (A1) and inter-chromosomal asymmetry index (A2) (Zarco 1986), asymmetric karyotypes percent (AsK%), asymmetry Index (AI) (Paszko 2006), total form percent (TF%), coefficient of variation of chromosome length (CV_{CL}), coefficient of variation of centromeric index (CV_{CI}), coefficient of variation of arm ratio (CV_r) and categories of Stebbins (1971).

Fluorochrome staining with DAPI and CMA

Giemsa stained slides of each landrace were destained with 70% methanol for 40 min and air dried.

DAPI and CMA staining was carried out separately following the protocol of Kondo and Hizume (1982) with required modifications. For DAPI staining, slides were kept for 30 min in Mcllvaine buffer and then stained with 0.1µg ml^{-1} solution of DAPI for 10- 30 min, mounted in non-fluorescent glycerol and observed under Carl Zeiss Axio Lab A1 fluorescent microscope using carl zeiss DAPI filter cassette. For CMA staining, the same slides were de-stained air-dried and then kept in Mcllvaine buffer for 30 min followed by McIlvaine buffer with 5mM $MgCl_2$ for 10 mins. Slides were stained with 0.1mg ml^{-1} solution CMA for 30- 60 mins followed and rinsed with Mcllvaine buffer containing 5mM $MgCl_2$. Finally, slides were mounted with non-fluorescent glycerol and kept for maturation at 4^0C for 72 hrs. CMA stained chromosomes were observed under the above-mentioned fluorescent microscope fitted with Carl Zeiss FITC filter cassette and signals were analyzed using software Prog Res 2.3.3.

Statistical analysis

Descriptive statistics including mean values were analyzed for all measured parameters and variability in the data was expressed as the mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was performed to detect significant differences ($p \leq 0.05$) in the mean (Rohlf 1998). Duncan's multiple range test (DMRT) was used for post hoc analyses using SPSS v 16.0 statistical package. To study the karyotypic relationships among the collected landraces of *S. indicum*, scatter diagram of A1 versus A2 was drawn following the descriptions of Paszko (2006). In order to further clarify the chromosomal relationship between each of the studied landrace, principal components analysis (PCA) was conducted according to McVean (2009). In this study, nine karyological variables (A1, A2, TF%, AsK%, CV_{CL}, CV_{CI}, CV_r, AI and TCL) were used to plot the principal components using the InfoStat version 2013d (free version).

RESULTS

In the present study, four landraces of *S. indicum* differing in seed coat colour viz., Black seeded til (BT), Dark brown seeded til (DBT), Light brown seeded til (LBT) and White seeded til (WT) were used for karyotype analysis (Fig. 1). Nearly 99% seeds of each sesame landrace germinated within 3- 6 days after imbibition. Distinct diversity in the floral morphology pertaining to four different landraces of *Sesamum* was noted. The length of the corolla was 15- 20 mm with characteristic pigmentations on the lower lip. The flowers in black seeded til (BT) showed intense purple pigmentation in lower lip of corolla while in other landraces (DBT, LBT and WT) the intensity of the pigmentation ranged from pale lavender/ purple to light pink to white respectively (Fig. 1).

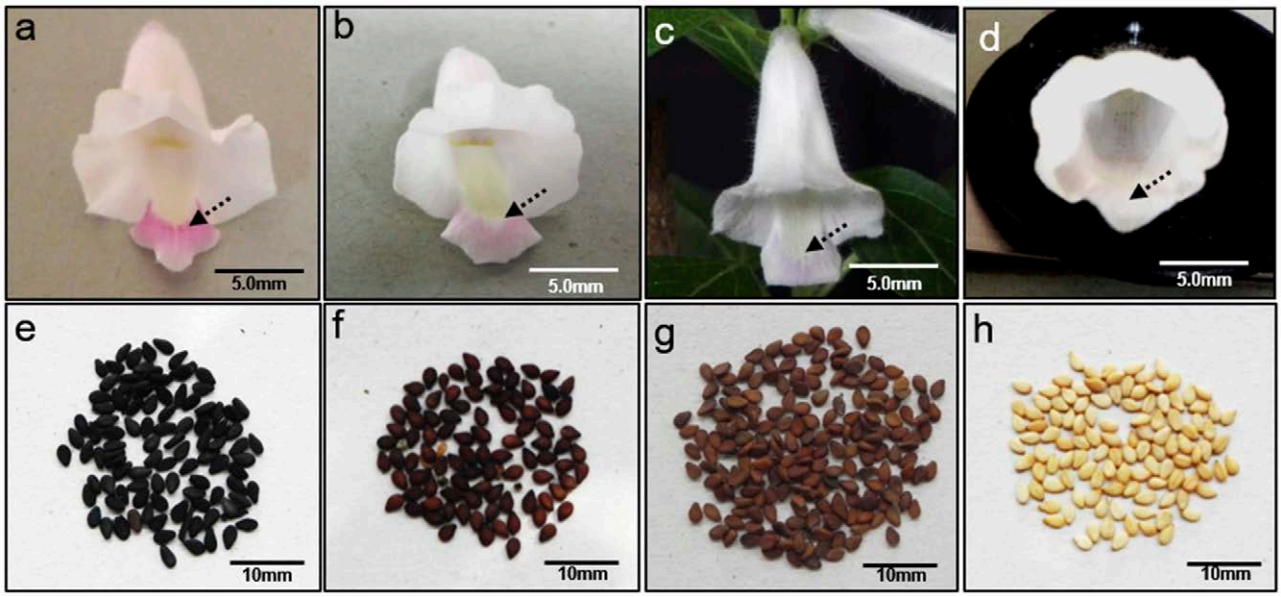

Figure 1. Flower and seed morphology of four Indian landraces of *S. indicum*. a & e) Black seeded til; b & f) Dark Brown seeded til; c & g) Light Brown seeded til; d & h) White seeded til. Dotted arrows indicate pigmentation patterns in lower lip of the corolla.

Karyo-morphometric analysis

Standardization of enzymatic maceration of root tip cells at 37 °C is the most crucial step to obtain well scattered metaphase chromosomes. In the present study, enzymatic maceration of root tips of all the collected landraces was performed for 55- 90 min and finally the time was optimized to 85- 90 min to obtain cytoplasm free well scattered chromosomes. The giemsa staining was done for 20 min. For each landrace, at least 20 countable metaphase plates were studied to determine diploid chromosome number.

Somatic chromosome number of 2n= 26 was observed in all the studied landraces of *S. indicum* (Fig. 2; Table 1). All the landraces were characterized by small sized chromosomes ranging from 1.24 ± 0.02 to 2.87 ± 0.09 µm (Fig. 2; Table 1). A significant variation in the total chromatin length was observed among the studied accessions. Black seeded til (BT) was characterized by highest total chromatin length (52.75 ± 0.24 µm), while the lowest (44.85 ± 0.35 µm) being found in Dark brown seeded til (DBT). The detailed karyotype analysis revealed nine pairs of chromosomes with nearly median primary constriction, three pairs with sub median primary constrictions and a single satellite pair in each of the studied landraces (Fig. 2). The ordering of satellite (sat) bearing pair was found to be constant (5th pair) in all the landraces having identical haploid karyotype formula: 3Sm + 9m + 1Sm.Sat (Fig. 2; Table 1).

In the present study, several karyo-morphometric variations were also noted among the studied landraces. Low values of intra-chromosomal asymmetry index (A1) and inter-chromosomal asymmetry index (A2) were observed in all the studied landraces (Table 2). Asymmetric Index (AI), the product of coefficient of variation in chromosome length (CV_{CL}) and coefficient of variation in centromeric index (CV_{CI}) was found to be low (ranging from 1.464 to 1.964) in all studied accessions of *Sesame* (Table 2). Whereas, Ask% and TF% showed moderate values for all the four *Sesamum* landraces (Table 2). Analysis of the karyotype asymmetric indices also revealed that all studied landraces except Black seeded til (BT) belong to the group 2A of Stebbins classification while Black seeded til (BT) belongs to group 2B (Table 2).

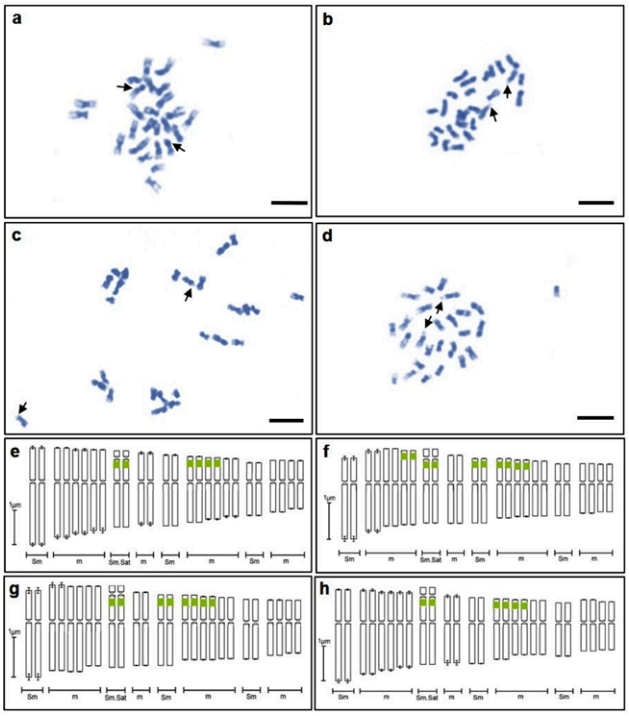

Figure 2. Panel A: EMA based giemsa stained mitotic metaphase plates of four Indian landraces of *S. indicum* showing 2n= 26 chromosomes. a) Black seeded til; b) Dark Brown seeded til; c) Light Brown seeded til; d) White seeded til. Arrows indicate secondary constricted chromosomes. Bar= 5 µm. Panel B: Comparative ideograms of the studied four landraces. Also showing positive CMA fluorescent band on respective chromosomes, Bar= 1 µm.

Fluorochrome banding analysis

In the present study, fluorochrome staining of *Sesamum* somatic chromosomes using DAPI and CMA was standardized for the first time. Chromosomes were stained properly with DAPI when incubated for 30 min, while for CMA, staining time was optimized at 60 min. The CMA staining of *Sesamum* chromosomes revealed the presence of distinct CMA positive (CMA^{+ve}) signals/ zones in all the studied landraces However, the number of chromosomes showing CMA^{+ve} signals varied among them (Table 3). Based on the CMA signalling patterns, chromosomes were grouped into two basic types: type A [chromosomes with no CMA^{+ve} signals] and type B [chromosomes (including one pair of sat-bearing chromosomes) with distal CMA^{+ve} signal on short arm]. Both the type A and B chromosomes were present in all the four landraces of *Sesamum* while, the number of each type was found to be landrace specific (Table 3). The Black seeded til (BT) and White seeded til (WT) were characterized by six chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3b and 3k), while the Dark brown seeded til (DBT) showed ten chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3e). The Light brown seeded til (LBT) was characterized by eight chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3h). However, we could not detect DAPI $^{+ve/-ve}$ signals on chromosomes of any of the landraces studied (Table 3).

Table 1. Chromosome morphometric analysis of four Indian landraces of *S. indicum**.

S. indicum landraces	Zygotic chromosome number (2n)	Length of longest chromosome (µm)		Length of shortest chromosome (µm)		Total chromatin length (µm) (Mean ± S.D.)	Ordering no. of SAT bearing pair	Karyotype formulae (n)
		Absolute (Mean ± S.D.)	Relative (Mean ± S.D.)	Absolute (Mean ± S.D.)	Relative (Mean ± S.D.)			
Black seeded Til	26	2.87 ± 0.09^{b}	5.44 ± 0.14^{b}	1.36 ± 0.02 ^{c}	2.58 ± 0.05^{a}	52.75 ± 0.24^{c}	5th	3Sm+9m+ 1Sm.Sat
Dark Brown seeded Til	26	2.20 ± 0.12^{a}	4.90 ± 0.24^{a,b}	1.24 ± 0.02^{a}	2.77 ± 0.06^{b}	44.85 ± 0.35^{a}	5th	3Sm+9m+ 1Sm.Sat
Light Brown seeded Til	26	2.15 ± 0.14^{a}	4.76 ± 0.28^{a}	1.28 ± 0.02^{a}	2.84 ± 0.07^{b}	45.20 ± 0.42^{a}	5th	3Sm+9m+ 1Sm.Sat
White seeded Til	26	2.62 ± 0.07^{b}	5.42± 0.06^{b}	1.33± 0.03^{b}	2.76 ± 0.03^{b}	48.29 ± 0.74^{b}	5th	3Sm+9m+ 1Sm.Sat

*Values followed by same letter are not significantly different according to Duncan's multiple range tests test (P=0.05).

Table 2. Comparative karyometric analysis of four Indian landraces of *S. indicum**.

S. indium landraces	A1	A2	TF%	AsK%	CV_{CL}	CV_{CI}	CV_{r}	AI	Stebbin's group
Black seeded Til	0.603	0.004	37.061	62.293	22.496	8.732	15.639	1.964	2B
Dark Brown seeded Til	0.619	0.007	37.747	61.360	19.056	10.218	17.877	1.947	2A
Light Brown seeded Til	0.628	0.009	38.228	60.886	17.369	8.433	14.562	1.464	2A
White seeded Til	0.636	0.015	38.163	61.007	21.370	7.523	12.311	1.607	2A

*Values followed by same letter are not significantly different according to Duncan's multiple range tests test (P=0.05). A1: Intra-chromosomal asymmetry index; A2: Inter-chromosomal asymmetry index; TF%: Total form percent; AsK%: Asymmetric karyotype percent; CVCL: Coefficient of variation of chromosome length; CVCI: Coefficient of variation of centromeric index; CVr: Coefficient of variation of arm ratio; AI: Asymmetry index.

Table 3. Fluorescent banding patterns in four Indian landraces of *S. indicum.*

S. indicum landraces	Maximum no. of chromosomes with CMA^{+ve} bands	Position of CMA^{+ve} bands in chromosome	CMA karyotypes (n)	Maximum no. of chromosomes with $DAPI^{+ve/ve-}$ bands
Black seeded Til	6	Distal part of short arm	20A+6B	Nil
Dark Brown seeded Til	10	Distal part of short arm	18A+8B	Nil
Light Brown seeded Til	8	Distal part of short arm	18A+8B	Nil
White seeded Til	6	Distal part of short arm	20A+6B	Nil

CMA^{+} signals in four studied landraces are incorporated in the Idiogram (Fig. 2)

Scatter plot and principal component (PCA) analyses

The scatter diagram of A1 versus A2 revealed that Dark brown seeded til (DBT) and Light brown seeded til (LBT) were placed close to each other, thereby forming a cluster, while the Black seeded til (BT) and White seeded til (WT) were positioned away from the cluster (Fig. 4). In the present study, PCA was further conducted to clarify the karyotypic relationship between the landraces using different karyo-morphometric parameters. In this eigenvector-based multivariate analysis, the component 1 (PC1) was found to be 59.6% of the total variation whereas component 2 (PC2) was 33.4% (Fig. 5). The obtained cophenetic correlation was 0.998, indicating a good fit between the eigenvalues and eigenvectors distance matrix. The PCA plot (Fig. 5) displayed the close positioning of Dark brown seeded til (DBT) with Light brown seeded til (LBT), which was similar to that of the scatter plot (Fig. 4). On the other hand, the Black seeded

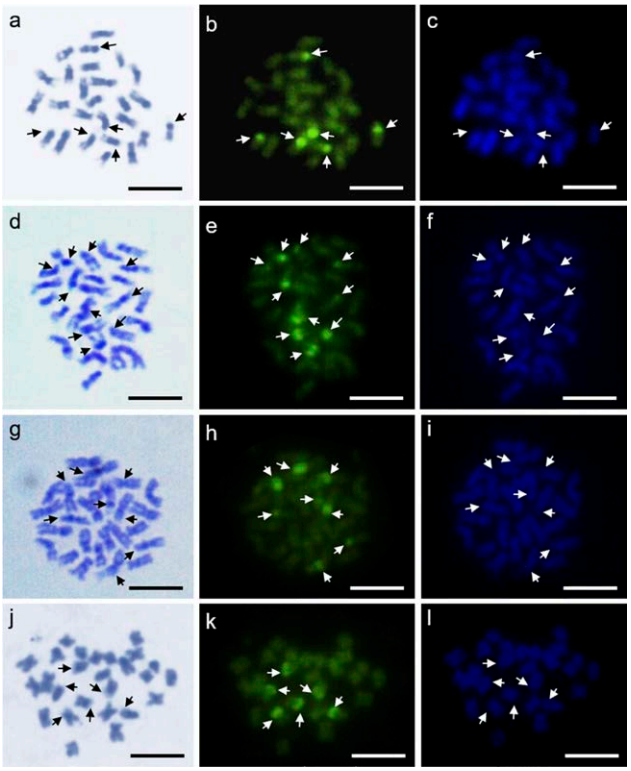

Figure 3. Somatic metaphase chromosomes (2n= 26) of four Indian landraces of *S. indicum* stained with Giemsa, CMA followed by DAPI. a- c) Black seeded til; d- f) Dark Brown seeded til; g- i) Light Brown seeded til; j- l) White seeded til. Bar= 5 µm. Arrows indicate the chromosomes showing CMA^{+ve} signals/ zones when stained with CMA fluorochrome.

til (BT) and White seeded til (WT) were located at a significant distance from each other (Fig. 5).

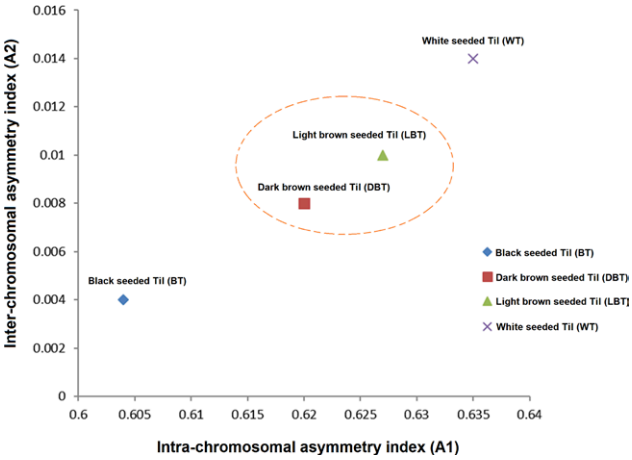

Figure 4. Scatter diagram of intra-chromosomal asymmetry index (A1) versus inter-chromosomal asymmetry index (A2) of four Indian landraces of *S. indicum*.

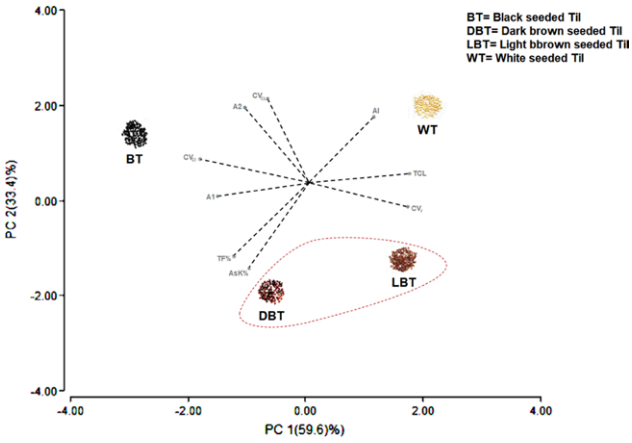

Figure 5. Principal component analysis (PCA) plot showing grouping of four Indian landraces of *S. indicum* based on nine karyomorphometric variables.

DISCUSSION

The present study demonstrates a comprehensive karyo-morphometric analysis of four Indian landraces of *S. indicum* based on giemsa, CMA and DAPI banding analysis. Due to its characteristic life forms and immense nutritive value, *S. indicum* has attracted the attention of researchers and breeders to plan a successful conservation strategies and improvements in breeding programs. However, only a few reports are available on the cytogenetics of Indian varieties of *Sesamum* till date may be owing to very small size of the chromosomes (< 3 µm) and technical limitations (Raghavan and Krishnamurthy 1947; Mukherjee 1959). In the present study, we have adopted the EMA based chromosome analysis to obtain cytoplasm free well scattered metaphase chromosomes of the species. The combination of enzymatic maceration and air dying methods is a very useful technique to analyze chromosome morphology, constrictions and types of chromosomes in detail (Fukui 1996). This method was instrumental in obtaining uniformly spread chromosomes against a cytoplasm free background in several crop species with small and medium sized chromosomes (Kurata and Omura 1978; Moscone 1996; Yamamoto 2007; Jha 2014; Jha and Halder 2016; Jha *et al.* 2017; Jha and Saha 2017; Ghosh *et al.* 2018).

The results obtained from the present analysis offers several insights into the karyological characterization of different *S. indicum* landraces viz. Black seeded til (BT), Dark brown seeded til (DBT), Light brown seeded til (LBT) and White seeded til (WT). The diploid chromosome number (2n= 26) in all studied landraces

of *S. indicum* is in agreement with the earlier reports (Morinaga *et al.* 1929; Kobayashi 1949; Raghavan and Krishnamurthy 1947). Raghavan and Krishnamurthy (1947) identified 13 pairs of somatic chromosomes with terminal constrictions in this species, while Kobayashi (1991) classified five pairs of median and eight pairs of submedian chromosomes including one pair (10^{th} pair) of sat-bearing chromosomes in *S. indicum*. In the present study, a significant variation in chromosome size (ranging from 1.24 µm- 2.87 µm) has been scored among the *Sesamum* landraces. Mukherjee (1959) reported two pairs of chromosomes having secondary constrictions in *S. indicum*, while the present EMA based analysis clearly revealed the presence of nine pairs of chromosomes with nearly median primary constriction, three pairs were submedian and a single pair (5^{th} pair) of sat-bearing chromosomes in all the studied landraces and which can be considered as the modal karyotype for Indian Sesame.

In addition to the EMA based giemsa staining, the fluorochrome banding patterns are documented here for the first time in four studied Indian landraces of *Sesamum*. The application of nucleotide specific fluorochromes i.e. GC-specific CMA, AT-specific DAPI in chromosome analysis has been reported to be very expedient in proper karyological characterization of many plant species (Schweizer 1976; Moscone *et al.* 1996). In the present study, CMA banding analysis provides a comprehensive cytogenetic characterization of four Indian landraces of *S. indicum*. Based on both karyomorphology and CMA signalling patterns, distinct homologies could be established between the studied landraces. Presently, we could not locate $DAPI^{+ve}$ bands in any of the studied samples. However, the differences in distribution of CMA^{+ve} signals/ zones clearly delimit each of the studied landraces of *Sesamum*.

In the present study, all the collected landraces of *S. indicum* exhibited symmetrical karyotypes based on categories of Stebbins (1971). However, the analyses of scatter diagram of A1 versus A2 and PCA plot unambiguously delimit each of the studied landrace (Fig. 4 and 5). PCA is a true eigenvector-based multivariate analysis, which can be used to project samples onto a series of orthogonal axes and to statistically clarify the genetic relationship among the studied samples (McVean 2009). The results obtained from scatter plot of A1 vs A2 and PCA analysis provide a strong relationship with that of the fluorochrome (CMA) banding analysis. The Dark brown seeded til (DBT) and Light brown seeded til (LBT) exhibited maximum CMA^{+ve} signals/ zones and appeared close to each other, while both the Black seeded til (BT) and White seeded til (WT) characterized by minimum CMA^{+ve} signals (i.e. six chromosomes with distal CMA^{+ve} signal on short arm) positioned distantly in the scatter diagram of A1 versus A2 and PCA plot.

As a whole, the present study involving EMA based giemsa staining techniques demonstrates an explicit karyo-morphometric characterization of four Indian landraces of *S. indicum* for the first time. Distinct landrace-specific variation in the distribution of CMA^{+ve} signals/ zones in somatic chromosomes was also established in the species. The grouping of the studied landraces was also corroborated by the analysis of scatter diagram of A1 versus A2 and PCA plot which revealed a strong relationship with that of the fluorochrome banding analysis. However, further studies employing in situ hybridization techniques like FISH/ GISH and DNA barcode analysis are required for clarification of evolutionary processes within the particular species.

In conclusion, the present karyo-morphometric analysis explicitly characterizes four important Indian landraces of *S. indicum* for the first time. Application of EMA method of chromosome analysis followed by giemsa and fluorescent dye CMA which targets GC rich constitutive heterochromatin regions on chromosomes has clearly demonstrated that the method may be used as useful tool to characterize and differentiate *S. indicum* at the varietal level.

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