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Fluorescent Chromosome Banding and Genome Size Estimation in Three Species of *Swertia*

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Summary Chromosomal attributes of the critically endangered medicinal plant *Swertia chirayita* along with its allied species *S. nervosa* and *S. bimaculata* are precisely essential to assess phylogenetic relationships. The present study deals with karyomorphometric analysis based on patterns of fluorescence chromosome banding and nuclear genome size estimation by flow cytometry reported for the first time in *S. chirayita* (2*n*=26), *S. nervosa* (2*n*=26) and *S. bimaculata* (2*n*=26). Fluorescent banding revealed the distinct differences in chromosomal $CMA^{+ve}/DAPI^{-ve}$ heterochromatic sites among the species. There were six chromosomes with CMA^{+ve} signals in *S. chirayita* and two chromosomes with CMA^{+ve} signals in *S. bimaculata* whereas all chromosomes of *S. nervosa* displayed CMA^{+ve} signals. Due to a difference in CMA banding pattern, proportion of GC rich regions differed among the chromosomes of *S. chirayita* (3.32%), *S. nervosa* (18.76%), and *S. bimaculata* (0.64%). *S. bimaculata* had the highest 2C DNA content (9.82 pg/2C) compared to *S. nervosa* (1.95 pg/2C) and *S. chirayita* (1.09 pg/2C). Lowest amount of GC-rich CMA sites along with highest DNA content distinguished *S. bimaculata* from the other two species. A nearly nine-fold difference in nuclear genome size among the homoploid species of *Swertia* (2*n*=26) and particular chromosomal discrimination by localized CMA bands allowed interpretation of karyotypic affiliations in the three species of *Swertia*.

Key words *Swertia*, Nuclear DNA content, Flow cytometry, Fluorochrome banding, Total chromatin length.

The genus *Swertia* L. (family Gentianaceae) occurs mainly in the temperate regions of the northern hemisphere. According to the Regionalization of World Flora by Takhtajan (1978), *Swertia* is said to be present in 14 regions of the world, with the highest species diversity in the Himalayas and South-Western China (Meusel *et al.* 1978). About 40 species of *Swertia* are found in India, distributed in the temperate Himalayan region (from Kashmir to Bhutan and also in the Khasia hills and Western Ghats). They are mainly found at high altitudes ranging from 1200 to 3000 m (Hooker 1885, Kirtikar and Basu 1984, Samaddar *et al.* 2014). The North West Himalayas are known to be the abode of 16 species of *Swertia* (Chopra *et al.* 1956, Garg 1987, Ho *et al.* 1994, Misra *et al.* 2010) and 13 species have been reported from the North Eastern states of India (Pradhan and Badola 2010, Samaddar *et al.* 2014).

Among the species of *Swertia* that are found in India, many have been described in different traditional systems of medicine, for their use in the treatment of various ailments (Kirtikar and Basu 1984, Misra *et al.* 2010). Of these species, *Swertia chirayita* is considered to be of high medicinal value owing to its hypoglycemic and antimalarial activities (Brahmachari *et al.* 2004). It is designated as critically endangered owing to excessive human exploitation (Rai *et al.* 2000), resulting in the use of substitutes/adulterants in "chirata" trade. *S. nervosa* and *S. bimaculata* are among the popularly used substitutes of *S. chirayita* in the herbal industry (Joshi and Dhawan 2007). Growing demand in commercial trade and destructive harvesting beyond sustainable limits have already changed the IUCN status of many *Swertia* species. Germplasm documentation, preservation and genetic characterization thus have become essential for medicinal plants like *Swertia* (Samaddar *et al.* 2014).

The species of *Swertia* are taxonomically disputed owing to some overlapping macromorphological characters. *S. chirayita*, *S. nervosa*, and *S. bimaculata* show similarities in certain morphological characters like opposite decussate arrangement of leaves, inflorescence panicles of cymes, and loculicidal capsule type of fruits. However, distinctive differences such as nature of stem, leaf morphology, and floral features, have been helpful to discriminate the species (Hooker 1885, Rijal 2009, Samaddar *et al.* 2014, 2015). Molecular markers such as AFLP and RAPD have been employed for identification of the species of *Swertia* (Misra *et al.* 2010, Samad-

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dar *et al.* 2015). In terms of biochemical constituents, content of Swertiamerin was found to differ among *S. chirayita*, *S. bimaculata*, and *S. nervosa* (Samaddar *et al.* 2013). Amarogentin was found in *S. chirayita* and *S. nervosa* but not detected at all in *S. bimaculata*. The content of magniferin was also shown to be different in *S. chirayita* and *S. nervosa* (Khanal *et al.* 2015).

Being identified as a highly heterogeneous and polyphyletic genus (Chassot *et al.* 2001), chromosome study is considered to have great potential in evaluation of taxonomic affinities and phylogenetic relationships. There are only limited reports on chromosome analysis on Indian *Swertia* species (Khoshoo and Tandon 1963, Roy *et al.* 1988). We have recently reported chromosome number, karyotype, and meiotic analysis in *Swertia* species from Eastern Himalayas following conventional staining techniques (Samaddar *et al.* 2015). Based on conventional methods, *S. chirayita* and *S. nervosa* revealed an overall similarity in chromosome features while *S. bimaculata* had some differences in chromosome size and karyotype. There are many scopes to investigate chromosomal physiognomy based on advanced cytogenetic methods and fluorochrome banding analysis (Bhowmick *et al.* 2012, Jha and Yamamoto 2012, Nath *et al.* 2015). Especially the implication of enzymatic maceration and air drying technique (EMA, Fukui 1996) can potentially aid karyotype analysis following fluorochrome staining of chromosomes. In addition to chromosome analysis, interspecific genome size variation is considered as a taxonomically informative marker for homoploid species (Kron *et al.* 2007) which has not been reported so far in the case of *Swertia*. The use of flow cytometric methods for estimation of genome size has been recognized as a progressive measure to assess interspecific relationships (Bennett and Leitch 1995) which is aimed to improve cytogenetic background of the three species of *Swertia*. In the present study, nuclear DNA content and karyotype analysis based on fluorescence chromosome banding have been reported for the first time in the critically endangered species *S. chirayita*, *S. nervosa*, and *S. bimaculata*, to explore patterns of karyoevolutionary trends within the genus.

Materials and methods

Plant materials

Swertia chirayita (Roxb.) H. Karst. (synonym: *Swertia chirata* Buch.-Ham. ex Wall), *Swertia nervosa* (Wall. ex G. Don) C. B. Clarke and *Swertia bimaculata* (Sieb. & Zucc.) Hook. f. & Thomson ex C. B. Clarke plants with flowers and green fruits were collected during the month of September–November from Lava, Darjeeling, India. Collected specimens were identified on the basis of morphological characters and herbarium specimens were prepared. The voucher specimens have been deposited in the Herbarium of Shivaji University, Kolhapur, (SUK) Maharashtra, India with the accession numbers SUK-5286, SUK-5288, and SUK-5287 for *S. chirayita*, *S. nervosa*, and *S. bimaculata*, respectively.

Seeds of each species were surface sterilized with 0.1% $HgCl_2$ (w/v) for 30 min, washed five times with sterilized distilled water and cultured on Murashige and Skoog's (1962) basal medium under 16 h photoperiod at 24°C and 60% relative humidity in a growth chamber. The roots and leaves obtained from *in vitro* derived plantlets were used for cytological study.

Chromosome preparation for fluorochrome staining

Healthy root tips (0.5–1 cm) obtained from two months old axenic seedlings were pretreated in 8-Hydroxyquinoline (2 mM) at 20–22°C for 3.5 h, fixed overnight in 1 : 3 glacial acetic acid : methanol and stored at –20°C. Fixed root tips were washed in cold distilled water and then incubated in enzyme mixture containing 1% cellulose (Onozuka RS, Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.75% macerozyme R-10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.15% pectolyase Y-23 (Kyowa Chemical Products Co., Ltd., Japan) and 1 mM EDTA (pH 4.2) for 60 min (Fukui 1996). Enzyme digested root tips were macerated in a drop of fixative (1 : 3 aceto-methanol) on glass slides and air dried. Slides were stained with 2% Giemsa solution (Giemsa azure eosine methylene blue solution, Merck, Germany) in 1/15 phosphate buffer for 15 min, rinsed with distilled water, air dried and mounted with xylene. Position of the metaphase plates were marked on slides and photographed using ProgRes®CT5 (Jenoptik, Germany) camera attached to Leitz GMBH microscope. Chromosome measurements [long arm length (*l*), short arm length (*s*), chromosome length (CL)] and total diploid chromatin length $\left(TCL = \sum_{i=1}^{2n} CL\right)$ were taken using the software ProgRes 2.3.3. For karyotype analysis, chromosomes were classified on the basis of centromeric indices [$Ci=(s/CL)\times100$] 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) ($Ci>12.5–25.0$). Chromosome morphometric data were taken from at least five different metaphase plates from three individual plants of each species to construct karyotypes.

Giemsa stained slides were destained in 70% methanol for subsequent staining of chromosomes with base-specific fluorochromes 4′-6-diamidino-2-phenylindole (DAPI) (AT-specific) and chromomycin A_3 (CMA) (GC-specific), following the protocol of Schweizer (1976) with minor modifications (Bhowmick *et al.* 2012, Jha and Yamamoto 2012, Nath *et al.* 2015). Chromosomes were stained with 50 $\mu g\,mL^{-1}$ DAPI solution with Actinomycin D as a counterstain and observed under fluorescent microscope Zeiss Axioscop 2 with a UV filter cassette. After observation, the same slides were stained

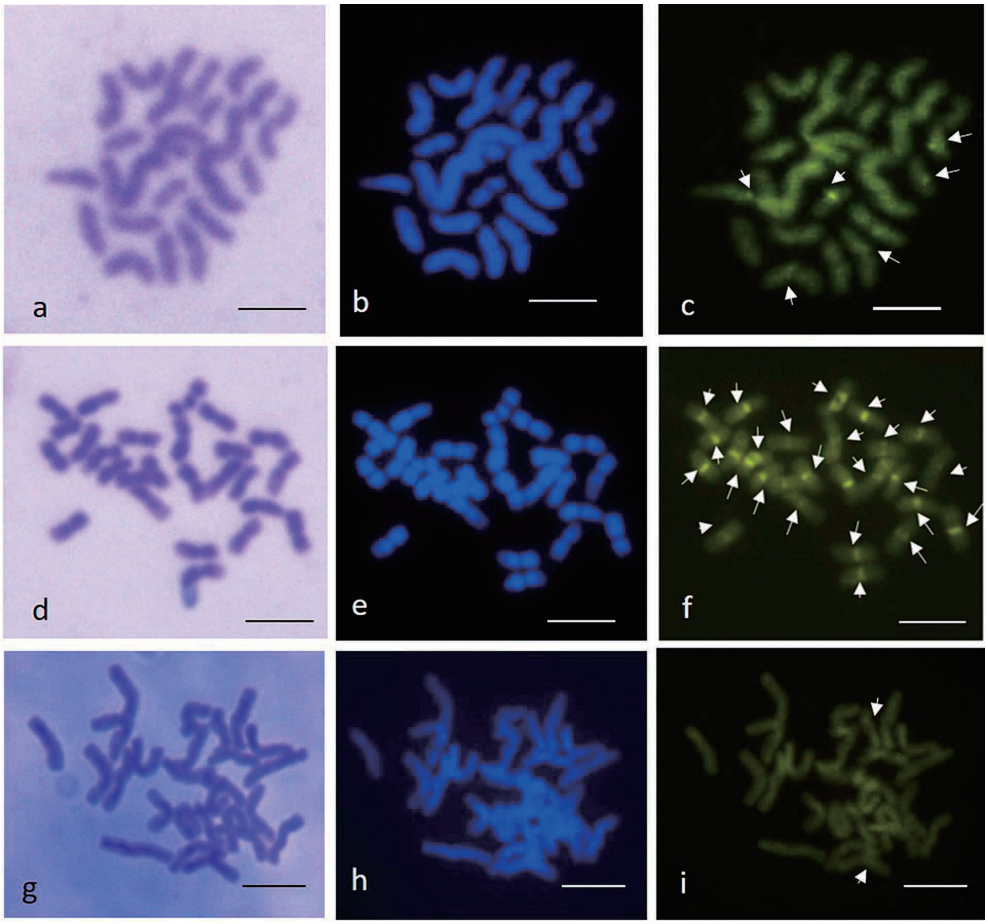

Fig. 1. Somatic metaphase chromosomes ($2n$=26) in the species of *Swertia*. (**a**–**c**) Chromosomes of *Swertia chirayita* stained with Giemsa (a), DAPI (b) and CMA3(c). (**d**–**i**) Chromosomes of *Swertia nervosa* stained with Giemsa (d), DAPI (e) and CMA3 (f). (**g**–**i**) Chromosomes of *Swertia bimaculata* stained with Giemsa (g), DAPI (h) and CMA3 (i). Arrows indicate CMA^{+ve} signals on chromosomes. Bar scale=10 μm.

with 0.1 mg mL^{-1} CMA solution and observed under BV filter cassette. Images were captured with the camera ProgRes MFscan (Jenoptik, Germany) attached to Zeiss Axioscop2. Lengths of chromosomal fluorescent signals were measured using the software ProgRes CapturePro2.8.8. Based on the number and length of the signals, the GC rich regions in chromosomes was expressed as the relative percentage of total chromatin length (Moscone *et al.* 1996, Guerra 2000) for each species.

Genome size estimation

Leaves from two months old axenic seedling derived plants of *S. chirayita*, *S. nervosa*, and *S. bimaculata* were used as source material for nuclei isolation and estimation of genome size. The standard *Zea mays* L. 'CE-777' and *Solanum lycopersicum* L. 'Stupické polní rané' were kindly provided by Dr. Jaroslav Doležel, Institute of Experimental Botany, Olomouc, Czech Republic, with reported 2C value of 5.43 and 1.96 pg, respectively (Doležel *et al.* 2007). Nuclei isolation was done according to the protocol of Galbraith *et al.* (1983) in Tris-$MgCl_2$ buffer (Pfosser *et al.* 1995) with 50 μg mL^{-1} RNase A. Leaves of standard and sample materials were co-chopped on ice beds with a sharp razor blade in a petridish, cell debris were precipitated by centrifugation and isolated nuclei were stained with propidium iodide (PI) (50 μg mL^{-1}). Nuclei suspensions of sample and standard material were simultaneously analyzed using a BD FACS Verse Flow Cytometer (BD Bioscience, U.S.A.) with a 488 nm solid state laser (50 mW). Samples were run at a medium speed (60 μL min^{-1}) and data were acquired and analyzed using the BD FACS Suite Software. A linear PI fluorescence Area (PI-A) *vs.* PI fluorescence Width (PI-W) plot was drawn to eliminate debris using qualitative gating. A PI fluorescence histogram (PI-A) was drawn to view nuclear DNA content. At least 3000 nuclei were run per sample and three replicates from three separate individuals were analyzed. Conversion of mass value into base-pair numbers was done according to the factor 1 pg=978 Mbp (Doležel *et al.* 2003).

Statistical analysis

Descriptive statistics were calculated and parameter studied included mean, standard deviation (SD) and minimum and maximum values. Results were expressed as mean and the Tukey's HSD test was used for *post-*

hoc analyses. The statistical analysis was conducted at 95% confidence level and *p* value less than 0.05 was considered statistically significant. Principal component analysis (Marghali *et al.* 2014) was conducted to see cytological relationships between the three species of *Swertia*. The variables used for this study included quantitative data like the estimated total chromatin lengths, amount of GC rich regions in chromosomes as measured from the length of CMA^{+ve} signals (Moscone *et al.* 1996, Guerra 2000) and 2C DNA content. InfoStat Version 2013d (free version) was used for correlation analysis using the non-parametric Spearman test (Sokal and Rohlf 1995) and principal component analysis.

Results

Karyomorphological analysis based on fluorescent banding pattern

The number of chromosomes in the diploid complement was $2n=26$. EMA-Giemsa method facilitated elimination of cytoplasmic background and clear visualization of chromosome morphology in the species of *Swertia* (Fig. 1). *S. chirayita* ($2n=26$) and *S. nervosa* ($2n=26$) are characterized by the presence of small chromosomes ($\leq 3.5\,\mu$m) while most of the chromosomes in *S. bimaculata* ($2n=26$) are of medium size (3.5–6 μm). The total chromatin length was found to display significant differences between *S. bimaculata* and *S. chirayita* while *S. nervosa* had a TCL of intermediate value (Table 1). Somatic metaphase complement of the three species of *Swertia* displayed distinct karyomorphology and all had a pair of chromosomes with two constrictions (Table 1).

Fluorescence banding of the EMA-Giemsa stained metaphase plates enabled clarification of chromosomal differences among the species of *Swertia*. Chromosomes stained with DAPI did not display any specific bands while a differential distribution of CMA^{+ve} signals was evident among the species (Figs. 1, 2). The CMA^{+ve} signals always corresponded with $DAPI^{-ve}$ areas on the chromosomes. On the basis of position and number of chromosomal CMA^{+ve} signals, six types of chromosomes were categorized which are differentially distributed in the three species of *Swertia*. The CMA^{+ve} signals always corresponded with $DAPI^{-ve}$ areas on the chromosomes. Chromosomes with CMA^{+ve} signals at the centromeric region were designated as type A, chromosomes with proximal CMA^{+ve} signals were designated as type B, chromosomes with CMA^{+ve} signals at centromeric and interstitial region on long arm was termed type C, nucleolar chromosomes with one CMA^{+ve} signal at the nucleolar constriction were designated type D, nu-

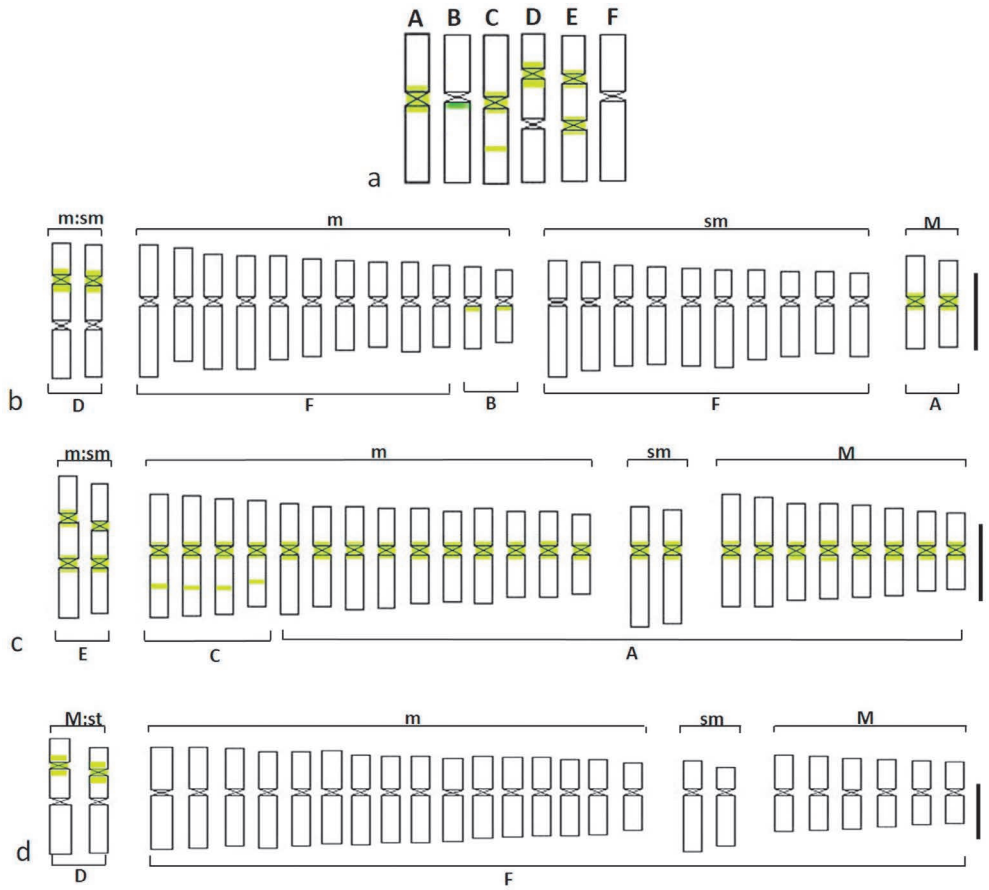

Fig. 2. (**a**) Chromosome types of *Swertia* species based on CMA^{+ve} signals. Ideograms of *S. chirayita* (**b**), *S. nervosa* (**c**) and *S. bimaculata* (**d**), chromosomes arranged according to descending length. Bar scale=1 μm.

Table 1. Comparative karyomorphology based on fluorescent banding and nuclear DNA content of three species of *Swertia*.

Species	Karyotype	Nuclear DNA content		TCL (μm)	No. of chromosomes with CMA^{+ve} signals	Karyotype on the basis of CMA^{+ve} banding pattern	Chromosomal GC rich regions (CMA^{+ve})
		pg/2C	Mbp/2C				
S. chirayita (2*n*=26)	2M+12m+10sm+2m:sm	1.09±0.16[a]	1062.76±152.87[a]	60.14±4.97[a]	6	2A+2B+2D+20F	3.32±0.21[a]
S. nervosa (2*n*=26)	8M+14m+2sm+2m:sm	1.95±0.14[a]	1910.36±137.04[a]	80.27±1.71[b]	26	20A+4C+2E	18.76±2.51[b]
S. bimaculata (2*n*=26)	6M+16m+2sm+2M:st	9.82±0.71[b]	9607.22±691.30[b]	133.16±13.03[b]	2	2D+24F	0.64±0.20[c]

Values followed by same letter are not significantly different according to Tukey's HSD test (p=0.05). TCL: total diploid chromosome length.

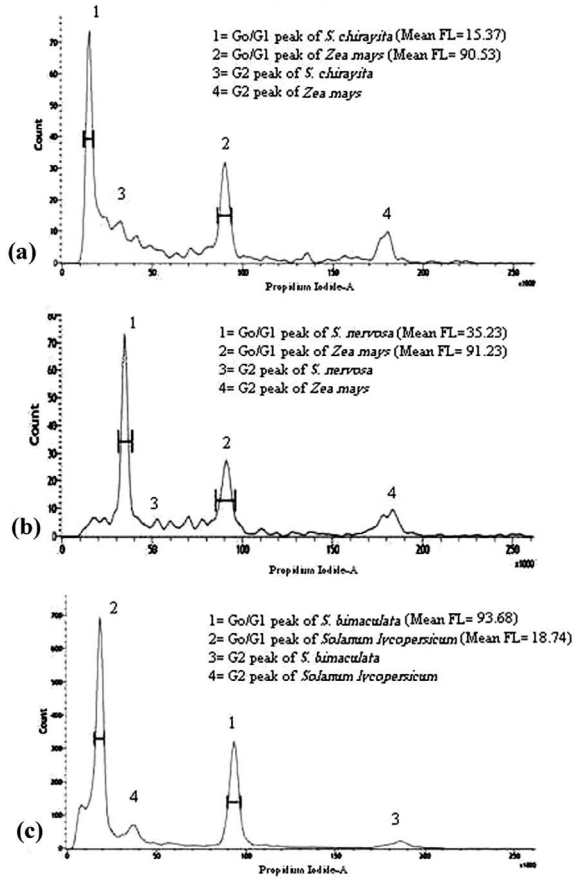

Fig. 3. Propidium iodide fluorescence intensity-area (PI-A) histograms of *Swertia chirayita* (**a**) and *Swertia nervosa* (**b**) with internal standard *Zea mays* L. 'CE-777.' (**c**) Propidium iodide fluorescence intensity-area (PI-A) histograms of *Swertia bimaculata* with internal standard *Solanum lycopersicum* L. 'Stupické polní rané. Mean FL=Mean fluorescence intensity.

cleolar chromosomes with CMA^{+ve} signal at centromeric as well as the nucleolar constriction were designated type E, and chromosomes with no detectable CMA signals were type F (Fig. 2a).

In *S. chirayita*, one pair of metacentric chromosomes had centromeric CMA^{+ve} signals (type A). Another pair of nearly metacentric chromosomes had proximal CMA^{+ve} signal on the long arm (type B). One pair of chromosomes with two constrictions (m.sm) showed the presence of CMA^{+ve} signal on the submedian constriction (type D) (Fig. 1a–c, Fig. 2b). Ten pairs of chromosomes did not possess any CMA^{+ve} signals (type F). Hence the karyotype of *S. chirayita* according to CMA banding pattern was 2A(2M)+2B(2m)+2D(2m.sm)+20F (10m+10sm) (Fig. 2b, Table 1).

In the case of *S. nervosa*, CMA^{+ve} signals were observed in all somatic chromosomes (Fig. 1d–f). Centromeric CMA^{+ve} signals (type A) were found in 20 chromosomes of which 8 were metacentric, 10 were nearly metacentric, and 2 were submetacentric. There were four nearly metacentric chromosomes with CMA^{+ve} signals at centromeric and interstitial region (type C). In the case of the nucleolar pair of chromosomes of *S. nervosa*, CMA^{+ve} signals were detected at both the nearly median and submedian constrictions (type E). Thus, on the basis of CMA banding pattern, the karyotype of *S. nervosa* was 20A(8M+10m+2sm)+4C(4m)+2E(m.sm) (Fig. 2c, Table 1).

CMA^{+ve} signals were observed only at the subterminal constriction of the nucleolar pair of *S. bimaculata* (type B1). The rest of the chromosomes did not possess any CMA^{+ve} signals and belong to Type F (Fig. 1g–i, Fig. 2d). Thus, on the basis of CMA^{+ve} banding pattern the karyotype of *S. bimaculata* is 2D (M:st)+ 24F(6M+16m+2sm).

Based on the presence of chromosomal CMA signals, the GC rich elements in the species of *Swertia* exhibited differential distribution (Table 1). The proportion of GC rich regions was highest in the chromosomes of *S. nervosa* (18.76%) followed by that of *S. chirayita* (3.32%) while lowest GC rich chromosomal region was found in *S. bimaculata* (0.64%) (Table 1).

Genome size of S. chirayita*,* S. nervosa*, and* S. bimaculata

Comparison of 2C values revealed interspecific difference in DNA contents which reached up to about nine fold in the case of *Swertia bimaculata*. The highest nuclear DNA content of 9.82 pg/2C was observed in *S. bimaculata* and was significantly different from that of the other two species: *S. nervosa* (1.95 pg/2C) and *S. chirayita* (1.09 pg/2C) (Table 1, Fig. 3). Nuclear DNA content was positively correlated with TCL (r=1.00, p=0.16).

Interspecific chromosomal relationship based on principal component analysis

Principal component analysis revealed the formation of three distinct locations for *S. chirayita*, *S. nervosa*, and *S. bimaculata* (Fig. S1) in PCA plot. *S. chirayita* and *S. nervosa* were relatively close in terms of the karyo-

type variables while *S. bimaculata* noticeably occupied a distant position than these two species (PC1 17.0% and PC2 83.0% values). Distant location of *S. bimaculata* indicated significant differences from *S. chirayita* and *S. nervosa*.

Discussion

The chromosome number ($2n$=26) and karyotypes studied in the *Swertia* species, corroborated to a previous report of conventional acetoorcein based study (Samaddar *et al.* 2015). Fluorochrome banding and genome size estimation in the critically endangered species *S. chirayita* and its adulterant species *S. nervosa* and *S. bimaculata* enabled cytological characterization of three species showing distinct differences within the genus. Nuclear DNA content of the *Swertia* species positively correlated with TCL (r=1.00, p=0.16) which is a general trend in plants (Acosta *et al.* 2012). In spite of being a homoploid species, *S. bimaculata* has significantly higher length of total chromatin and nuclear DNA content than *S. chirayita* and *S. nervosa* and hence can be distinguished based on 2C DNA values. Such a difference in nuclear DNA content between homoploids indicates taxonomic heterogeneity in rapidly diverging species (Loureiro *et al.* 2010). Especially, the remarkable divergence in genome size of *S. bimaculata* without any change in chromosome number had exceeded genome size divergence in many other homoploid species (Loureiro *et al.* 2010). Apart from the difference in 2C DNA content, unique fluorochrome banding pattern enabled cytological distinction between these species.

Previously, karyotype differences between species was accomplished after a thorough analysis of chromosome morphometric parameters based on conventional acetoorcein staining (Samaddar *et al.* 2015). In the current study, staining of the somatic chromosomes with chromomycin A3 clarified species identification at a single glance (Fig. 1) based on presence and absence of CMA bands and mode of distribution of CMA signals. EMA method had been central in the implication of fluorochrome banding technique since the method results in well spread metaphase chromosomes with representable morphology (Fig. 1). *S. nervosa* exhibited the greatest preponderance of GC rich CMA^{+ve} signals on the chromosomes. The physical positioning of GC-rich bands in *S. nervosa* appeared in the centromeric regions in all chromosomes, corresponding to an equilocal pattern of distribution (Fig. 1d–f, Fig. 2c). Although uncommon, centromeric occurrence of GC rich CMA^{+ve} sites were previously detected in the chromosomes of *Coccinia grandis* (Bhowmick *et al.* 2012). The occurrence of CMA^{+ve} signals on nucleolar constrictions perhaps constitutes the NOR- GC elements in *S. nervosa* like in many other plant species (Guerra 2000). Apart from the centromeric and nucleolar regions, interstitial GC rich blocks are also found in two pairs of chromosomes remaining as a distinctive chromosomal marker for *S. nervosa* (Fig. 2c). Although CMA-positive signals commonly represent GC-rich heterochromatin (Guerra 2000), the exact nature and function of the GC rich CMA^{+ve} sites is subject to further characterization.

In the case of *S. chirayita*, centromeric CMA^{+ve} signals were detected in one pair. Another pair of chromosomes had proximal CMA^{+ve} site just below centromere. The nucleolar pair had CMA^{+ve} signals only at the nucleolar constriction (Fig. 2b). However, the proportion of GC rich sites in *S. chirayita* was considerably low compared to the adulterant species, *S. nervosa*. The pair of nucleolar chromosomes that have been reported to be alike in *S. chirayita* and *S. nervosa*, could be distinguished by CMA banding patterns as type D in the former and type E in the latter species.

It is interesting to note that *S. bimaculata* with largest TCL and genome size had the lowest heterochromatin (0.64%) among the three species. The nucleolar constriction had a CMA signal of type D (Fig. 2d) and rest of the chromosomes had a uniform pattern of CMA staining. The reduction in chromosomal CMA signals might result from dispersed nature of GC rich sequences in the genome. A correlation between the largest genome size in *S. bimaculata* and the very limited amount of GC rich CMA^{+ve} sites remains to be a stimulating concern. Moscone *et al.* (2003) suggested that the increase in repetitive sequences not only at particular chromosomal sites but also at dispersed locations can lead to expansion in genome size as found in *Capsicum* which may be plausible also in *Swertia bimaculata*.

Presently, a combination of cytological features such as karyotype, fluorochrome banding pattern, and nuclear genome size had been employed to assess interspecific relationships by PCA. Distinct positions of the three species in a PCA plot indicated the utility of the present approach for cytological characterization of each species. A relative proximity between *S. chirayita* and *S. nervosa* probably indicates karyotype affinity as was reported earlier (Samaddar *et al.* 2015). However, *S. bimaculata* was found to have profound divergence in terms of chromosomal features and DNA content as evident after principal component analysis (Fig. S1).

Our study is the first report on fluorochrome banding pattern and genome size estimation in the genus *Swertia*. The current study provides reference for further evaluation of other *Swertia* species to understand the trend of chromosomal GC-rich element distribution in relation to nuclear DNA contents in the genus. Such molecular cytogenetic analysis can furnish valuable information to consolidate the existing knowledge on the evolution and systematics of the group and can be used as a platform for further studies.

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