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Karyotype Analysis and 4C Nuclear DNA Estimation in Different Cultivars of *Clitoria ternatea* L.

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Summary The present article has reported a detailed karyotype analysis and 4C nuclear DNA estimation in different cultivars and populations of *Clitoria ternatea* L. of Fabaceae. This plant, widely distributed in tropical regions in India, is a climber and is an economically important medicinal plant. The karyotype study has revealed a constancy in the diploid somatic chromosome number ($2n=16$) in all these cultivars and populations. However, the nature and number of nucleolar chromosomes varied. The centromeric chromosomes were either metacentric or sub-metacentric in all these cultivars. The total chromosome length and volume differed among these cultivars and populations having the same somatic chromosome number. The differential condensation of chromosomes and the association of variable amounts of proteins in chromosome composition might have been responsible for such differences. *In situ* cytophotometric studies revealed an intermediate genome size in this species. This is the first report on the detailed karyotype and genome size of cultivars of *Clitoria ternatea* L.

Key words *Clitoria ternatea* L., Fabaceae, Genome size, *In situ* cytophotometry, Karyotype, Nuclear DNA amount.

Clitoria ternatea L. or "Butterfly Pea", a member of the Leguminosae (Fabaceae) family, Phaseoleae tribe, and Clitoriinae subtribe, is an indigenous, hard, perennial climber, found in the hedges and thickets in many countries worldwide including India, up to an altitude of 1500 m (Morris 2009, Wealth of India 1962). They are extensively grown in gardens for their flowers. It is also grown as a forage crop. The plant is a tall, slender, climbing herbaceous vine with five leaflets. Flowers are usually papilionaceous, white, bright blue or violet/mauve in colour with yellowish centre. However, in some cultivars, the flowers are actinomorphic or irregular and termed as "double" form (Gandhi and Patil 1993). Butterfly pea is usually a self-pollinated plant. However, partial outcrossing probably exists as segregating genotypes have been identified (Cook *et al.* 2005).

The plant has several medicinal properties. In Ayurveda, the roots, seeds and leaves of *C. ternatea* have long been widely used as a reputed nervine tonic and is believed to promote memory and intelligence (Taranalli and Cheeramkuzhy 2003, Govindarajan *et al.* 2005, Mukherjee *et al.* 2007). It is also used for the treatment of leucoderma. It alleviates swelling and pain. It has haemostatic action, hence it is used in bleeding piles and other haemorrhagic disorders. Leaf juice provides relief for headaches when used as nasal drops. The boiled leaf and root extracts are very effective in rheumatoid arthritis. It has a tranquillizing effect on the brain and is used in the treatment of vertigo and brain weakness.

Clitoria ternatea L. has a diploid chromosome number of $2n=16$ (Joson and Ramirez 1991). Although there are reports on the determination of the chromosome number of this plant species, no detailed cytological analysis of the different cultivars of this species is available. The analysis of genetic diversity within and among different species, cultivars and populations of different plant species is necessary for crop improvement, conservation and management. Karyotype analysis, based on chromosome number and structural parameters, is an important and effective way to study the origin, evolution and classification of different plant genomes. Moreover, there are no reports on genome size of this species. Knowledge of genome size is quite important as the data are used in diverse fields including cell and molecular biology, ecology, phytogeography and systematics (Bennett and Leitch 1995, Bennett 1998, Leitch *et al.* 1998). Several studies have suggested that genome size is an important character for predicting how different plant species would respond to climate change and other environmental factors such as ionizing radiation (Sparrow and Miksche 1961), global warming (MacGillivray and Grime 1995, Grime 1996) and increased atmospheric CO_2 concentration (Jasienski and Bazzaz 1995). Therefore, understanding the patterns of C-value distribution and evolution is very important (Leitch *et al.* 1998).

In view of the above facts, the present investigation

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was undertaken on a detailed karyological analysis of different cultivars of *Clitoria ternatea* L. and estimation of genome size.

Materials and methods

Materials

The plants of *C. ternatea* (blue, single), *C. ternatea* (mauve, single, Population 1) and two cultivars of *C. ternatea* (white, single and mauve, double, Population 2) were collected from the Experimental Garden, Department of Botany, University of Calcutta, Globe Nursery, Kolkata and The Agri-Horticultural Society of India, Alipore, Kolkata respectively. Another cultivar (mauve, single, Population 3) was collected from the North 24-Pgs district of West Bengal, India. All the plants, collected from different regions, were maintained in the Experimental Garden, Department of Botany, University of Calcutta for further studies.

Methods

Cytological study

For karyotype analysis, roots attaining a length of about 0.5″ to 1.0″ were collected separately from each of the cultivars. The maximum mitotic activity was found between 10:30 a.m. to 12:30 p.m. Root tips were pre-treated in saturated aqueous solution of *p*-dichlorobenzene (PDB). Pre-treatment was carried out at 18°C for three hours, after an initial shock treatment at 0°C for 5 min (Mukhopadhyay and Banerjee 1989, Mukhopadhyay *et al.* 1989), followed by washing in distilled water. These were then fixed in chilled Propionic-Carnoy's fixative (propionic acid : chloroform : absolute ethanol=1 : 3 : 6) at 18°C for 24 h and finally stored in 70% ethanol at 18°C for future use. Prior to staining, the root tips were hydrolyzed in 1 N HCl for 10 min at 60°C. After thorough washing in distilled water, root tips were put in 45% propionic acid for 5 min and further stained in 2% propionic-orcein solution for at least three hours. The stained root tips were taken on clean, thin, grease-free glass slides and squashed in a drop of 45% propionic acid and observed under the microscope. Well-scattered somatic metaphase plates were drawn from the slides with the help of drawing prism at a table magnification of approximately ×2300 using an Olympus microscope with 15× eyepiece and 100× oil immersion objective lens.

The chromosomes were classified into different types according to Levan *et al.* (1964), based on their *i*-values. Total chromosome length in each material was calculated by adding the length of all chromosomes. The chromosome volume was calculated using the formula $\pi r^2 h$, where π=3.142, r=radius of the chromosome (width/2) and h=height of the chromosome (Mukhopadhyay and Sharma 1987, Lahiri *et al.* 2010). Intra-chromosomal asymmetry index (A_1) and inter-chromosomal asymmetry index (A_2) values were calculated according to the method suggested by Romero Zarco (1986).

Genome size estimation

For microspectrophotometric studies, the root tips were pre-treated and fixed as described before. The root tips were hydrolyzed in 1 N HCl for 10 min at 60°C. After thorough washing in distilled water to remove all traces of acid, root tips were stained in Schiff's reagent at 18°C for one hour. These were then squashed in 45% acetic acid and readings were taken instantly to avoid error (Lahiri *et al.* 2010). The root tips of the standard material (*Allium cepa* var. *rosette*) were treated maintaining the condition similar to that of the samples to avoid experimental error, if any.

Cytophotometric analysis in 25 randomly selected somatic cells (late prophase, early metaphase and metaphase stages) from root tips was carried out with a Leitz Wetzlar Aristophot with microspectrophotometer at a wavelength of 550 nm (Sharma and Sharma 1980). The radius of the aperture was kept constant for all the readings. The amount of nuclear DNA was measured in arbitrary units of relative absorbance on the basis of optical density. The relative arbitrary units of absorbances were converted to absolute units (picogram) by considering the 4C nuclear DNA amount of *Allium cepa* var. *rosette* (67.1 pg) as the standard (Van't Hof 1965).

Results and discussion

Cytological analysis of all the cultivars and populations of *Clitoria ternatea* L. studied in the present investigation revealed $2n$=16 somatic chromosomes. The individual chromosome morphology studied in these cultivars and populations of *Clitoria ternatea* showed quite distinct karyotypes with two different size ranges of chromosomes. The differences in karyotypic details are principally the number and combination of different chromosome types including those with secondary constrictions. In the white (single) cultivar, there are 2 pairs of nucleolar chromosomes, 3 pairs of sub-metacentric chromosomes (sm) and 3 pairs of metacentric chromosomes (m) (Table 1; Fig. 1a–c). The blue (single) cultivar revealed the presence of 3 pairs of nucleolar chromosomes, 2 pairs of sub-metacentric chromosomes and 3 pairs of metacentric chromosomes (Table 1; Fig. 1d–f). Both the populations of the mauve, single cultivar (populations 1 and 3) showed 2 pairs of nucleolar chromosomes, 3 pairs of sub-metacentric chromosomes and 3 pairs of metacentric chromosomes. However, the types of nucleolar chromosomes varied among these two populations with respect to the positions of the two constrictions (Table 1; Fig. 1g–i, m–o). In population 1, the nucleolar chromosomes had one constriction in the sub-telocentric (st) position. The mauve, double cultivar (population 2) also possessed 2 pairs of nucleolar

Table 1. A comparative analysis of chromosome characteristics and genome sizes of different cultivars of *Clitoria ternatea* L.

Cultivar/Population	2*n*	RCL (μm)	TCL (μm)	RCV (μm^3)	TCV (μm^3)	No. of nucleolar chromosomes	Karyotype asymmetry index		Karyotype formula	4C nuclear DNA amount (pg)
							A_1	A_2		
Clitoria ternatea (White, single)	16	1.95–3.91	47.60	1.13–9.08	80.10	4	0.368	0.306	$C_2\ D_2\ E_6\ F_6$	25.90±0.56
Clitoria ternatea (Blue, single)	16	2.10–5.40	59.70	2.78–10.90	91.10	6	0.286	0.325	$C_2\ D_4\ E_4\ F_6$	26.38±0.54
Clitoria ternatea (Mauve, single, Pop. 1)	16	1.95–5.0	53.04	1.30–6.60	63.02	4	0.348	0.332	$A_2\ B_2\ E_6\ F_6$	23.60±0.48
Clitoria ternatea (Mauve, double, Pop. 2)	16	1.73–3.91	42.60	1.26–6.50	54.80	4	0.248	0.291	$C_4\ E_2\ F_{10}$	27.62±0.58
Clitoria ternatea (Mauve, single, Pop. 3)	16	1.73–4.34	44.70	2.88–11.10	82.80	4	0.334	0.299	$D_4\ E_6\ F_6$	22.17±0.54

RCL: Range of Chromosome Length, TCL: Total Chromosome Length, RCV: Range of Chromosome Volume, TCV: Total Chromosome Volume. A, B, C, D: Nucleolar chromosomes; E, F: Centromeric chromosomes [A: st, sm; B: st, m; C: sm, sm; D: sm, m; E: sm; F: m].

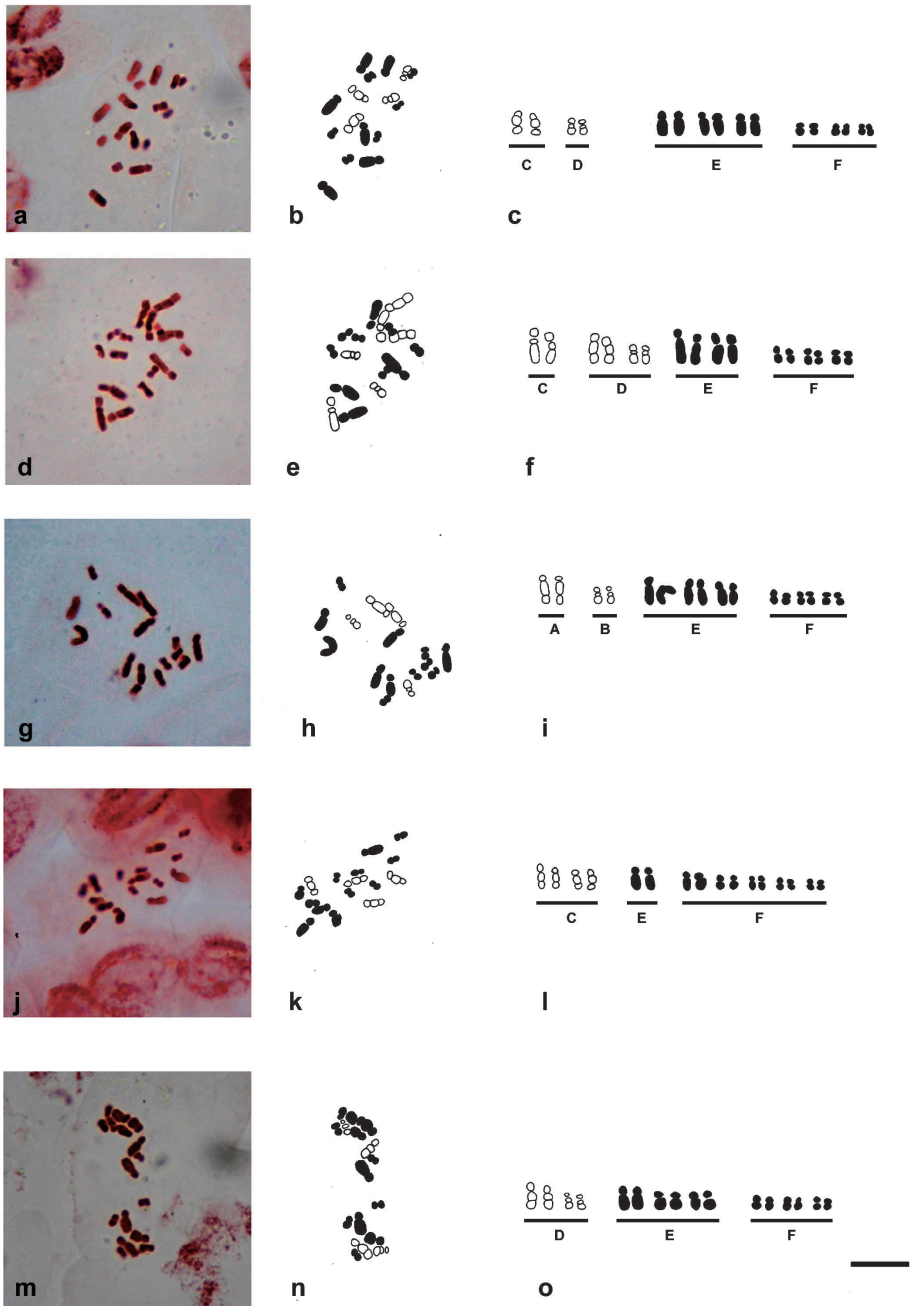

Fig. 1. Photograph of somatic metaphase plates and their respective drawing and karyograms of different cultivars and populations of *Clitoria ternatea* L. (bar=10 μm). (a–c) White, single. (d–f) Blue, single. (g–i) Mauve, single (Population 1). (j–l) Mauve, double (Population 2). (m–o) Mauve, single (Population 3).

chromosomes. However, it had only a single pair of sub-metacentric chromosome and 5 pairs of metacentric chromosomes (Table 1; Fig. 1j–l). The increase or decrease in number of secondary chromosomes might be attributed to duplication of such chromosome or translocation between the chromosomes with or without secondary constrictions at a very early stage of evolution (Mukhopadhyay and Sharma 1987, Mukhopadhyay and Ray 2013, Kumari *et al.* 2014) All these findings clearly indicated the role of cryptic structural alterations of chromosomes in the evolution of these varieties (Mukhopadhyay and Sharma 1987, Lahiri *et al.* 2010, Samanta *et al.* 2015).

The data on chromosomal parameters for each sample were obtained after examining ten cells at metaphase stages. Length of individual chromosome ranged from 1.73 to 5.40 μm while the chromosome volume ranged from 1.13 to 11.10 μm^3 among the different cultivars and populations. The Total Chromosome Length (TCL) ranged from 42.60 μm in the mauve, double cultivar (population 2) to 59.70 μm in the blue, single cultivar. The Total Chromosome Volume (TCV) ranged from 54.80 μm^3 in the mauve (double) cultivar (population 2) to 91.10 μm^3 in the blue, single cultivar (Table 1). A moderate degree of correlation (r=0.52) has been found between the TCL and TCV (Fig. 2a) which may be attributed to the differential condensation and spiralization

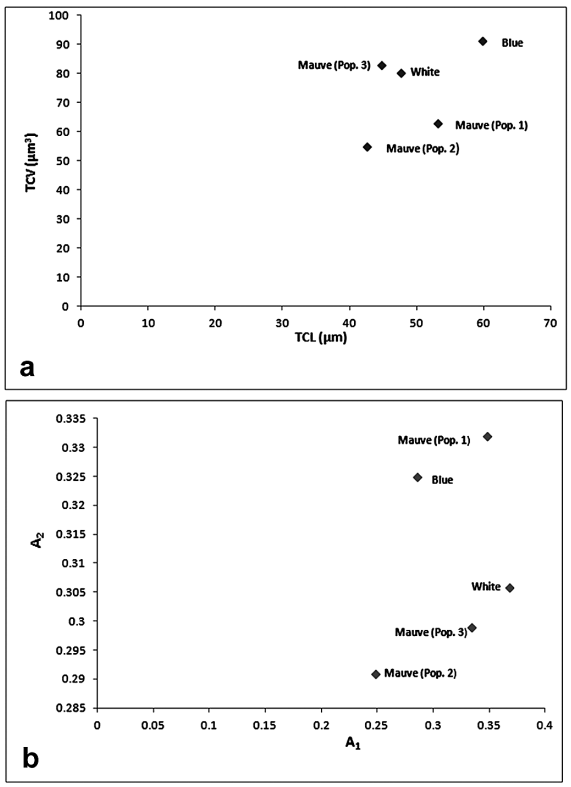

Fig. 2. Scatter diagrams of (a) total chromosome length and total chromosome volume relationship and (b) inter- and intra-chromosomal karyotype asymmetry in different cultivars and populations of *Clitoria ternatea* L.

of the chromosome arm (Sharma and Mukhopadhyay 1984, Lahiri *et al.* 2010) among these cultivars and populations.

Among the different cultivars and populations of *C. ternatea* analysed in the present investigation, the intra-chromosomal asymmetry index (A_1) varied from 0.248 to 0.368 while the inter-chromosomal asymmetry index (A_2) varied from 0.291 to 0.332. Karyotype asymmetry for the relation between the chromosome arms is estimated by A_1 while karyotype asymmetry due to relation between size of different chromosomes is estimated by A_2 (Romero Zarco 1986). A higher value of A_1 denotes a higher degree of karyotype asymmetry (Sheidai and Jalilian 2008). In the present study, the white (single) cultivar shows maximum karyotype asymmetry (Table 1; Fig. 2b), indicating an advanced feature.

The genome size (4C nuclear DNA value) was estimated through *in situ* cytophotometry from Feulgen-stained nuclei. The lowest amount was recorded in the population 3 of the mauve (single) cultivar (22.17 pg) while the highest amount was recorded in the population 2 of the mauve (double) cultivar (27.62 pg). The white, single and the blue, single cultivars showed intermediate values (Table 1). All the cultivars/ populations revealed intermediate genome size (Rewers and Sliwinska 2012). There is very weak correlation between TCL and 4C nuclear DNA amount (r=0.04) and a weak negative correlation between TCV and 4C nuclear DNA amount (r=−0.23). Both chromosome length and volume are associated with the degree of chromosome spiralization during cell division which is under genetic control (Sharma and Mukhopadhyay 1984). The variation of these two parameters among the cultivars and populations of this plant indicates the role of specific genomic constitution (Mukhopadhyay and Ray 2013). Moreover, in a eukaryotic system the chromosome volume is not only determined by DNA alone but also by both histone and non-histone proteins. A detailed cytochemical assay utilizing specific enzymes and interpherometry for protein estimation may provide positive clues to this direction (Sharma and Mukhopadhyay 1984, Mukhopadhyay and Ray 2013). The evidences indicate that each population/cultivar has its own karyotype and distinctive range in chromosome length and volume. Therefore, these characters, which are under genetic control, can be utilized as suitable parameters for classification and identification of cultivars/populations of *Clitoria ternatea* L. (Mukhopadhyay and Sharma 1987, Mukhopadhyay and Ray 2013, Samanta *et al.* 2015).

The present investigation on chromosome and genome analyses revealed that, in spite of numerical constancy, the cultivars and populations of *Clitoria ternatea* L. differed with respect to the chromosome characteristics. The different chromosome parameters studied showed differences in these cultivars and populations with both intra-chromosomal and inter-chromosomal asymmetry.

However, a moderate genome size was observed in *Clitoria ternatea* L. as revealed from *in situ* micro-spectrophotometry. The genomic specificity may be correlated with the flower characteristics of the cultivars.

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