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Genome Size Estimation in Aneuploid Accessions of *Urginea indica* Kunth. Hyacinthaceae

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ABSTRACT

Indian squill, Urginea indica is a very important and rare medicinal plant. It is found growing in India, Africa and Mediterranean regions. Due to its medicinal properties the bulb of Urginea has found its place in British and European pharmacopeia. In the present study, the existing variation in the DNA content among six accessions of Urginea indica, collected from diverse regions of Karnataka, India has been assessed using flow cytometry. Flow cytometry is a convenient and rapid method that has been used extensively for estimation of nuclear genome size in plants. Flow cytometry analysis of propidium iodide (PI) stained nuclei isolated from young root tissues of U. indica were used to estimate its genome size, using Allium cepa as reference standard with a reported 2C value of 33.5 pg of DNA. The nuclear DNA content ranged from 36.689 ± 0.17 pg in Magadi accession to 69.73 ± 0.29 pg of DNA in Nagarhole accession, with the DNA content of other accessions being Ranganathittu with 48.642 ± 0.4 pg, Karighatta with 53.02 ± 1.152 pg, Channamallipura with 48.84 ± 0.20 pg and B R Hills with 44.59 ± 0.17 pg of DNA. The known genome size of U. indica together with other genomic data would be utilized in advancing biochemical and evolutionary studies of the genus Urginea.

Key words: *Flow cytometry, Propidium Iodide, nuclear DNA content, Galbraith's buffer, intra-specific variation.*

INTRODUCTION

Urginea indica, commonly called as Indian squill or wild onion, is a very important medicinal plant found growing in India, Africa and Mediterranean regions (Fig. 1). It has magical potential to heal many human diseases with cardiotonic, anti-carcinomic, anti asthmatic, anti epileptic, dermatological and diuretic properties. Besides it has abortifacient effects and affects menstrual cycle. Wide genetic and chromosomal variations were also still being under research to differentiate the different accessions of *Urginea indica*. The bio-diversity and germplasm collection is also a major area of emphasis to protect the rare genus. The basic taxonomic work on higher molecular developmental studies is being explored in this genus.

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Fig. 1: Vegetative bulbs of *U. indica*

It is also a great source for many organic compounds yet to be characterized and discovered for its extensive possibility as potential bio-active molecule. The genetic variability and genomic studies are being a hot topic in research. *Urginea indica*, being a plant with high therapeutic values, offers an excellent candidate for future medicinal applications. Analysis of the characteristics of different species and their populations at the molecular level may reveal genetic basis of variations in the plant properties, thus helping to study and improve these attributes in the plant[1].

Determination of an accurate amount of nuclear DNA content is extremely important to understand the hereditary constituent of an organism. Flow cytometry, originally developed for medical studies, is an easy, rapid, accurate and convenient tool for estimating plant genome size, ploidy level, assessing DNA content and analysing cell cycle[2,3,4]. In addition to determining nuclear DNA content, flow cytometry in higher plants is used for studying chromosome size, centromeric index, sorting of large quantities of chromosomes of single type for gene isolation and mapping[5].

Genome size is a fundamental parameter in functional genomics studies to have an estimate on a species' genome size. It is an important first step towards deciding and strategizing for its sequencing. Over recent years, flow cytometry has become a preferred technique in estimating nuclear DNA content of plants because it is fast, easy and accurate compared to other classical methodologies such as Feulgen micro-spectrophotometry. The research on nuclear DNA content in plants focuses on observations of genome size variation between species possessing different morphological and ecological features. Genome sizes are typically given as haploid gametic nuclear DNA contents (C-values) either in units of mass (picograms, where 1pg = 10^{-12} g) or in number of base pairs (in eukaryotes, most often in megabases, where 1Mb = 10^6 bases). These are directly inter-convertible as 1pg = 978 Mb or 1Mb = 1.022 X 10^{-3} pg[6]. The first genome size estimates were conducted in the plant *Lillium longiflorum* in 1951.

Knowing the genome size of *U. indica* will expand the C-value database of Hyacinthaceae family and will also provide the information that would progress functional and structural genomic research of this important medicinal plant and together with other genomic data advance studies on biochemical and evolutionary divergence of *Urginea* species. The intra-specific changes in genome size, being therefore subject to natural selection, are naturally related with those leading to divergence and evolution species.

Therefore a thorough appraisal of such changes is required for the application of genome size data to demarcate intrageneric taxa and in microsystematics.

The bulbous monocots of Hyacinthaceae are characterized by the presence of copious mucilage that interferes largely with the nuclei isolation thus making flow cytometry difficult. As a result there are few genome size estimates made in Hyacinthaceae using Flow cytometry.

Flow cytometry may be characterized as a dynamic fluorescence microphotometry, with a focused stream of nuclei. The first successful isolation of nuclei for plant cytometry was made from *Vicia faba* root tips by Heller (1973). Ten years later, the work by Galbraith *et al.* (1983) paved way for the technology by reporting a simple technique for making nuclear suspensions. This chopping technique became the method of choice finally for preparation[8]. The slurry of the suspension is sieved and fluorochrome is added. Within minutes the nuclear isolate is ready for running on the flow cytometer. It is widely accepted that in work involving different species intercalating dyes without base preference should be used, principally, Propidium iodide (PI)[10]. Choice of isolation buffers plays a pivotal role in flow cytometric estimations.

From this background, the objective of the study was to detect the genome size of six accessions of *U. indica* sampled from different geographical regions of Karnataka using flow cytometry.

MATERIALS AND METHODS

Bulbs of *Urginea indica* were collected from six different parts of Karnataka, South India (Table 1). Voucher specimens have been deposited in the herbarium of Botany Department. Plants are grown and maintained in the green house of Department of Botany, Bangalore University. The standard material used was *Allium cepa*, with reported 2C-value of 33.5 pg of DNA. In 1965, Van't Hof published an estimate of the nuclear DNA amount of *Allium cepa* (2C = 33.5 pg) carefully obtained using chemical methods. This was later used as a calibration standard by his colleagues from Brookhaven and by other groups including Rees and colleagues at Aberystwyth, Bennett and Smith (1976) in Cambridge and Greilhuber (1977) in Vienna.

Table 1: Populations selected for flow cytometry

S. No.	Accessions of *U. indica*
1	Ranganathittu
2	Magadi
3	Karighatta
4	Channamallipura
5	B. R. Hills
6	Nagarhole

Isolation buffers

The popular nuclear isolation buffers are based on organic buffers such as MOPS, Tris and 4-(hydroxymethyl) piperazine-1-ethanesulfonic acid (HEPES) that stabilize the pH of the solution and keep nuclei in an intact or even sub-vital state[2,7,13,14,15,16]. Non-ionic detergents, such as Triton X-100 and Tween 20, are used to facilitate the release of nuclei from cells and prevent nuclei from clumping and attachment of debris, while the nuclear chromatin is stabilized by Mg^{2+} or spermine[2,7,15]. In some buffers, chelating agents (e.g. EDTA, sodium citrate) are added to bind divalent cations, which serve as cofactors of DNases; inorganic salts (*e.g.*, KCl, NaCl) are used to achieve proper ionic strength[17]. Some buffers are supplemented with reducing agents such as β-mercaptoethanol, metabisulfite and dithiothreitol to prevent the action of phenolic compounds, while PVP is added to bind the phenolics kept in a reduced state[16].

Sample preparation for Flow cytometry

Approximately 50 mg of young root tissue (~ 1-3cm long) from the bulbs of *Urginea indica* was used for sample preparation. Leaves, bulb scales, inflorescence axis and older roots (>3 cm) are not suited for flow cytometric sample preparations because of high mucilage content. Nuclei suspensions were prepared in Galbraith's buffer, with the composition of 45 mM Magnesium Chloride, 30 mM Sodium citrate, 20 mM 3-(N-morpholino) propansulfonic acid and 0.1% (v/v) Triton X-100. Plant tissue was chopped in 1 ml of buffer

solution using a sharp razor blade for ~ 60 s at 4^0 C. The homogenate was filtered through a 40 µm nylon filter to remove large debris. Nuclei suspension was stained with 50 µg/ml Propidium iodide (PI). 50 µg/ml RNase was added to the suspension to prevent the staining of double stranded RNA. Samples are incubated on ice and analysed within 10 minutes[7,8,11].

For genome size estimation, approximately 50 mg of the young root tissue of *Allium cepa* was used for sample preparation following the same protocol.

Flow cytometry

A BD FACS Calibur Flow Cytometer equipped with air-cooled argon-ion laser, 15 milliwatt, 488 nm and CellFIT software was used for analysis of samples. Prior to running samples, linearity of the instrument was checked using the BD DNA QC Particles kit (BD Bioscience, San Jose, CA). Doublets, disintegrated nuclei, and other cell debris were eliminated from analysis by gating forward and side scatter profiles of samples. The gate was uniformly maintained across all samples in each run. For each sample 5,000 to 12,000 events (nuclei) were collected and the resulting histograms were analyzed using CellFIT software (San Jose, CA). The absolute DNA amount of a sample is calculated based on the values of the G_1 peak means:

Sample 2C DNA content = [(sample G_1 peak mean) / (standard G_1 peak mean)]
X standard 2C DNA content (pg DNA).

Absolute DNA amounts are traditionally reported in pg DNA. However, with the advent of molecular biology and progress in genome sequencing projects, there has been a trend to express DNA amounts in terms of base pairs (bp) and to use the term "genome size"[8,11,14].

Statistical analysis

Statistical analysis was carried out by calculating the mean and standard errors of the genome size of the populations. Data was expressed as Mean ± Standard Error of Mean (SEM). Differences between the control and the different populations were analyzed by means of Student's unpaired *t*-test and one-way ANOVA test. The level of significance for testing the statistical significance was $P<0.05$.

RESULTS

Flow cytometric analysis of nuclei isolated from young *Urginea indica* roots showed only a single peak corresponding to the 2C ($G_0 + G_1$ phase) peaks corresponding to the 4C level were not detected (Fig.2). This indicates that there are no dividing cells in the portions of the roots sampled.

Fig. 2 a-g shows histograms of relative fluorescence of nuclei from various *Urginea* accessions processed using *Allium cepa* as reference standard. The genome sizes of the various accessions showed significant differences (Fig. 2; Table 2). Magadi population had the smallest genome size (36.68 ± 0.17 pg), while the Nagarhole population had the largest values (69.73 ± 0.298 pg). The genome size of the populations evaluated are Ranganathittu with a value of 48.642 ± 0.4 pg of DNA, Karighatta with a genome size of 53.015 ± 1.152 pg of DNA, Channamallipura had 48.74 ± 0.204 pg of DNA and B. R Hills had a value of 44.598 ± 0.1748 pg DNA.

Variations in nuclear DNA content estimates were noted among *U. indica* plants used; however, those variations were statistically significant ($P > 0.05$). Haploid genome size of *A. thaliana* (model plant) and *O. sativa* are 157 and 490 Mbp, respectively, whereas that of *U. indica* evaluated ranged from 17937 Mbp to 34132 Mbp[18,19].

Table 2: Values are means from three replicate measurements per plant sample

Sample	Avg. G_1 Mean	CV	2C DNA Content (pg DNA)	Haploid (C) DNA content (pg DNA)	Mbp
Control – *Allium cepa*	186.5633333	3.38	33.5	16.75	16381
Ranganathittu	270.9366667	3.91	48.65 ± 0.40	24.33	23795
Magadi	204.3266667	3.0	36.68 ± 0.17	18.34	17937
Karighatta	295.2466667	3.77	53.01 ± 1.152	26.51	25927
Channamallipura	286.5666667	3.96	48.84 ± 0.20	24.42	23883
B. R. Hills	248.4733333	3.06	44.61 ± 0.17	22.31	21819
Nagarahole	409.55	3.29	69.80 ± 0.29	34.9	34132

Values are significantly different from each other at $P \leq 0.05$, 1 pg DNA = 978 Mbp

Fig. 2 a - g: Histograms of flow-cytometric genome size measurements in *Urginea.* Relative fluorescence intensity of propidium-iodide stained nuclei of the populations of *Urginea* measured with the internal standard *Allium cepa*

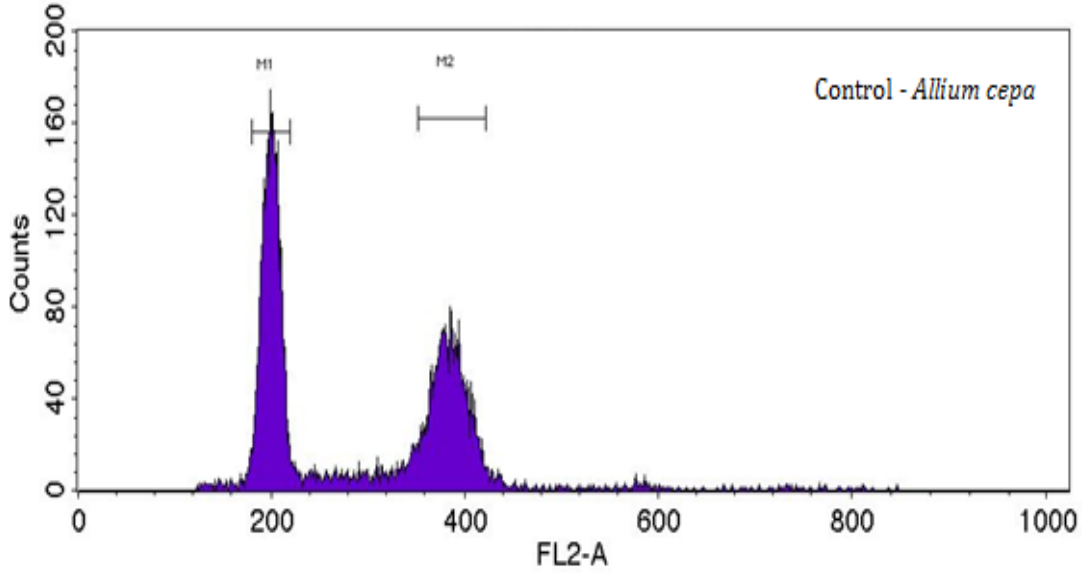

Fig. 2: a - Histograms of flow-cytometric genome size measurements in *Allium cepa*

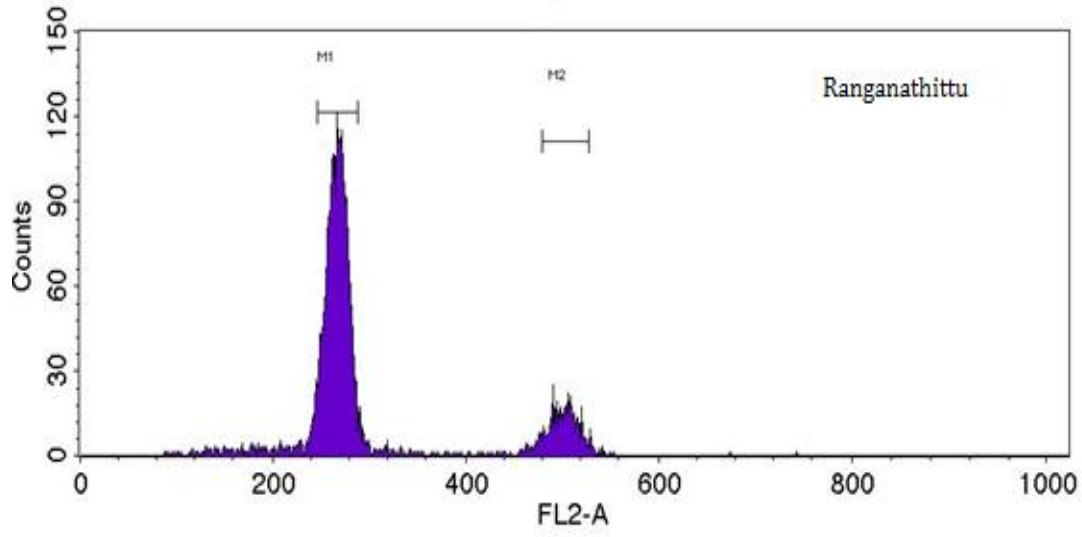

Fig. 2: b - Histograms of flow-cytometric genome size measurements in *Urginea indica* in Ranaganathittu accession

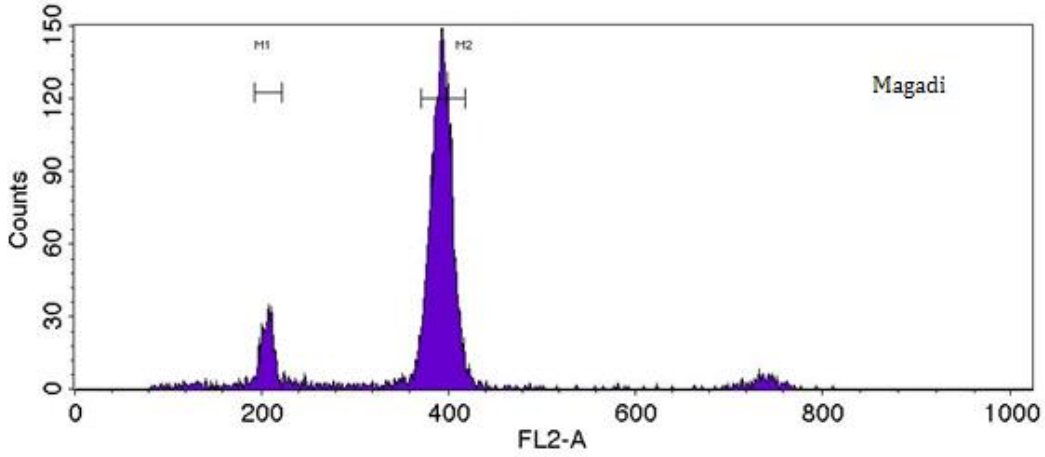

Fig. 2: c - Histograms of flow-cytometric genome size measurements in *Urginea indica* in Magadi accession

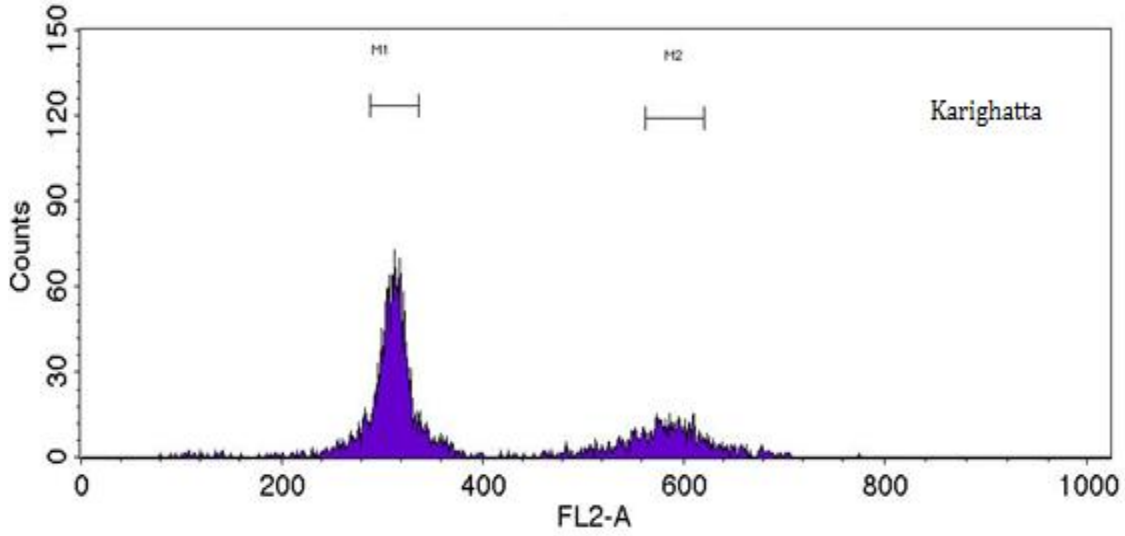

Fig. 2: d - Histograms of flow-cytometric genome size measurements in *Urginea indica* in Karighatta accession

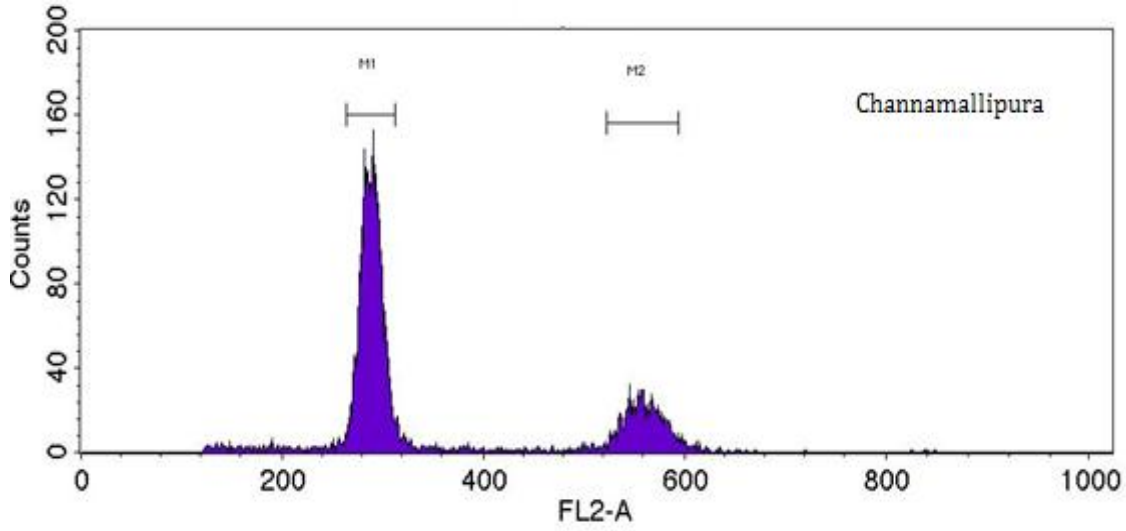

Fig. 2: e - Histograms of flow-cytometric genome size measurements in *Urginea indica* in Channamallipura accession

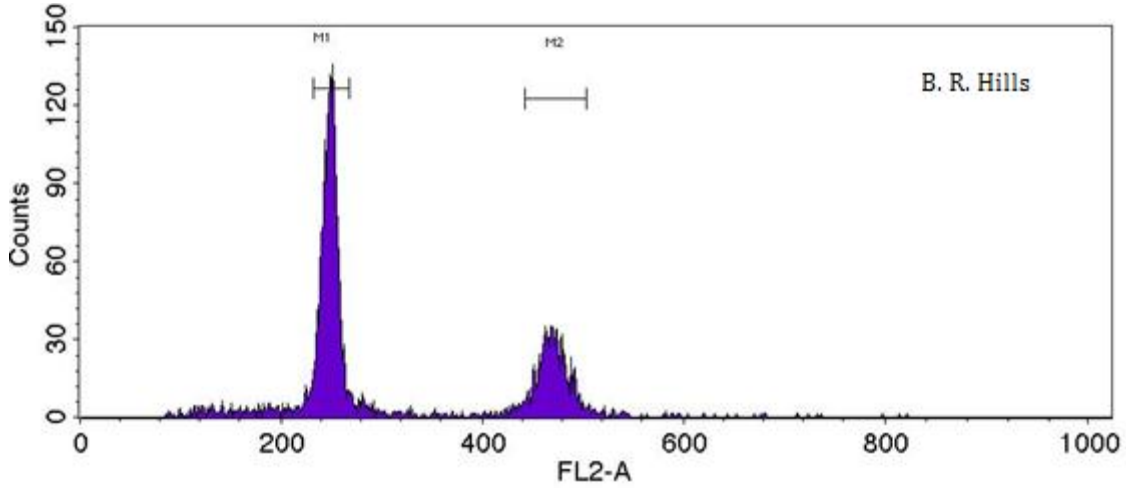

Fig. 2: f - Histograms of flow-cytometric genome size measurements in *Urginea indica* in B. R. Hills accession

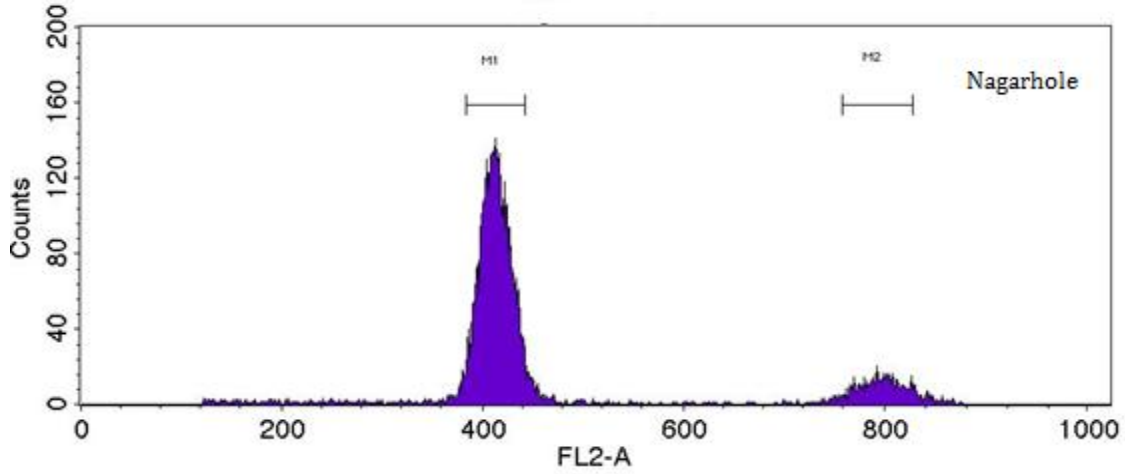

Fig. 2: g - Histograms of flow-cytometric genome size measurements in *Urginea indica* in Nagarhole accession

DISCUSSION

Intraspecific variability in genome size among plants is of great interest. Reports in many plant species show a striking range of variation which was previously thought to occur only between species. It is now being increasingly considered that the changes in genome size may not only be restricted to species divergence but also associated with various environmental conditions and developmental stages affecting different populations or individual plants[20]. The intraspecific changes in genome size, being therefore subject to natural selection, are naturally related with those leading to the divergence and evolution of species. C-value variation is mentioned to be due to heterochromatin polymorphism, B-chromosomes, aneuploidy, polyploidy or hybridity is well understood. To obtain perfect, absolute C-values the ideal calibration standard should exhibit no variation between individuals. *Allium cepa* has been informally adopted by common usage as the main calibration standard for C-value estimations in related species. Van't Hof's (1965) estimate for *Allium cepa* (2C = 33.5 pg) has been widely accepted as a calibration standard.

Plant species where intraspecific variation in genome size has been reported include soyabean, sunflower, pea and maize[15,21,22,23,24]. Twenty years after the breakthrough paper of Galbraith *et al.,* (1983), plant DNA flow cytometry is a very popular method with applications ranging from basic and applied research to industry. Taxonomy, population biology and ecology require the

analysis of large populations of plants, for which flow cytometry is ideally suited. It seems highly probable that a growing number of applications will be seen in these areas. While the estimation of relative DNA amounts for ploidy screening and some other applications usually do not represent serious problems, the use of flow cytometry for estimation of genome size is a greater challenge[17]. According to Deepak Ohri (1998), C-value variation may be due to heterochromatin, polymorphism, B-chromosomes, aneuploidy and polyploidy.

In the present study, the populations of *Urginea* showed statistically significant differences when compared with the reference standard *Allium cepa.* The six populations studied showed differences in four accessions (Magadi, Nagarhole, Karighatta, B.R. Hills) amongst each other except Ranganthittu and Channamallipura, with almost similar genome size (48.64 & 48.74 pg of DNA respectively).

The genomes of plants have frequently been labelled as 'fluid', 'dynamic' and 'in constant flux', due in large part to the seemingly common observation of pronounced intraspecific variation in their nuclear DNA contents. In some cases, real variation within species can be explained by the differential presence of supernumerary B chromosomes. Strictly speaking, this does not refute the notion of DNA constancy because the A chromosome complement remains unchanged. In other examples, however, intraspecific variation in DNA content can be attributed to recognizable polymorphisms in the A chromosomes themselves, as with heterochromatic knobs in maize or differentially deleted transposable element remnants in barley[26]. In species of *Urginea*, the differences in genome size can be attributed to the aneuploid status of the populations, but the variations in genome size within diploids and tetraploid accessions have been reported by Shivkameshwari.

In conclusion, the noted large genome size of *U.indica* will have important implications on structural and functional genomics research and provides information for consideration in genome sequencing undertakings of this economically important medicinal plant. The now known genome size of *U. indica* together with other genomic data would be utilized in advancing biochemical and evolutionary studies of the genus *Urginea.*

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