

*Full Length Research Paper*

# Optimized flow cytometric protocol and genome size estimation of Sabah snake grass (*Clinacanthus nutans*)

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***Clinacanthus nutans* is an economically important medicinal plant that can be found grown in many countries in the Asian region. Useful medicine properties such as anti-cancer, anti-bacteria, and anti-viral, backed by its high content of phytochemical compounds such vitexin, isovitexin, stigmasterol and lupeol has increased the demand for *C. nutans* in the market. Extensive work had been carried out on its content and pharmacological activity using fresh samples but limited studies for *in vitro* cultures of *C. nutans*. No genome size estimation has been done for *C. nutans*. Objective of this study was to analyse nuclear DNA content of *C. nutans* using flow cytometry. Preparation of different nuclei isolation buffer and stoichiometric DNA staining using propidium iodide was carried out. The genome size of *C. nutans* was estimated using *Glycine max* cv. Polanka as internal standard and its genome size was compared with *in vitro* plantlets of *C. nutans*. Flow cytometry analysis revealed that nuclear 2C DNA of *C. nutans* content is estimated at  $1.75 \pm 0.006$  pg. Coefficient of variation in flow cytometric analysis was within the limit of 5% implying that the results were reliable with the Tris.MgCl<sub>2</sub> being the best nuclei isolation buffer. No significant difference was observed from field grown and *in vitro* *C. nutans*. This finding will assist further in genome size evolution analysis of *Clinacanthus* spp. and to determine polyploids for increased active compounds and biomass.**

**Key word:** Flow cytometry; Nuclear DNA content, *Clinacanthus nutans*.

## INTRODUCTION

*Clinacanthus nutans*, also known as Sabah Snake Grass or Daun Belalai Gajah is a shrub belonging to the Acanthaceae family. This plant has recently gained much economic value in South Asian region mainly in Malaysia, Indonesia, and Thailand due to its pharmacological properties (Zulkipli et al., 2017). Its secondary content, rich in phytochemicals such as betulin, lupeol, vitexin, isovitexin and stigmasterol are beneficial to mankind.

Based on the active compounds, pharmacological activities such as anti-oxidative (Pannangpetch et al., 2007; Arullappan et al., 2014), anti-proliferative (Yong et al., 2013; Ghazemzadeh et al., 2014), anti-tumorigenic (Huang et al., 2015), anti-bacterial (Chomnawang et al., 2009; Arullappan et al., 2014), anti-viral (Kunsorn et al., 2013) and anti-inflammatory (Wanikiat et al., 2008) have been reported with *C. nutans*. Owing to the growing

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demand, conventional propagation methods through stem cutting and micropropagation have been proposed as an alternative way to cultivate this plant commercially. In line to that, a few *in vitro* protocols since then had been established and explored (Chen et al., 2015; Haida et al., 2020). Besides cultivation, field management, clone selection and polyploidization are being constantly explored to cater the growing demand.

Knowledge of nuclear DNA content, both size and molecular details have become essential in determining genetic evolution of plants besides gaining a better understanding at molecular and cellular level (Yan et al., 2016). Since the report of *Lilium longiflorum* genome size (Ogur et al., 1951), more than 12,200 plant species genomes have been estimated (Pellicer and Leitch, 2014). Genome size refers to its nuclear DNA content in a cell and is measured in picograms (pg) or megabase pairs (Mbp, 1 pg = 987 Mbp) (Dolezel et al., 2003). Estimation of genome size is crucial in many biological fields such as evolution, ecology, population genetics and plant breeding (Kron et al., 2007; Loureiro et al., 2010). Its size change is associated with environmental factors, climatic variation, and geographical plant distribution (Bennett, 1976; Levin and Funderburg, 1979) and (Ohri and Khoshoo, 1986). Continuous subculturing of plantlets in *in vitro* may lead to genetic disturbance and cause somaclonal variation (Sliwinska and Thiem, 2007). It is crucial to ensure genome stability in *in vitro* plantlets especially medicinal plant such as *C. nutans* that is highly sort after for its pharmaco-properties. Moreover, limited studies have evaluated the effect of *in-vitro* conditions on genome size.

Genome size evaluation is constantly being optimized both at the process level as well as use of improved instruments. The estimate of genome size in plants started with Feulgen densitometry (Bennett and Leitch, 1997) and re-association kinetics (Dhillon et al., 1980) followed by the popular flow cytometry (FCM) (Dolezel et al., 2007). The FCM requires a small quantity of sample and can analyse many cells in a shorter time with higher precision compared to other spectrophotometry approaches (Moghbel et al., 2015; Dhooghe et al., 2010; Omidbaigi et al., 2010).

However, the accuracy of FCM in some plants was subjected to the presence of endogenous fluorescence inhibitors such as tannins (Loureiro et al., 2006) and anthocyanins (Bennett et al., 2008). This demands the need to optimize Galbraith et al. (1983) simple method of plant nuclei isolation technique, chopped leaf tissues in a lysis buffer. The selection of appropriate nuclei isolation buffer according to the plant and inhibitors present is considered crucial in genome size determination. As the appropriate buffer ascertains the quality of the sample maintained and stoichiometric errors during DNA staining (Sadhu et al., 2016). Among the common buffer used in nuclear DNA estimation in plants are Galbraith buffer (Galbraith et al., 1983), LBO1 buffer (Dolezel et al.,

1989), Otto buffer (Otto, 1990; Dolezel and Gohde, 1995), Tris.MgCl<sub>2</sub> buffer (Pfosser et al., 1995) and Tris.MgCl<sub>2</sub> with 1% PVP (Dolezel et al., 1989). Although the chemical component of the mentioned buffers varies, each of the nuclei isolation buffer contains an organic pH-stabilizing chemical such as MOPS, TRIS or HEPES, chromatin stabilizers such as MgCl<sub>2</sub>, MgSO<sub>4</sub> or Spermine, and divalent cation binding metal chelators such as EDTA or sodium citrate as nuclease cofactors. Inorganic salts such as KCl or NaCl are also added to attain proper ionic concentration and non-ionic detergents such as Triton X-100 or Tween 20 which further aid in releasing nuclei and remove debris from the surface of the nuclei (Coba and Brown, 2001).

Studies have reported that concurrent evaluation of both target plants and standards, use of internal and external standards (Price et al., 2000; Noirot et al., 2005), use of appropriate nuclei isolation buffer and addition of anti-oxidative compounds (Dolezel and Bartos, 2005; Dolezel et al., 2007) reduces experimental error. The genome size is reflected and estimated based on known DNA content that act as standards. Plants such as *Solanum lycopersicum* L. Stupicke polni rane (2C DNA content = 1.96 pg), *Glycine max* Merr. Polanka (2C DNA content = 2.50 pg), *Zea mays* L. CE-777 (2CDNA content = 5.43 pg) and *Pisum sativum* L. Citrad (2C DNA content = 9.09 pg) (Dolezel et al., 2007) are commonly used as an estimator or standard to estimate the sample plant genome size. It is recommended that the standard should have a genome size close to target plant species (Dolezel et al., 1998).

No genome size estimation had been carried out for *C. nutans* which is highly sort after for its medicinal values as this can help in further exploration of clone selection and polyploidization sector to enhance active compounds. At present, to cater the growing demand, stem cutting has been the propagation method used to cultivate this valuable medicinal plant which could soon lead to extinction (Zulkipli et al., 2017). Besides, genetic disturbance and somaclonal variation is reported in *in vitro* cultures (Sliwinska and Thiem, 2007). Therefore, this study explores the impact varying nuclei isolation buffer to determine the genome size of *C. nutans* besides determining the variation, if any, in *in vitro* grown and field grown plants using laser sourced flow cytometer (BD Accuri C6) along with *Glycine max* cv. Polanka (Dolezel et al., 1992) as an internal standard.

## MATERIALS AND METHODS

### Plant materials

Plant material for field grown *C. nutans* was obtained from the medicinal plant garden, at the Forest Research Institute Malaysia (FRIM), Kepong, Selangor, with identity confirmed by the FRIM's botanist, at the Ethnobotanical Department. These samples were collected in the month of September 2017, and grown in pots, at Taylor's Garden, and thereafter explants were collected to establish



**Figure 1.** Plant materials used in this experiment (a) *In vitro* *C. nutans*; (b) field grown *C. nutans*; (c) *G. max* used as standard.

*in vitro* culture. Both, field grown *C. nutans* and standard plant were grown under similar condition at the greenhouse or *in vitro*. Greenhouse plants were grown in 30 cm pots under natural light source at Taylor's Garden. *Glycine max* cv. Polanka (2C DNA content = 2.50 pg) was chosen as the standard reference as its genome size was larger than of the target sample. Seeds of *G. max* was obtained from Cytogenetic lab, MPOB (Malaysian Palm Oil Board) which was procured from Dr. Jaroslav Dolezel, Institute of Botany, Olomouc, Czech Republic. The ambient growth room condition of  $25 \pm 2^\circ\text{C}$  and 60 to 65% relative humidity, at 12 h photoperiod, with light intensity at 3000 lux were maintained for *in vitro* grown *C. nutans* cultures in Plant Tissue Culture room at Taylor's University. These plantlets were sub-cultured every 8 weeks using nodal cutting approach. The samples used for analyses were maintained for a duration of over two years (Figure 1).

### Sample preparation

Plant samples (*C. nutans*) and standards (*G. max*) were selected randomly for nuclei isolation using different isolation buffer and genome size evaluation with FCM. Three nuclei extraction buffer were prepared according to modified procedure from Dolezel et al. (1989), first being Tris.MgCl<sub>2</sub> containing 200 mM Tris, 4 mM MgCl<sub>2</sub>. 6H<sub>2</sub>O, 0.5% (v/v) Triton X-100 and 1% PVP (Dolezel et al., 1989), LBO1 buffer containing 15 mM Tris, 2 mM Na<sub>2</sub>EDTA, 80 mM KCL, 20 mM NaCl, 0.5 mM spermine, 15 mM β-mercaptoethanol, 0.1% Triton X-100, pH 7.4) (Dolezel et al., 1989) and Otto buffer comprising of Otto I (0.1 M citric acid and 0.5% (v/v) Tween 20; pH 2 to 3) and Otto II (0.4 M Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O; pH 8-9) (Otto, 1990). Fully formed young leaf from sample and standard was excised. Each sample, weighing approximately 30 to 50 mg were placed on ice cold 6 mm petri dish to decrease the nuclease activity and was chopped into tiny segments (0.5 to 1.0 mm) in 1 ml of ice-cold buffer using a sharp clean razor blade (Treet Corporation Ltd) to form a homogenate. About 500 μl of the homogenate was then pipetted out and filtered through a 40 μm nylon mesh (Fisher

Scientific) into a 1.5 ml microcentrifuge tube to remove cell fragments and large debris. The filtrate was then added with 2.5 μl of RNase (10 mg/ml; Sigma-Aldrich) to denature RNA and 25 μl of Propidium iodide (1 mg/ml; Sigma-Aldrich) to stain the DNA. Final concentration of propidium iodide was maintained at 50 μg/ml. Samples and standard were incubated on ice in the dark for 30 min prior to analyse with flow cytometer. Four replicates were carried out for each buffer on different days and debris factor and yield factor were calculated based on the formula as below (Loureiro et al., 2007):

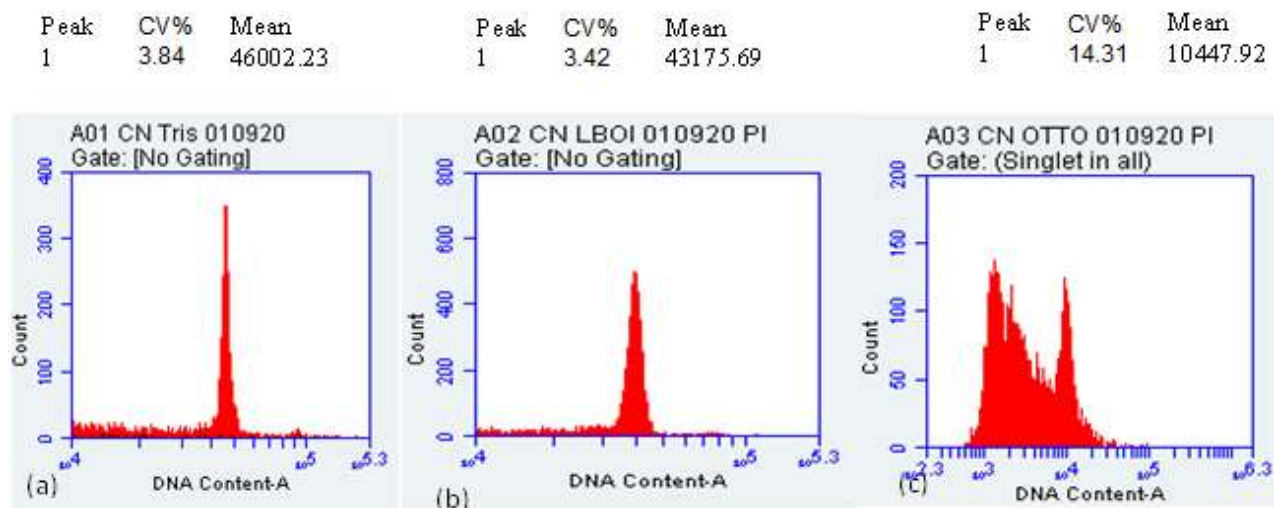
$$\text{DF (\%)} = \frac{\text{Total number of particles} - \text{total number of nuclei}}{\text{Total number of particles}} \times 100$$

$$\text{YF (nuclei s}^{-1} \text{ mg}^{-1}) = \frac{\text{Total number of nuclei / number of seconds of run(s)}}{\text{Weight of tissue (mg)}}$$

### Flow cytometric analysis

Estimation of nuclear DNA content was performed using BD Accuri<sup>TM</sup> C6 flow cytometer. Samples and standard were stained with propidium iodide and were excited at 488 nm with 20-mW laser illumination. The fluorescence signal also known as the pulse area measurement was screened using two different filters. FL2 with a 585/40 nm bandpass filter was used for propidium iodide-stained nuclear DNA content. Auto fluorescence due to chloroplast was excluded from the analysis by selecting a region using FL3 (670 nm long pass filter). Flow rate was kept constant at low pace (14 μl sample per minute) throughout the experiment and a run time for each sample was approximately 3 to 5 min. Each sample, at least 5000 nuclei were evaluated.

Resolution, linearity, and doublet discrimination capability of the instrument was evaluated using Accuri C6 DNA QC Particles Kits which includes the usage of chicken erythrocyte nuclei (CEN) and cow thymocyte (CTN) as 8 beads and 6 beads. This was carried out



**Figure 2.** Histogram of relative fluorescence intensity derived from nuclei of *C. nutans* isolated in (a) Tris.MgCl<sub>2</sub> with 1% PVP; (b) nuclei of *C. nutans* isolated from LBO1 buffer; (c) nuclei *C. nutans* isolated from Otto buffer.

prior to running the samples and the standard. The nuclei were gated to obtain a region around the signals due to intact single nuclei and to eliminate the background fluorescence due to debris, fragmented DNA and auto-fluorescence from chlorophyll. The gate was consistently maintained across all the samples during the run using FCM. Histogram was plotted to compare median fluorescence intensity (MFI) of the peaks derived from the sample and standard used as external and internal. The DNA content per cell is proportional to the fluorescence intensity of PI measured by the flow cytometry (Yan et al., 2016). Therefore, the 2C DNA content was calculated based on the value of fluorescence intensity of the peaks for both sample and standard based on the calculation as below (Dolezel et al., 2007):

$$\text{Sample 2C DNA } C. \text{ nutans (pg)}: \frac{\text{MFI of sample peak} \times 2.5\text{pg (2C DNA } G. \text{max)}}{\text{MFI of standard peak (} G. \text{max)}}$$

(MFI: Mean fluorescence intensity)

### Presence of inhibitors

Test sample and standard were chopped together to assess the presence of inhibitors in the *C. nutans*. This is due to the presence of phenolic compounds in the cytosol of plants that inhibits the propidium fluorescence. Therefore, it is required to test for inhibitors for all sample plants that are run using FCM to determine the nuclear DNA content (Price et al., 2000). Presence of inhibitors are confirmed if the mean peak position of the standard plant is lower in the presence of the target sample compared to the mean of the standard plant chopped alone. In this experiment, the sample and standard were examined separately and chopped together and processed as one sample.

### Data analysis

Total of four replicates from *C. nutans* were examined using the three buffers (Tris.MgCl<sub>2</sub>, LBO1 and Otto buffer). Each replicate was run on different days. For genome size estimation, total of six *C. nutans* plants were analysed with four from wild type *C. nutans* and the other two *in vitro* propagated *C. nutans* plantlets. Each

plant sample was analysed in triplicates with each replicate being performed on different days. In all the experiments, the fluorescence of at least 5000 nuclei to 10000 nuclei was measured. Conversion from picograms (pg) to base pair numbers was done as follows: 1 pg DNA is equivalent to  $0.978 \times 10^9$  bp (Dolezel et al., 2007). The results were analysed using one way analysis of variance (ANOVA) using SPSS version 25. For significant effect, Tukey's pairwise comparison was carried out with p value  $\leq 0.05$ .

## RESULTS

### Comparative analysis of different buffers

The choice of nuclei isolation buffer is a crucial step in genome size estimation in plants. Three different buffers were examined here for *C. nutans* as these three buffers (Tris.MgCl<sub>2</sub>, LBO1 and Otto buffer) differ in their ingredients to extract the nuclei from the plant. The effectiveness of these buffers in *C. nutans* nuclei isolation is presented in Figure 2. It is seen, Tris.MgCl<sub>2</sub> with 1% PVP buffer (a) and LBO1 buffer (b) shows low CV value compared to Otto buffer (c) and with a narrower peak. The result also shows that no noticeable nuclear aggregation particles was seen in FCM histogram of nuclei suspension extracted using Tris.MgCl<sub>2</sub> (a) and LBO1 buffer (b). However, Otto buffer (c) showed cell clumping and increased CV value ( $\geq 5\%$ ) plus relatively higher variance in mean fluorescence intensity in all the replicates.

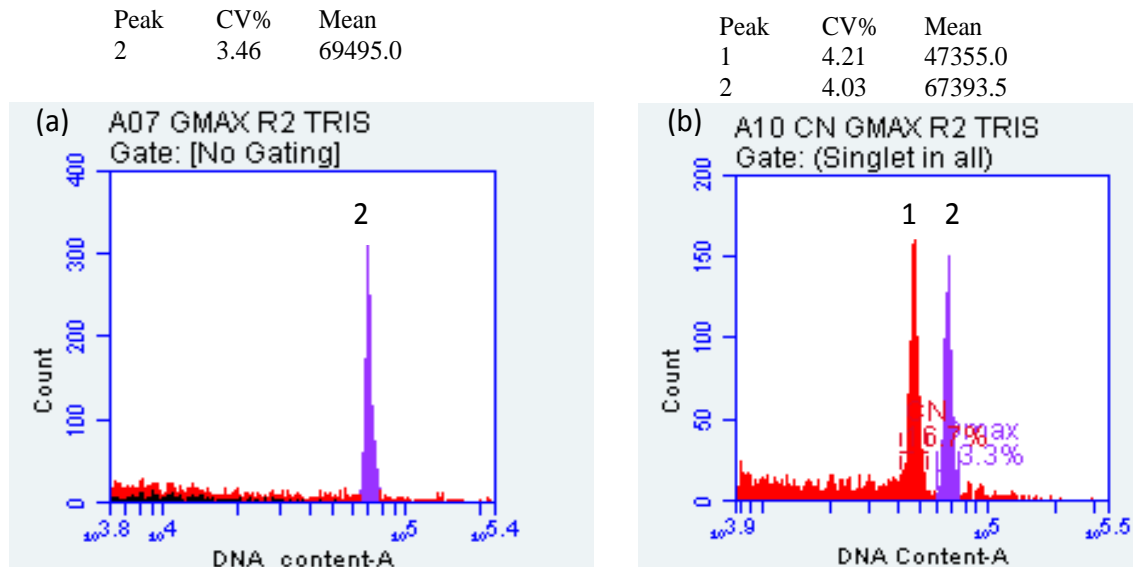
The efficacy of the buffers was further evaluated based on the calculation obtained from a combination of high yield factor (YF), low debris factor and low CV (%) peaks (Table 1). Based on the FCM analysis of *C. nutans*, there was no significant difference between the debris factor in all three-buffer tested. No significant variation too was



**Table 1.** Flow cytometric parameter analysed using different buffers Tris.MgCl<sub>2</sub>, LBO1 and OTTO. Each treatment was replicated four time.

Buffer	CV (%)	DF (%)	YF (nuclei/sec/mg)
Tris.MgCl <sub>2</sub>	4.745 ± 0.978 <sup>a</sup>	8.411 ± 4.489 <sup>a</sup>	39.930 ± 5.618 <sup>b</sup>
LBO1	4.495 ± 1.140 <sup>a</sup>	8.685 ± 5.179 <sup>a</sup>	6.846 ± 1.542 <sup>a</sup>
Otto	10.82 ± 2.601 <sup>b</sup>	12.275 ± 5.655 <sup>a</sup>	7.150 ± 3.482 <sup>a</sup>

Mean values followed by same letter are not significantly different at  $p \leq 0.05$  (Tukey's test).



**Figure 3.** Histogram of relative fluorescence intensity derived from (a) nuclei isolated from leaves of *G. max* only; (b) nuclei isolated from wild type *C. nutans* and *G. max* cv Polanka (standard) chopped, stained, and analysed simultaneously.

seen with total number or particles, total number of singlet nuclei and the weight of the *C. nutans* leaf cut. However, the time required to run the sample differed in these buffers. LBO1 buffer and Otto buffer took an average of 25 to 28 min to run the analysis compared to Tris.MgCl<sub>2</sub> buffer, which was much faster, an average of 5 min. As such, LBO1 and OTTO buffer gave a significantly low yield factor compared to Tris.MgCl<sub>2</sub> buffer (Table 1). In this study, significantly highest yield factor (YF), lowest debris factor (DF) and low CV peaks are seen with Tris.MgCl<sub>2</sub> supplemented with 1% PVP. Therefore, Tris.MgCl<sub>2</sub> with added 1% PVP buffer was further used for genome estimation in *C. nutans* plant.

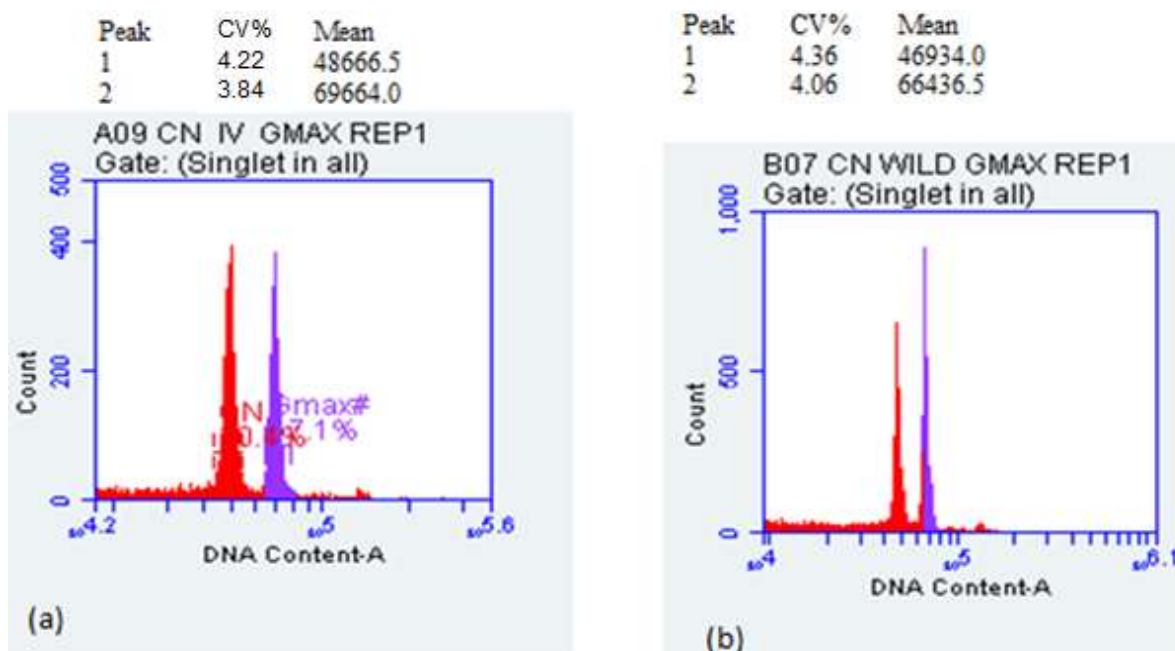
### 2C Nuclear DNA content analysis in *C. nutans*

Genome estimation for *C. nutans* along with *G. max* cv. Polanka as its internal reference was carried out using Tris.MgCl<sub>2</sub> with 1% PVP. The mean fluorescence intensity peak for *G. max* (as external) and *C. nutans* presented in Figure 3, shows distinct and well separated

2C peak of *G. max* and 2C peak of *C. nutans*. The coefficient of variation (CV) for the mean fluorescence intensity varied between 3.5 to 4.5%. The 2C nuclear DNA content of *C. nutans* was estimated by comparing DNA content of the standard (*G. max*), to be  $2C = 1.75 \pm 0.005$  pg. For the 2C-value obtained in this study, the CV value (standard deviation divided by average number of channels) is crucial. The CV values being above 5% indicates that the extracted nuclei are not concentrated. This leads to larger deviation in the 2C DNA value which will not be precise. Therefore, CV value of  $\leq 5\%$  is taken as reliable factor for genome size estimation (Cao et al., 2014).

### Genome size comparison between *in vitro* and field grown *C. nutans*

Figure 4 shows the comparison of mean fluorescence intensity between the wild type and *in vitro* grown *C. nutans*, as well as the DNA values (2C) of *C. nutans* along with 1C calculated (bp) using Tris.MgCl<sub>2</sub> buffer. The



**Figure 4.** Histogram of relative fluorescence intensity obtained after analysis of (a) *C. nutans in vitro* and (b) *C. nutans* wild type nuclei isolated simultaneously with *G. max* cv Polanka (2C=2.5pg) as an internal standard. The peak number 1(*C. nutans*); peak no.2 (*G. max*), mean fluorescence intensity and coefficient of variation percentage (CV) is stated.

**Table 2.** Nuclear DNA content and genome size (mean + SD) of *C. nutans* estimated with flow cytometry using *G. max*.

Plant material	n(R)	2C Nuclear DNA content mean $\pm$ SD (pg)	1C Genome size $\pm$ SD ( $10^9$ bp)	CV%
Wild Type	4(3)	1.7455 $\pm$ 0.009574 <sup>a</sup>	0.8535 $\pm$ 0.004 <sup>a</sup>	4.4
<i>In vitro</i>	2(3)	1.7495 $\pm$ 0.002121 <sup>a</sup>	0.8555 $\pm$ 0.001a	3.8
All plants	6(3)	1.7475 $\pm$ 0.005848	0.8736 $\pm$ 0.003a	

Mean values followed by same letter are not significantly different at  $p \leq 0.05$  (Tukey's test). *G. max* cv Polanka (2C=2.5 pg) as internal standard. Sample number (n), replicate measurements per sample (R) are as indicated. 1pg DNA=0.978  $\times 10^9$  bp according to Dolezel et al. (2003).

estimated nuclear DNA content of field grown *C. nutans* was compared to its *in vitro* plantlets using *G. max* cv Polanka as internal standard (Figure 3). It shows no significant difference in genome size, between the field grown and *in vitro* grown *C. nutans* chopped together with *G. max* (Table 2).

## DISCUSSION

Establishing the genome size and determining the best nuclear isolation protocol is among the preliminary, but crucial step in description of a plant species, and constructing pathways for genome sequencing (Gregory, 2005). DNA analysis using FCM involves preparation of

intact nuclei suspension which is stained using PI fluorochromes prior to analysis. It is crucial for the suspension buffer to protect the nuclear DNA from degradation and to provide a suitable environment for specific stoichiometric staining of the nuclear DNA including reduction in the negative effect of cytosolic compound present in plants during DNA staining (Loureiro et al., 2007). Although there are several nuclear isolation buffers available, published data shows that no one buffer works for all plant species (Bainard et al., 2010). Similarly, prominent yield of nuclei was seen for *C. nutans* isolated using Tris.MgCl<sub>2</sub> buffer with 1% PVP compared to OTTO and LBO1 buffer. Tris being an organic compound helps in stabilizing the pH of the solution, thus keeping the nuclei intact (Greilhuber et al.,

2007). Besides the presences of Triton X -100 further helps to ease the nuclei release from the cells and isolates them away from debris (Galbraith et al., 1983). The addition of polyvinylpyrrolidone (PVP) that is known to bind to phenolics and prevents interaction with DNA (O'Brien et al., 1996) has been crucial. The absences of PVP in both OTTO and LBO1 buffer could be a factor for high CV or low yield (Griehuber et al., 2007). PVP have been reported to eliminate the intervention of inhibitory compounds in many studies (Veselska et al., 2014; Nath et al., 2014; Sadhu et al., 2016). However, the performance of the Tris.MgCl<sub>2</sub> with 1% PVP did not exhibit good isolation of nuclei cells in *Garcini mangostana* (Midin et al., 2017) and *Drimia indica* (Nath et al., 2014). In certain plant species, the nuclei released from the plant cells adheres to the precipitate that is formed from citric acid in Otto I buffer as it forms mucous substance (Dolezel et al., 2005) thus giving a high background debris as seen in the histogram plot of this study. But Otto buffer was successfully used as isolation buffer in other plant species such as *Erianthus arundinaceus* (Yan et al., 2016), *Cucurma* species (Skornickova, et al. 2007) and *Festuca pallens* (Smarada and Bures, 2006). The presence of beta-mercaptoethanol in LBO1 reduced the CV value but did not give a high yield for the nuclei of *in vitro* *C. nutans*. But this buffer is reported applicable in evaluation of genome size in other plant species such as *Rosa macrophylla* (Idrees et al., 2020) and *Primula genus* (Wang et al., 2015).

Based on the FCM analysis, the difference between the mean fluorescence intensity of *G. max* prepared separately and those prepared together with *C. nutans* was not significant (Figure 2). This suggests that the addition of 1% PVP as antioxidant in the Tris.MgCl<sub>2</sub> buffer (Dolezel et al., 1989) was able to minimize the cytosolic effect of *C. nutans* cell debris or secondary compounds in the PI staining of *G. max*. Thus, giving consistent results of nuclear DNA content of *C. nutans* against *G. max* as internal standard. Based on the mean fluorescence values, the nuclear DNA content of *C. nutans* was approximately 0.7 times of *G. max*. Therefore, it's estimated that the nuclear DNA content of *C. nutans* to be 1.75 pg /2C and the 1C value to be 0.835 ×10<sup>9</sup> bp. With this known genome size of *C. nutans*, evolutionary pattern of other species within *Clinacanthus* genus can be explored in the future besides being a basic guidance in polyploidization work.

In this study, micropropagation of *C. nutans* was carried out using nodal segments which facilitates lower risk of gene instability (Kesari et al., 2012). FCM analysis of 2C DNA carried out on *in vitro* plantlets of *C. nutans* showed a similar pattern compared to the field grown *C. nutans* indicating genome stability after continuous subculture over a period of two years. This suggests that the genome of the *C. nutans* plantlets is stable after prolonged subcultures. Similarly, genome stability was recorded in six medicinal plants that were propagated *in*

*vitro* by Sliwinska and Thiem (2007). Stable genome sizes were also observed in *Hydrasis canadensis* L. (Obae et al., 2010) and *Pongamia pinnata* L. (Choudury et al., 2013) too. Thus, this paves the way for mass propagating *C. nutans* via tissue culture, to meet commercial-scale cultivation demands, while preserving the threatened wild population of this high value medicinal plant.

The recent release of C values in the plant data base has a compilation of more than 12,000 plant species with Angiosperm covering more than 10,000 species (Leitch et al., 2019). The Kew-C value database reports the smallest angiosperm genome size belongs to *Fragaria viridis*. Duch with 1C value at 0.1 pg and the largest (95 pg) belongs to *Trillium apetalon* using the same method of estimation, i.e. flow cytometer. Based on the data, *C. nutans* genome size falls at the lower end of the C-value distribution under the family of Acanthaceae, which ranges from 0.4 to 2.91 pg/1C. From this experiment, *C. nutans* genome size can be estimated to be approximately two times larger than *Hygrophilla violacea* with 1C DNA value at 0.40 pg and 0.8 times smaller than *Acanthus mollis*, which has 1C DNA value at 0.97 pg (Leitch et al., 2019). Genome size is directly associated to cell size and cell size is significantly related to cell division (Yuan et al., 2021). From the genome size comparison, cell size of *C. nutans* is larger than *H. violacea* and smaller than *A. molis*. With the variation found in genome size, correlation between the genome size and other phenotypical traits can be investigated to further understand the evolution drift, if any exists among a family (Yuan et al., 2021).

## Conclusion

2C nuclear DNA content of *C. nutans* is estimated at 1.75 pg using flow cytometry analysis. This report is the first to estimate the nuclear DNA content of *C. nutans* and therefore provides important information required to assist further genomic and molecular studies of this economically important medicinal plant.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Glycine max* cv. Polanka as the reference plant for this experiment. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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