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SHORT COMMUNICATION

Flow Cytometric Based Estimation of Genome Size in Arecanut (*Areca catechu* L.) Varieties

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Abstract

The present work was undertaken with the objective to estimate the nuclear DNA content in arecanut varieties using flow cytometry. Released varieties of arecanut maintained in the field gene bank at ICAR-Central Plantation Crops Research Institute (ICAR-CPCRI), Regional Station, Vittal, Karnataka, were used for the estimation of DNA content by using BD AccuriC6 flow cytometer. Nuclei isolated from leaves of *Pisum sativum* cv. Citrad of known genome size was used as an external reference standard. Significant differences were observed between the arecanut varieties for 2C DNA content. Among the arecanut varieties, 2C DNA content (genome size) was found to be ranging from 6.025 (2.946 Gb/1C) to 6.710 pg (3.281 Gb/1C), with mean value of 6.472 pg (3.164 Gb/1C). The highest DNA content was recorded in the variety Swarnamangala (6.710 pg/2C or 3.281 Gb/1C), followed by Sumangala (6.695 pg/2C or 3.273 Gb/1C) and Shatamangala (6.623 pg/2C or 3.238 Gb/1C), while the lowest DNA content of 6.025 pg/2C or 2.946 Gb/1C was recorded in the variety Sreemangala. The results of this study indicate the potential of using flow cytometry for studying genome size diversity in arecanut.

Keywords: Flow cytometry, *Areca catechu*, Varieties, DNA content, Genome size.

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Introduction

The areca palm which is highly cross-pollinated is an allotetraploid with chromosome number 2n = 32 (Venkatasubban, 1945). The palm is an unbranched, erect, monoecious tree growing in hot and humid tropical region of the world and its centre of origin is considered to be South East Asia (Bavappa *et al*., 1982). It is the lone commercially cultivated crop majorly used for masticatory purposes and also in socio-religious purposes since thousands of years (Balasimha and Rajagopal, 2004). The arecanut chewed along with betel leaf and slaked lime for their effect as a mild stimulant (Rao, 1982), increases stamina, and alertness, kills worms, removes/subdues bad odor, beautifies the mouth, induces purification and causes mild sensation in the body and stimulates flow of saliva to aid digestion. The nuts contain 8 to 12% fat which can be mixed with cocoa fat for confectionery products and also substituted for vanaspati in preparations of sweets and biscuits (Murthy, 1957).

Extensive research works in arecanut was carried out on morphological and biochemical characterization in order to make use of these characters in crop improvement programmes. However, the genetic diversity information provided by morphological characters is limited and environmental and physiological factors can influence these parameters. Therefore, researchers are using molecular markers. In arecanut, molecular markers like random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs) etc. have been used to study the genetic relationships, diversity and geographical correlation among different arecanut germplasm,

varieties, wild species and related genera. However, no work on genome size diversity has been undertaken in arecanut.

Flow cytometry studies have been undertaken in different crop species in different parts of the world. There are very few reports are available on such studies in the family Arecaceae, while reports of such works are not available in arecanut as per our knowledge. Therefore, the present work was undertaken with the objective to estimate the DNA content or genome size in arecanut varieties using flow cytometry.

To estimate 2C DNA content, nine released arecanut varieties (five varieties developed by evaluation of indigenous germplasm and four varieties developed by evaluating exotic accessions), maintained in the field gene bank at ICAR-Central Plantation Crops Research Institute, Regional Station, Vittal, Karnataka (ICAR-CPCRI) (Table 1) were used. Spindle leaves collected from three palms in each variety were used to isolate the intact nuclei. The leaves from *Pisum sativum* cv. Citrad of known genome size (2n = 9.09 pg) was used as a reference standard (Dolezel and Bartos, 2005).

Estimation of nuclear DNA content was done by using BD Accuri C6 flow cytometer available at Division Crop Improvement, ICAR-CPCRI, Kasaragod, Kerala. Galbraith *et al.* (1983) explained procedure used for the flow cytometry analysis. To isolate intact nuclei, 100 mg of leaf tissue of individual palms from each arecanut variety and 50 mg of pea leaves (external reference standard) were chopped separately with a sharp razor blade for 90 seconds in a petri dish containing 1.5 mL of cold nuclear isolation buffer supplemented with β-mercaptoethanol and 1% PVP (in order to reduce the effect of phenolic compounds). After chopping, the buffer containing cell constituents and large tissue remnants was passed sequentially through nylon filters with mesh size of 50 µm followed by 20 µm to separate nuclei from the cell debris. To the buffer with nuclei, 2.5 µL of 10 mg/mL of DNAse free RNAse A and flurochrome propidium iodide were added to a final concentration of 50 µg/mL. Then the samples were incubated at 4°C under dark condition for 15 minutes before flow cytometric analysis. During the preparation of reference standard (pea) and arecanut samples same experimental conditions are maintained to avoid the error since pea is used as an external reference standard. External standardization was followed since internal standardization yielded almost overlapping histogram peaks and higher coefficient of variation (CV).

Before estimating the nuclear DNA content in arecanut varieties, two nuclei isolation buffers such as Galbraith's buffer (Galbraith *et al.*, 1983) and LB01 buffer (Dolezel *et al.*, 1989) were screened in order to isolate quality nuclear suspension for flow cytometry analysis.

The samples were analyzed on a BD AccuriC6 flow cytometer with an excitation wavelength of 488 nm. In each variety, three samples with three replications were analyzed with low speed and counted up to 1500 nuclei per sample using C6 flow plus software. Gunn *et al.* (2015) and Ranjini *et al*. (2020) counted up to 500 and 1500 nuclei per sample, respectively. Based on these references in the present experiment up to 1500 nuclei are counted for each sample. Reference standard (pea) was analyzed each time before analyzing every arecanut sample. Further, mean fluorescent units for both reference standard (pea) and arecanut were recorded based on the position of the G0/G1 peak (non-replicated phase of the cell cycle) and 2C nuclear DNA content was calculated as per the Dolezel *et al.* (2003) and expressed in pg/2C.

$$\text{2C value of the arecanut sample} = \frac{\text{G0/G1 peak value of the arecanut}}{\text{G0/G1 peak value of the reference standard}} \times \underset{(\text{2C=9.09 pg})}{\text{Genome size of the reference standard}}$$

The 2C DNA content was converted to mega base-pairs (Mbp) by a conversion factor of 1 pg = 978 Mbp (Dolezel *et al.*, 2003). The values were statistically tested and a post hoc test like Duncan's multiple range test (DMRT) was done by employing IBM SPSS Statistics version 20 Software.

In tropical perennial tree species like arecanut, such studies are rare, hence this study was carried out to determine genome size in this palm using a flow cytometric procedure in arecanut varieties, comprising of five varieties developed and released by evaluating indigenous germplasm and four varieties developed and released by evaluating exotic accessions, maintained in the field gene bank at ICAR-CPCRI, Regional Station, Vittal, Karnataka.

Among the two nuclei isolation buffers tested for obtaining quality nuclear suspension *viz*., Galbraith's buffer (Galbraith *et al*., 1983) and LB01 buffer (Dolezel *et al*., 1989), arecanut samples prepared using LB01 buffer gave better results in terms of sufficient nuclei yield, lesser background noise and lowest coefficient of variation of histogram of G0/G1 peak (2.5%) as compared to the samples prepared in the Galbraith buffer. Galbraith *et al.,* (1983) opinioned that in most cases histograms with peak CVs below 3% is considered fully acceptable. Hence for the estimation of 2C DNA content in arecanut varieties, intact nuclei isolated from the LB01 buffer was used (Figure 1). CVs of histogram of G0/G1 peak in all the arecanut varieties were maintained below 2.5%.

Among all the nine arecanut varieties studied in the present investigation, the 2C DNA content (genome size) varied from 6.025 pg (2.946 Gb/1C) to 6.710 pg (3.281 Gb/1C). Mean DNA content was observed to be 6.472 pg/2C (3.164 Gb/1C) (Table 1). The analysis of variance (ANOVA) indicated significant differences among arecanut varieties for nuclear DNA content (Table 2). The highest DNA content (genome size) was observed in the variety Swarnamangala, 6.710 pg/2C (3.281 Gb/1C), followed by Sumangala 6.695 pg/2C (3.273 Gb/1C), Shatamangala (6.623 pg/2C or 3.238 Gb/1C) and the lowest DNA content (genome size) of 6.025 pg/2C (2.946 Gb/1C) was recorded from the variety Sreemangala.

Table 1: Arecanut varieties used in the study and DNA content of arecanut varieties estimated by flow cytometry

Varieties	*Source of germplasm*	*DNA content (pg/2C)*	*Genome size (Mbp/haploid set)*
Mohitnagar	India (IC 557422)	6.557 ± 0.019^{bc}	3206.536
Kahikuchi	India (VTL-64)	6.340 ± 0.045^{e}	3100.423
Madhuramangala	India (IC 593737)	6.491 ± 0.007^{cd}	3173.936
Nalbari	India (IC 593736)	6.363 ± 0.015^{e}	3111.670
Shatamangala	India (IC 557397)	6.623 ± 0.036^{b}	3238.810
Mangala	China (IC 557417)	6.439 ± 0.006^{d}	3148.671
Sumangala	Indonesia (IC 557418)	6.695 ± 0.043^{a}	3273.692
Sreemangala	Singapore (IC 557420)	6.025 ± 0.072^{f}	2946.225
Swarnamangala	Vietnam (IC 557419)	6.710 ± 0.002^{a}	3281.190
Mean		6.472 ± 0.215	3164.573

Table 2: ANOVA of DNA content (pg/2C) in arecanut varieties

Source	*Degrees of freedom*	*Sum of squares*	*Mean sum of squares*	*F*	*Significance level (p-value: 0.05)*
Between Groups	8	1.100	0.138	89.849	0.000
Within Groups	18	0.028	0.002		
Total	26	1.128			

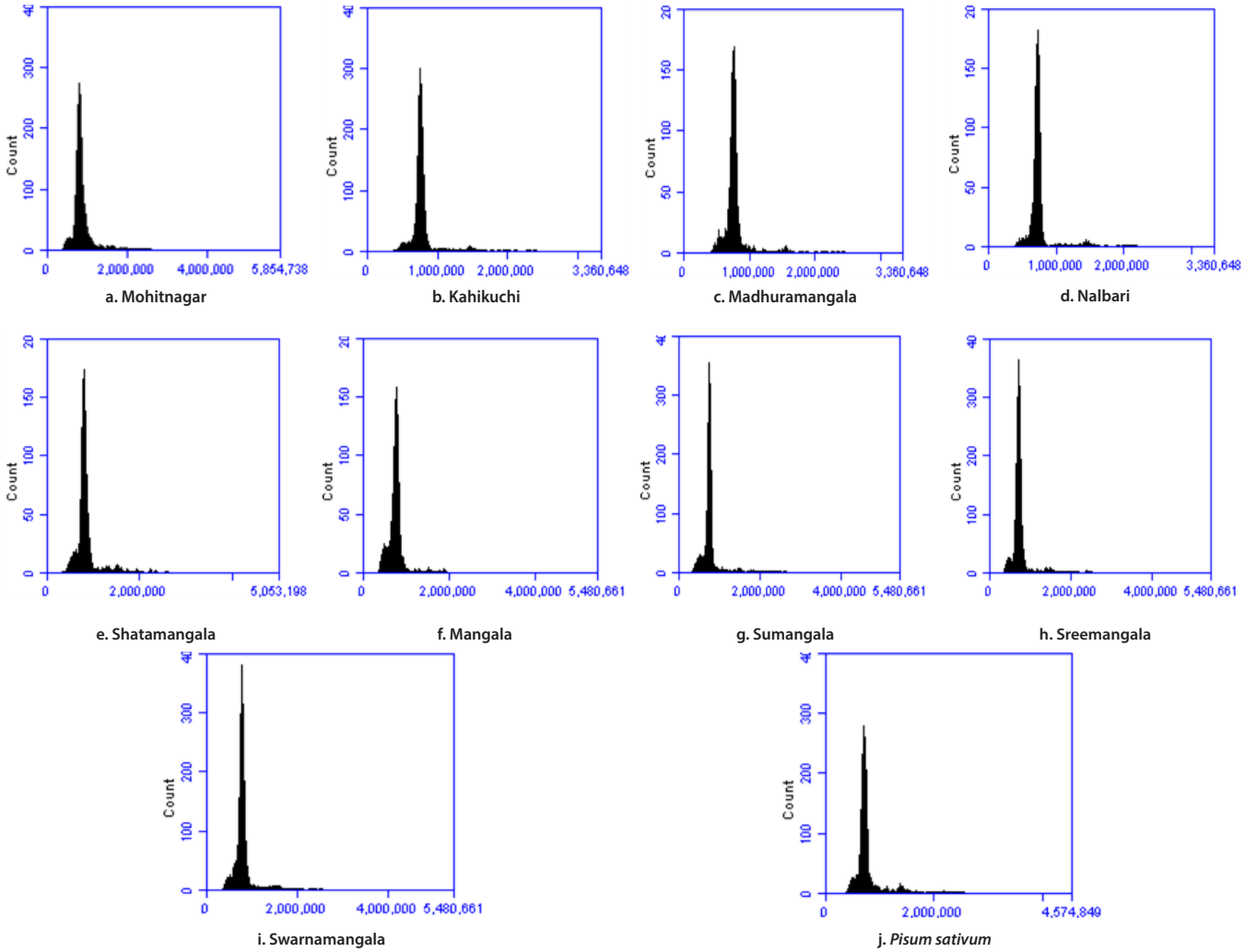

Figure 1: Flow cytometry histogram of arecanut varieties and reference standard.

DMRT post hoc test also indicated significant differences among arecanut varieties for 2C DNA content. The DNA content of the Sreemangala variety is significantly different from that of all other remaining varieties. DNA content of Swarnamangala and Sumangala is found to be significantly different from all other arecanut varieties. Similarly, 2C values of Shatamangala and Mohitnagar, Mohitnagar and Madhuramangala, Madhuramangala and Mangala and Nalbari and Kahikuchi were significantly different from remaining varieties (Table 1).

The intraspecific variation observed for nuclear DNA content in arecanut corresponds to intraspecific variation reported for other palm species. Genome size of 23 coconut populations covering most of the genetic diversity of the genus *Cocos nucifera* was analyzed by flow cytometer and reported the presence of intraspecific variation for nuclear DNA content in coconut with mean nuclear DNA content of 5.966 ± 0.111 pg/2C (Gunn *et al.,* 2015). Further, similar observations were made in coconut by Neto *et al*. (2016). In oil palm, Madon *et al*. (2008) estimated the genome size of *Elaeis sp.* and its intra-specific and inter-specific hybrids using *Glycine max* as external standard. The average 2C DNA content was found to be 3.86 ± 0.26 pg. They found that there was a great variation between intra and inter-specific hybrids of *Elaeis* in their nuclear DNA content. Similarly, Camillo *et al*. (2014) observed that African oil palm has a nuclear DNA content of 4.32 ± 0.173 pg and American oil palm a nuclear DNA content of 4.43 ± 0.018 pg, and concluded that the genome size of American oil palm is larger than that of African oil palm.

Among the varieties of arecanut developed from indigenous germplasm, 2C DNA content (genome size) ranged from 6.340 pg/2C (3.100 Gb/1C) to 6.623 pg/2C (3.238 Gb/1C) with a mean DNA content of 6.475 pg/2C (3.166 Gb/1C). The highest DNA content (genome size) was observed in the variety Shatamangala, i.e., 6.623 pg/2C (3.238 Gb/1C) followed by Mohitnagar 6.557 pg/2C (3.206 Gb/1C) and the lowest DNA content (genome size) was recorded from the variety Kahikuchi, 6.340 pg/2C (3.100 Gb/1C).

Among the arecanut varieties developed from exotic germplasm, the mean DNA content (genome size) was 6.467 pg/2C (3.162 Gb/1C). The DNA content was varied from 6.025 pg/2C (2.946 Gb/1C) to 6.710 pg/2C (3.281 Gb/1C). The highest DNA content was observed in the variety Swarnamangala, 6.710 pg/2C (3.281 Gb/1C), followed by Sumangala 6.695 pg/2C (3.273 Gb/1C) and the lowest DNA content was recorded from the variety Sreemangala, 6.025 pg/2C (2.946 Gb/1C).

As reports on estimation of genome size in arecanut using flow cytometry analysis are not available, this study appears to be the first report in arecanut. The results of this study indicate the potential of using flow cytometry for studying genome diversity in arecanut. The results from the present investigation can be applied for further analysis of DNA content/genome size in large number of arecanut varieties/germplasm, wild species, related genera, inter-specific hybrids and rare arecanut mutant/abnormal types to better understand the intra-specific, inter-specific variation in the crop and arrive at meaningful conclusion for better utilization in arecanut improvement programmes. The possibility of using this for varietal discrimination/ identification of true to type planting material also needs to be explored.

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