Original Research Article

Assessment of Genome Size Variation in Tall and Dwarf Coconut Accessions by Flow Cytometry

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A B S T R A C T

Nuclear DNA content and genome size variation in 20 phenotypically divergent coconut accessions including 14 tall and 6 dwarfs of different geographical origin were analyzed by flowcytometry. A flow cytometric protocol amenable to the crop was standardized by comparing four buffer compositions, three sample sizes and four incubation periods for isolated nuclei using *Pisium sativum* cv. Citrad (2C=9.09pg) as an internal reference standard. The study revealed significant differences for 2C DNA content among the accessions ranging from 5.73pg to 6.25pg, with 8.79% difference. The mean 2C DNA content of tall accessions has 6.07pg/2C and 5.89pg/2C in dwarfs, corresponding to 1C genome size of 2968.23 Mb and 2880.21Mb, respectively. Among the accessions, Guam II Tall (6.258 pg/2C) had the highest nuclear DNA content whereas Chowghat Orange Dwarf had the lowest (5.732pg/2C). The results of the present study suggest existence of intra specific variation for genome size in this species, independent of the place of origin of the distinct accessions.

Introduction

Coconut (*Cocos nucifera* L.) is a member of the monocotyledonous family Arecaceae (2n=2x=32), distributed throughout the humid tropics between 20ºN- 20ºS latitude (Beccari 1916). At present, coconut is cultivated in 93 countries in an extent of 12.19 million ha.

with an annual production of 69.83 million tons of nuts (CDBSTAT2014).Coconut is a monotypic species with no known wild relatives, in spite of this the crop presents considerable diversity in forms. This high level of intraspecific variability is the result of natural evolution and adaption as well as human intervention in the exploitation of the

species. The major classification of coconut populations are based on the plant habit and breeding behavior, i.e. tall and dwarf types. Tall palms are late to flower, normally cross pollinated and hence, highly heterozygous. In contrast, dwarf palms are early flowering, more homozygous due to high degree of self pollination (Narayana and John, 1949; Menon and Pandali, 1958).

Generally, both types are present across the tropical areas of Indian Ocean Islands, South and South East Asia, Pacific Ocean islands, Central and South America, Atlantic and African regions and show high variability for morphological characters and for other quantitative traits between the populations as well as within the population (Balakrishnan and Namboodiri, 1987; Ratnambal *et al.,* 2005; Arunachalam *et al.,* 2005; Niral *et al.,* 2007).

The nuclear DNA in plant cells is a source for most of the hereditary material and hence, always subjected to comprehensive studies. The total amount of nuclear DNA in the unreplicated reduced nucleus regardless of ploidy of the taxon is referred as genome size and it is expressed as C-value in picograms (1 pg DNA= 978bp) (Dolezel *et al.,* 2003).

In plants the knowledge on genome size variation is important in many areas of research ranging from evolutionary studies to genome sequencing and gene cloning projects. In flowering plants, variation for 1C nuclear DNA content is nearly 2400- fold high ranging from 0.06 pg in *Genlisea margaretae* to 152.23 pg in the *Paris japonica* (Ohri 1996). Indeed, variation in genome size has also been well documented between closely related species or within the species even in the absence of the alteration in chromosome number and structure (Greilhuber, 1998, Ohri 1996 and Knight *et al.,* 2005).

Subsequently, it was found that, such difference is predominantly associated with the changes in the repetitive or non coding DNA sequences and found to have influence on many plant morphological and physiological traits (Lavergne *et al.,* 2010). Correspondingly, over the past decade a number of studies correlated variation in economically important traits such as flower size (Meagher *et al.,* 2005), seed mass (Aliyu *et al.,* 2014) and growth rate (Jian *et al.,* 2017) with the relative variation in their DNA content and inferred that selection of breeding materials can be aided by selecting germplasms based on variation in genome size.

Moreover, several workers have also been reported within species correlation between the genome size and ecological variables such as altitude, latitude and temperature (Knight *et al.,* 2005).

With this context, present study is outlined at understanding whether the intraspecific variations observed at the morphological level in tall and dwarf coconuts are reflected by relative variations in their genome size and their possible correlation with the place of origin.

Materials and Methods

Twenty coconut accessions (Table 1) of which 14 tall and 6 dwarf accessions maintained in the coconut field gene bank at ICAR- CPCRI, Kasaragod, representing four different place of origin were included in the assessment of genome size variation.

Standardization of sample preparation protocol

Prior to the estimation of genome size in different coconut accessions, sample preparation protocol has been standardized to

isolate quality nuclear suspension for flow cytometry analysis.

Different buffer systems screened on coconut

Screened commonly used four buffer compositions on coconut, including Galbraith's buffer (Galbraith *et al.,* 1983), LB01 buffer (Dolezel *et al.,* 1989), Tris MgCl$_2$ buffer (Pfosser *et al.,* 1995) and Woody Plant buffer (Loureiro *et al.,* 2007).

Leaf sample size and incubation period for coconut and internal reference standard

Three sets of leaf sample sizes (50, 100 and 150mg) from the young leaf tissue of coconut were tested to determine the suitable quantity of leaf sample size required to release sufficient number of nuclei to the isolation buffer. Leaf sample of 50 mg was used for the internal reference sample as reported in the literature (Dolezel *et al.,* 1998). For the analysis, the leaves of *Pisium sativum* cv. Citrad of known genome size (2n=9.09pg) was used as an internal reference standard (Dolezel and Bartos, 2005). Prepared sample were incubated in four different incubation periods ranging from 0, 15, 30 and 60 minutes.

Sample preparation for flow cytometric analysis

Intact nuclei were isolated by chopping the tender leaf tissue of coconut and internal

reference standard using a razor blade, approximately for 90 seconds in a petri dish containing 1.5 ml cold isolation buffer supplemented with 1% PVP-10 in order to reduce the effect of phenolic compounds released from the coconut leaves. Filtered the nuclei suspension through a 50µ filter to remove larger leaf debris followed by filtration with 20µ nylon net filter to separate the intact nuclei. To the filtered lysate 2.5µL of 10mg/mL of DNAse free RNAse A and Propidium iodide to a final concentration of 50µg/mL was added and the mixture was then incubated at 4ºC under dark condition before the analysis.

Experimental design and data collection

The relative florescence intensities of PI-stained nuclei were measured with a BD accuri C6 flow cytometer at laser excitation 488 nm. The treatment factors (isolation buffer, sample size and incubation period) were applied to the leaves of Chowghat Orange Dwarf (COD) coconut accession and the analysis was repeated on three different days. Further, quality of nuclear suspension was determined based on the values of three variables such as coefficient of variation of G0/G1 peak (%) (a measure of nuclear integrity and variation in DNA staining), background factor (%) (a measure of a sample quality) and nuclear yield factor (nuclei sec^{-1}mg^{-1}) (a proportion of nuclei in suspension in respect to the sample size), calculated as follows (Aliyu, 2012).

$$\text{Background factor (BF)} = \frac{\text{Total number of particles - Total number of intact nuclei}}{\text{Total number of particles}} \times 100$$

$$\text{Yield factor (YF)} = \frac{\text{Total number of intact nuclei/ Number of run time (s)}}{\text{Leaf sample size (mg)}}$$

Performed Analysis of Variance (ANOVA) of three nuclei suspension variables using three factor analysis using SPSS Software (Table 2).

Sample preparation and data analysis for the estimation of 2C DNA content in coconut accessions

Intact nuclei were isolated from 20 coconut accessions following the developed sample preparation protocol. For each accession, three individual palms were selected and analysis was carried out in 2 replicated trials. A total of six samples per accession was measured at a low speed and counted about 1500 nuclei per sample using C6 flow plus software. Parameters for data acquisition were kept constant for all samples. The mean florescence units for both coconut and internal reference standard were recorded based on the positions of histogram G0/G1 peaks (non-replicated phase of the cell cycle) to calculate nuclear DNA content by Dolezel *et al.,* 2003.

2c value of the coconut sample = G0/G1 peak value of the coconut/ G0/G1 peak value of the internal reference standard x genome size of the reference standard (2C= 9.09 pg)

The genome size calculated was expressed in pg/2C and converted to mega base-pairs (Mbp) by a conversion factor of 1pg = 978 bp (Dolezel *et al.,* 2003). The values were then summed up and statistically tested using IBM SPSS version 2.0 software.

Results and Discussion

Standardization of sample preparation protocol for flowcytometry analysis of coconut

In the present study, four nuclei isolation buffer, three sample size and three incubation periods were compared in order to isolate quality nuclei suspension for the estimation of 2C DNA content in coconut by flow cytometry. The analysis of variance of the three nuclear suspension quality parameters i.e. coefficient of variation of G0/G1 peak (%), background factor (%) and nuclear yield factor (nuclei sec^{-1}mg^{-1}), showed significant variation in the performances of the 4 nuclei isolation buffers, 3 leaf sample size and 3 incubation period (Table 2). All combination interactions of these three factors such as buffer x sample size, buffer x incubation time, sample size x incubation period, buffer x sample size x incubation time were statistically significant. The CV values of the histogram peaks vary from 3.88 to 9.29%., background factor of the nuclear suspension was between 20.43 to 44.10%.Nuclear yield for the coconut leaf samples ranged from0.27 to 0.54 nuclei sec^{-1}mg^{-1}. Among the four buffers tested Galbraith's buffer was found comparatively efficient followed by LB 01 buffer. Leaf tissue of about 100 mg with incubation period for 15 min gave best quality in terms of sufficient nuclei yield (0.54 nuclei sec^{-1}mg^{-1}), lesser background noise (20.43%)and lowest coefficient of variation of histogram of G0/G1 peak (3.88%). During the sample preparation slight modification was done by adding 1% PVP-10 and 15µmβ-mercaptoethanol to the chopping buffers in order to reduce the effect of phenolic compounds released from the coconut leaves. This helped the analysis by increasing the sharpness of the peaks, nuclei yield and reduced CV values.

Estimation of 2C DNA content in coconut accessions

The 2C values of coconut accessions varied from 5.73pg to 6.25pg with about 8.79% difference between the smallest and largest values recorded in the accessions under study. Based on the mean DNA content, 1C genome

size of coconut accessions was estimated to be 5885.16 Mb. The analysis of variance indicated significant difference in DNA content among the coconut accessions as well as between the dwarfs and talls (Table 3). The accessions belong to Pacific Ocean Islands such as Guam II Tall and Fiji Longtongwan

Tall had the highest DNA content of 6.258 pg/2C or 3.06 GB/1C and 6.208pg/2C or 3.03 GB/1C respectively. Whereas, Chowghat Orange Dwarf native to India had the smallest (5.732pg/2C or 2.802 GB/1C) (Table.4) (Fig. 1).

Table.1 List of coconut accessions investigated in this study

Place of Origin	Accessions
South Asia	West Coast Tall; East Coast Tall; Andaman Ordinary Tall; Benaulim Tall; Sri Lanka Tall; Chowghat Orange Dwarf; Gudanjali Green Dwarf; Sri Lanka Yellow Dwarf
South East Asia	Java Tall; Philippines Ordinary Tall; Nu HimiKupien Tall; Malayan Orange Dwarf; Malayan Green Dwarf
Pacific Ocean Islands	Fiji Longtongwan Tall; Fiji Tall; Guam II Tall; New Guinea Orange Dwarf
Central & S. America & Africa	Jamaica Tall ; Nigerian Tall; Blanchisseuse Tall

Table.2 ANOVA for quality nuclear suspension variables of coconut for buffer composition, leaf tissue sample size and incubation time of isolated nuclei

Source	Degrees of freedom	Back ground factor (%)	Coefficient of variation (%)	Nuclear Yield factor (nuclei sec^{-1}mg^{-1})
Replication	2	5.7761NS	0.7372*	0.0006NS
Buffer	3	4351.4867*	264.7182*	0.4839*
Sample size	2	302.7204*	7.9942*	0.2198*
Incubation time	3	635.1279*	13.8540*	0.0739*
Buffer x Sample size	6	7.5412NS	0.5408*	0.0174*
Buffer x Incubation time	9	73.5734*	1.3224*	0.0109*
Sample size x incubation time	6	57.6787*	2.5381*	0.0035NS
Buffer x Sample size x incubation time	18	13.6539*	1.0026*	0.0046*
Error	94	5.5609	0.2264	0.0026

* - Significant at 5% ,NS - Non Significant, p-Value < 0.05 - Significant at 5%, p-Value < 0.01 - Significant at 1%

Table.3 ANOVA of estimated genome size (pg/2C) in coconut accessions

Source	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F Prob
Within tall accessions	13	0.513054	0.039466	11.85	6.5161
Within dwarf accessions	5	0.141221	0.028244	8.48	1.5374
Between Tall and Dwarf accessions	1	0.467004	0.467004	140.19	1.1927
Error	40	0.133252	0.003331		
Total	59	1.254531			

Table.4 Absolute DNA content of coconut accessions, estimated by flow cytometry

Accessions	International Abbreviation	Place of origin	Absolute DNA content(2C) in pg	Estimated genome size (Mbp/haploid set)
Talls				
West Coast Tall	WCT	India	6.051±0.039	2958.939
East Coast Tall	ECT	India	5.978±0.077	2923.242
Andaman Ordinary Tall	ADOT	India	6.053±0.070	2964.807
Benaulim Tall	BENT	India	6.130± 0.026	2997.57
Java Tall	JVT	Indonesia	5.872± 0.026	2871.408
Philippines Ordinary Tall	PHOT	Philippines	5.937± 0.065	2903.193
NuHimiKupien Tall	NHKT	New Caledonia	6.136± 0.078	3000.504
Fiji Longtongwan Tall	FJLT	Fiji	6.208± 0.069	3035.712
Fiji Tall	FJT	Fiji	5.924± 0.050	2896.836
Guam II Tall	GUBT- II	Guam	6.258± 0.033	3060.162
Jamaica Tall	JMT	Jamaica	6.108± 0.092	2986.812
Nigerian Tall	NIT	Nigeria	6.062± 0.045	2964.318
Blanchisseuse Tall	BLIT	Trinidad &Tabago	6.138± 0.025	3001.482
Sri Lanka Tall	SLT	Sri Lanka	6.198± 0.044	3030.822
Dwarfs				
Chowghat Orange Dwarf	COD	India	5.734± 0.018	2803.926
Gudanjali Green Dwarf	GDD	India	5.890± 0.029	2880.21
Malayan Orange dwarf	MOD	Malaysia	5.925± 0.031	2897.325
Malayan Green Dwarf	MGD	Malaysia	5.954± 0.033	2911.506
Sri Lanka Yellow Dwarf	CYD	Sri Lanka	5.991± 0.011	2929.599
New Guinea Orange Dwarf	NGOD	Papua New Guinea	5.804± 0.022	2838.156
CD (5%)			0.095	

Figure.1 Flow cytometry histogram of dwarf coconut accession, Chowghat Orange Dwarf. Peak a. represent the G0/G1 peak of coconut accession, peak b. represents the G0/G1 peak of internal reference standard pea
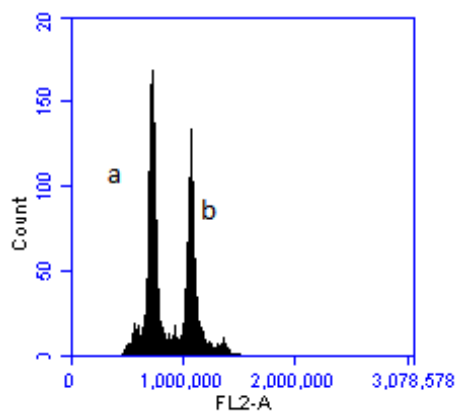
Coconut leaves is known to contain high cytosolic compounds particularly a high level of phenolics and tannins. Such compounds generally interfere with the staining of the isolated nuclei along with the fluorochrome thereby causes errors during the flowcytometry analysis by increasing histogram CV value and background noise. In

order to avoid these problems it is advised to modify isolation buffer by adding reducing agents such as PVP and β-mercaptoethanol which preserve chromatin proteins and counteract the interference of phenolic compounds with DNA staining ((Dolezzel and Barto 2005). Moreover it is also important to test various buffers to identify the best one which is suitable for the crop of our interest because there is no single isolation buffer which works well with all species (Aliyu 2012). Hence, in this investigation,4 nuclei isolation buffers (Galbraith's buffer, LB01 buffer, Tris MgCl$_2$ buffer and Woody Plant buffer) supplemented with the reducing agents (1% PVP-10 and15 mμ β-mercaptoethanol) were tested for the preparation of quality nuclei suspension from coconut leaves. Among the 4 buffers tested, Galbraith buffer with PI flurochrome has shown to be most appropriate lysis medium for coconut in order to estimate the relative nuclear DNA because it gave the G0/G1 histogram peak with lesser CV of 2.90% and higher nuclei yield of 0.54 (nuclei sec^{-1}mg$^{-1)}$ with least background noise (20.43%). Galbraith *et al.,* (1998) opinioned that in most cases histograms with peak CVs below 3% are considered fully acceptable. Lower quality of nuclear suspension was derived from Tris MgCl$_2$ buffer which shows the susceptibility of the buffer to confounding effect of secondary metabolites.

The improved quality of the nuclear suspension extracted using Galbraith buffer over the Tris MgCl$_2$ buffer may be due to the presence of MOPS in the buffer which has a pKa of 7.2 and a better buffering capacity than TRIS with a pKa value of 8.1. Excellent performance of the Galbraith buffer over Tris MgCl$_2$ buffer has also been reported in several plant species (*Sedum burrito, Oxalis pes-caprae, Lycopersicon esculentum, Celtis australis, Pisum sativum, Festuca rothmaleri and Vicia faba*) (Loureiro *et al.,* 2007).

In addition to the composition of the buffer, size of the leaf sample used for the sample preparation and incubation period of the suspension with flourochrome also influence the quality of the nuclear suspension (Aliyu 2012).In coconut we found that leaf sample size of 100 mg followed by incubation period of 15 minutes was ideal to get quality histogram with lesser background noise compared to higher sample size of 150 mg and incubation period for 60min.This observation had proved the opinion of earlier reporters, more the leaf sample more will be the release of cytosolic compounds to the isolation medium. Similarly, delay the flowcytometric analysis causes rapid degradation of intact nuclei in the suspension and poor quality measurements (Aliyu 2012). The flurochrome propidium iodide (PI) a DNA intercalator is used in the study which is known as best dye and produces histograms with lower variation. Moreover, for the sample preparation choice of proper internal reference standard of known DNA content is important since, it should not overlap with the histogram peaks of the samples under study. Therefore, *Pisium sativum* cv. Citrad with known genome size of 9.09 pg/2C was selected as an internal standard. Generally nuclear genome of the peas is seem to be stable and ranged from 8.84 to 9.39pg DNA, which is in the middle of the known range of genome size in plants (Dolezzel and Bartoss, 2005).

Based on the mean DNA content of all the coconut accessions analyzed 1C genome size was estimated to be 5885.16 Mb (2C value is 6.01 pg).The 2C values for 20 coconut accessions are ranged between 5.73 to 6.25pg.The first study on genome size of coconut, estimated using Feulgen densitometry reported mean 2C value of coconut as 7.10pg (Roser *et al.,* 1997). Subsequently, Gunn *et al.,* (2015) recorded mean 1C genome size as 5829.73 Mb based

on the flow cytometry analysis. Our result is almost concurrent with the Gunn *et al.,* (2015) results even though the authors used different internal reference standard [*Pisium sativum* (2C= 9.09pg) and *Petunia xhybrid* (2C=2.85pg)] as well as different flurochrome [Propidium iodide and DAPI (4,6- diamidino-2-phenylindole, dihydrochloride)] for the analysis.

The inter accession variation for DNA content was about 8.79% between the largest and smallest values reported. This variation might be the result of addition/deletion of the DNA sequences within the chromosomes. Such differences observed for genome size in coconut accessions correspond to intraspecific variation reported for many plant taxa including maize (Maria et.al. 2015); Sorghum (Laurie D.A. and Bennett M.D., 1985); Coffee (Noirot *et al.,* 2003); Cardamom (Anjali *et al.,* 2016); Tea (Suman *et al.,* 2019)etc.

Further, there is a significant difference for 2C DNA within the tall and dwarf coconut groups. For 14 tall accessions 2C values ranged between 5.87pg to 6.25pg, it is about 6.35% difference; in case of 6 dwarfs it ranges from 5.73pg/2C to 5.99pg/2C with only 3.91% difference. Even though, the values of DNA content in dwarf accessions studied is significantly different, the percentage variation is comparatively less and this indicating more homogeneous nature of the dwarf coconut forms than talls (Narayana and Jhon 1949). Eilam *et al.,* (2007) and Ozkan *et al.,* (2010) inferred that cross pollinated crops possess higher variation for nuclear DNA content compared to self pollinated crops. Even in present study, observed greater variation for 2C DNA content in allogamous tall coconut accessions than autogamous dwarf accessions.

Some of the researchers reported the presence of a clear geographical gradient in 2C DNA content within the distribution area of the plant species (Knight *et al.,* 2005, Smarda and Bures 2006, Lysak *et al.,* 2009). In Coconut, such distinct differences for nuclear DNA content based on the geographical distribution of accessions was not found, in contrast observed variation for DNA content in the accessions of same place of origin.

Recent advances in genomic research in plant species, successfully correlated the significant differences in genome size, with the economically important traits (Jian *et al.,* 2017) and inferred that in addition to impact of structural genes on quantitative traits, these minor changes in DNA sequences may also have influence on some QTLs in plants. Similarly, in the coconut accessions under study had variation for most of the phenotypic characters including vegetative as well as fruit component traits and which vary significantly for 2C DNA content. Hence, the data furnishing on genome size variation in coconut accessions helps to understand the evolutionary relationship, extend of intraspecific variation and it may pave the way for the crop improvement programme by means of association studies.

Acknowledgement

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