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APPLICATION OF FLOW CYTOMETRY FOR ESTIMATION OF NUCLEAR DNA CONTENT IN *Elaeis*

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ABSTRACT

*A method was established and proposed to be the standard protocol for estimating the genome size of oil palm [*Elaeis guineensis *and its intraspecific hybrids,* E. oleifera *and* oleifera *x* guineensis *(O x G) interspecific hybrids]. The method used is laser-sourced flow cytometry. Oil palm Frond -1 leaflets, LBO1 lysis buffer and* Glycine max *cv. Polanka (as external standard) were used for the DNA content estimation. The 2C DNA contents of* E. guineensis *for the different fruit types were:* dura *(D) = 4.10 ± 0.20,* pisifera *(P) = 3.64 ± 0.28 and* tenera *(DxP) = 3.83 ± 0.31 pg. A simple average gave the DNA content for* E. guineensis *as 3.86 ± 0.26 pg.*

Keywords: Elaeis guineensis, Elaeis oleifera, intra- and interspecific hybrids, DNA content, flow cytometry.

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INTRODUCTION

Elaeis guineensis and *E. oleifera* (subfamily Cocoineae) both belong to the family Palmae that contains over 225 genera and 2600 species. Madon *et al*. (1998) reported the chromosome numbers of *E. guineensis* and *E. oleifera* to be 2n = 32, and found that in both species the chromosomes can be divided into three groups on the basis of length: Group I of only Chromosome 1 (the longest), Group II of Chromosomes 2-9 (medium length) and Group III of Chromosomes 10-16 (medium/short).

E. guineensis exhibits polymorphism in its fruits with three recognized fruit types - *dura*, *pisifera* and *tenera*. The thick-shelled *dura* (D), homozygous for the major gene (Sh Sh) determining the presence of shell, is characterized by large fruits with large kernels (endosperm) and thick shells with only a thin layer of oil-bearing mesocarp. The shell-less *pisifera* (P) is homozygous recessive for the shell gene (sh sh) and has smaller fruits with no shell, only a distinct fibre ring around a very much reduced kernel. In the commercial production of oil palm planting materials, *dura* (D) is used as the maternal parent and *pisifera* (P) the paternal parent, the cross producing the hybrid, *tenera* (DxP). The *tenera* (T) is heterozygous for the shell gene (Sh sh) and therefore has a thinner shell, in smaller fruits with smaller kernels but with a thicker mesocarp than *dura*. The *tenera* fruit also has a distinct fibre ring around its shell (Beirnaert and Vanderweyen, 1941). Work on TxT crosses by the above authors led to the discovery of the genetic control of the oil palm fruit type, which is based merely on a single gene for shell thickness. By switching from planting *dura* to *tenera* (DxP), the oil yield was increased dramatically by 30%.

Another species of oil palm is *Elaeis oleifera* (HBK) Cortes, with slower height growth and lower oil yield than *E. guineensis*, but which oil is less saturated and richer in carotene (Rajanaidu, 1983; Tan *et al*., 1995; Mohd Din, 2000). Interest in *E. oleifera* has increased with discovery of its resistance to Lethal Bud Rot in Colombia (Hartley, 1988). Crossing *E. oleifera* with *E. guineensis* (OxG hybrid) improves the oil yield of the former, but it nevertheless remains inferior to that of *E. guineensis* (*tenera*, DxP) (Hardon, 1969; Hardon and Tan, 1969; Sharma and Tan, 1989).

The knowledge of DNA content in absolute units (genome size) is important in many areas of research ranging from evolutionary studies to genome mapping (Galbraith *et al*., 1997). The genomic DNA

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content of *E. guineensis* (*tenera*) as reported by Rival *et al*. (1997) using flow cytometry was 2C=3.786 ± 0.125 pg. However, Jones *et al*. (1982) reported 2C=2.4 ± 0.4 pg using the microdensitometric method on root tips of oil palm regenerants. Bennett and Smith (1991) and Röser *et al*. (1997) reported the genome size for *E. guineensis* as 4C = 4.00 and 4.8 pg, respectively, using also the microdensitometric method. However, there is no value reported for *E. oleifera*.

Flow cytometry (FCM) is a rapid and convenient technique that allows accurate determination of the nuclear DNA content, or genome size, in plants (Dolezel, 1991). The analysis is based on the use of propidium iodide (DNA intercalating fluorochrome) and analysis of the relative fluorescence intensity emitted by the stained nuclei (Dolezel *et al*., 1994). To determine the nuclear DNA content in absolute units, the fluorescence intensity of nuclei with unknown DNA content is compared with the fluorescence intensity of nuclei isolated from a species with known nuclear genome size (Dolezel *et al*., 1994). The objective of this study is to establish a standard protocol to estimate the genome size and to observe any variation in the intra- and interspecific hybrids of the oil palm by using a laser-sourced flow cytometer with *Glycine max* cv. Polanka (2C=2.5 pg) (soyabean) as the external standard, as well as to identify the most suitable oil palm frond and lysis buffer to be used.

MATERIALS AND METHODS

Plant Material

The intraspecific hybrids used were *tenera* (DxP) from crossing Deli *dura* x AVROS *pisifera* and Nigerian *dura* x AVROS *pisifera*. Deli indicates the provenance of oil palm material, Nigerian *dura* was prospected by MPOB while AVROS was the company that imported the original consignment of oil palm seeds into Sumatra and, after a few crossing programmes, produced the *pisifera* palms. For the interspecific hybrids, 7 F_1 OxG hybrids were selected, each for slow height increment and high oil yield, from *E. oleifera* (Suriname) x AVROS *pisifera*. '*Suriname*' indicates the origin of oil palm material prospected by MPOB.

Leaf samples (about 1 cm x 1 cm square) were taken from above 10-year-old palms for the estimation of DNA content - from the base, middle and end regions of three leaflets from the base, middle and end regions of Fronds –1 and 0. Frond +1 is the youngest fully opened frond with the leaflets fully detached from each other, so Fronds 0 and -1 are the next two successively younger fronds, *i.e.* not yet fully opened, or spear leaves.

Nuclei Preparation

All the samples from each leaflet were bulked and sliced into small pieces (1 mm x 1 mm) using a sharp clean blade, then placed in a 50 μm Medicon blender container (Becton Dickinson, USA) containing 1 ml LBO1 lysis buffer (made up of 15 mM Tris, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 0.1% Triton X-100, pH 7.5) (Dolezel *et al*., 1989) supplemented with 25 or 50 μg propidium iodide (PI) and RNaseA depending on the conditions listed in *Table 1* and blended using a Medimachine (Becton Dickinson, USA) for 5 min. The suspension nuclei were then collected from the Medicon container using a 1 ml syringe, filtered through a 70 μm filcon (Becton Dickinson, USA) into a 10 ml falcon tube followed by FCM analysis or further incubation at 4°C for 12/24/48 hr for method development. Incubation at 48 hr was observed to yield the best histogram peak. *Table 1* summarizes the different conditions used in the analysis in an attempt to obtain high resolution histogram peaks with 1%-3% coefficients of variation (CV). The external standard used was *Glycine max* cultivar Polanka (2C = 2.5 pg, kindly provided by Dolezel, J from the Czech Republic) with the suspension nuclei prepared shortly before the FCM analysis.

TABLE 1. INCUBATION CONDITIONS USED FOR EXTRACTION OF OIL PALM NUCLEI BY FLOW CYTOMETRY ANALYSIS

No.	Incubation [time (hr) at 4°C]	Conc. of RNaseA used (μg ml^{-1})	Conc. of used propidium iodide (μg ml^{-1})
1.	0	25	25
2.	0	50	50
3.	12	25	25
4.	12	50	50
5.	24	25	25
6.	24	50	50
7.	48	25	25
8.	48	50	50

Determination of Nuclear DNA Amount

A FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with an argon ion laser (15 mW) at 488 nm was used to analyse the fluorescent intensity of the nuclei. Histograms were collected over 1024 channels, and for each sample, 25000 events were captured. The peak means from the oil palm and soyabean histograms were obtained by using the CellQuest software. Using these values,

the 2C DNA content was calculated by the ratio of oil palm to soyabean fluorescent intensity multiplied by 2.5 pg, the genome size of soyabean. A one-way ANOVA (Minitab ver 13.1) and Tukey's test were performed to detect any significant differences between fronds and palm samples.

RESULTS

Leaf Samples for Nuclei Preparation

To prepare the nuclei from *E. guineensis*, the lamina of Frond -1 was found to be more suitable, being softer, less green and easier to process for nuclei suspension than that from Frond 0 which was more fibrous. However, for *E. oleifera* (Suriname) even the lamina from Frond -1 was already quite lignified but it was still possible to obtain the nuclei suspension.

Optimizing Nuclei Preparation

The nuclei suspensions from individual leaflets were processed under different conditions in an attempt to obtain highly resolved fluorescent histogram peaks with CV of 1% to 3% (*Figure 1*). The incubation conditions used are listed in *Table 1*. LBO1 lysis buffer supplemented with 50 mg PI and RNaseA followed by incubation for 48 hr at 4°C yielded the sharpest histogram peaks with 1%-3% CV. All the other conditions produced broader peaks with CV >3%. The sharp peaks produced by the LBO1 buffer might have been due to the efficiency of the PI in intercalating with the oil palm genome and to the majority of cells being in the same phase, *i.e.*, G1 and/or G2 phase of the cell cycle.

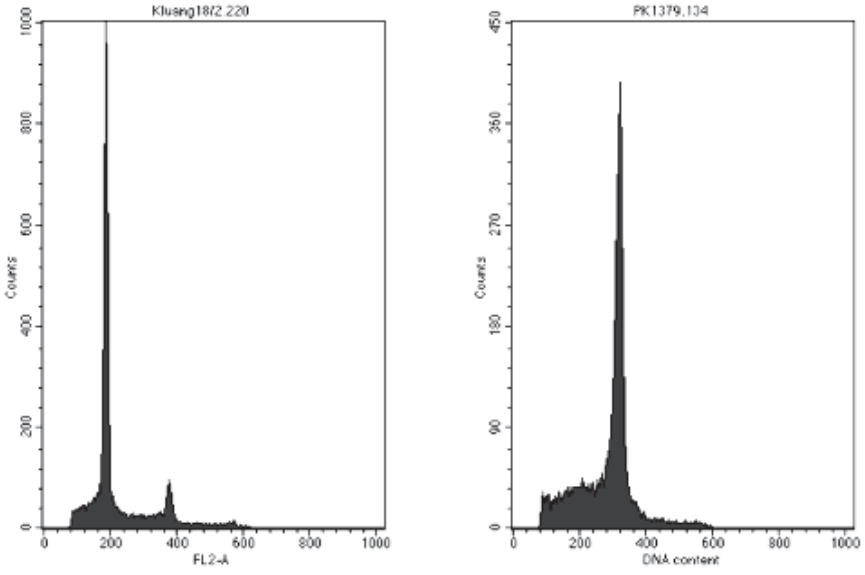
2C DNA Content

Tables 2 and *3* list the average 2C DNA contents in Frond –1 of Deli *dura* (DD), Nigerian *dura* (ND), AVROS *pisifera* (AP), *tenera* (DDxAP), *tenera* (NDxAP), *E. guineensis* (*dura*, *pisifera*, *tenera*), *E. guineensis, E. oleifera* (Suriname) and OxG hybrids (OxAP). The DNA contents in Frond –1 were generally higher and more consistent than those in Frond 0. For *E. guineensis* (*dura*, *pisifera* and *tenera*), the 2C DNA contents were 4.10 ± 0.20, 3.64 ± 0.28 and 3.83 ± 0.31 pg, respectively, giving an average 3.86 ± 0.26 pg through all the fruit types.

TABLE 2. THE 2C DNA CONTENTS IN FRONDS -1 AND 0 OF *E. guineensis* FOR DIFFERENT FRUIT TYPES - *Dura*, *Pisifera* AND *Tenera* (intraspecific hybrids)

Sample	Frond -1	Frond 0
Deli *dura* (DD)	4.12 ± 0.23[a]	3.57 ± 0.23 [a]
Nigerian *dura* (ND)	4.08 ± 0.22[a]	3.92 ± 0.09[a]
E. guineensis (*dura*)	4.10 ± 0.20 [a]	3.74 ± 0.25 [a]
AVROS *pisifera* (AP)	3.64 ± 0.28[b]	3.52 ± 0.34 [a]
E. guineensis (*pisifera*)	3.64 ± 0.28[b]	3.52 ± 0.34 [a]
DDxAP (*tenera*)	3.98 ± 0.31[ab]	3.62 ± 0.41 [a]
NDxAP (*tenera*)	3.67 ± 0.23[ab]	3.36 ± 0.29[a]
E. guineensis (*tenera*)	3.83 ± 0.31[ab]	3.49 ± 0.37 [a]
E. guineensis	3.86 ± 0.26 pg	3.58 ± 0.32 pg

Note: Figures in the same column with the same superscript are not significantly different while different superscripts indicate significant difference at P≤0.05 by Tukey's test.



a b

Figure 1. Examples of fluorescent intensity peaks for (a) standard Glycine max cv. *Polanka (2n) and (b)* E. guineensis *(2n).*

TABLE 3. THE 2C DNA CONTENTS IN FRONDS -1 AND 0 OF *Elaeis oleifera* (Suriname), *E. guineensis* (*Pisifera*) AND F_1OxG (interspecific hybrids)

Palm	Frond -1 2C (pg)	Frond 0 2C (pg)
E. oleifera (Suriname) - O	2.08 ± 0.04[a]	2.04 ± 0.03[a]
E. guineensis (*pisifera*) - G	3.64 ± 0.28[b]	3.52 ± 0.34[b]
F_1 OxG hybrids	4.16 ± 0.32[c]	4.19 ± 0.18[c]

Note: Figures in the same column with the same superscript are not significantly different while different superscripts indicate significant difference at P≤0.05 by Tukey's test.

DISCUSSION

Nuclei Preparation of Samples and Standard

Blending leaf tissues with lysis buffer released more nuclei than manual chopping for both oil palm and soyabean – as many as 25000 nuclei in fewer than 3 min. *Glycine max* cv. Polanka was chosen as the reference standard since its genome is about one-third less than that of the oil palm. Its leaves are also easy to process. However, internal standardization yielded almost overlapping histogram peaks with high CV. Hence, external standardization following Hendrix and Stewart (2005) was used instead.

Comparison of 2C DNA Contents

The 2C DNA contents for Deli *dura* and Nigerian *dura* (both from Frond -1) were 4.12 ± 0.23 and 4.08 ± 0.22 pg, respectively, with no significant difference between them; from Frond 0, the contents were lower at 3.57 ± 0.23 and 3.92 ± 0.09 pg, respectively, again without any significant difference between them. AVROS *pisifera*, a common male parent for producing *tenera* and OxG interspecific hybrids, had 2C DNA contents of 3.64 ± 0.28 and 3.52 ± 0.34 pg for Fronds -1 and 0, respectively. There was no significant difference between the DNA contents of *tenera* with either *dura* or *pisifera* parent with the values for Frond –1 being 2C=3.98 ± 0.31 pg (DDxAP) and 3.67 ± 0.23 pg (NDxAP), which were intermediate between both the parental values. However, there was a significant difference between the DNA contents of the parental *dura* and *pisifera* (Frond -1). By averaging the 2C DNA contents of *dura*, *pisifera* and *tenera*, the average obtained for *E. guineensis* was 3.86 ± 0.23 pg, close to the value obtained by Rival *et al*. (1997) of 3.786 ± 0.125 pg using laser-sourced flow cytometry with *Petunia hybrida* (2C=2.85 pg) as the reference standard. These values, however, differ from those of Jones *et al*. (1982), Bennett and Smith (1991) and Röser *et al*. (1997), possibly because of the different methods of estimation and standards used (*Table 4*).

For the interspecific hybrids, significant differences were observed between the progenies and parents from both Fronds -1 and 0. The female parent, *E. oleifera* (Suriname), had 2C=2.08 ± 0.04 and 2.04 ± 0.03 pg for Fronds –1 and 0, respectively, and crossing the AVROS *pisifera* mentioned previously (as male parent) with *oleifera*, the F_1 OxG hybrids had 2C=4.16 ± 0.32 (Frond -1) and 4.19 ± 0.18 pg (Frond 0), both higher than those of their parents. This may have been due to heterosis, or hybrid vigour (Snustad and Simmons, 2000), as OxG hybrids have more vigorous vegetative growth than their parental palms. The possible causes of the higher DNA content will be further discussed in the next section.

Intra- and Interspecific Variation

There was great variation in the estimated genome size with intra- and interspecific hybridization of *Elaeis*. At the intraspecific level, there was a significant difference between the *dura* and *pisifera* parents; hence, the genome size may be an indicator of the fruit phenotype. At the interspecific level, the F_1 OxG hybrids had significantly larger genomes than either of their parents by one-way ANOVA and Tukey's test. Hence, FCM on the DNA content may be useful in determining whether interspecific hybridization had occurred in the progenies derived from any cross.

Intraspecific variations in genome size have been observed in many species including *Medicago* (Blondon *et al*., 1984), *Coffea* (Barre *et al*., 1996), *Helianthus* (Michaelson *et al*., 1991; Natali *et al*., 1993), *Allium* (Bennett *et al*., 2000) and *Sorghum* (Laurie and

TABLE 4. THE 2C DNA CONTENTS IN DIFFERENT TISSUES OF *E. guineensis* AS REPORTED IN THE LITERATURE

Reference	2C DNA Content (pg)	Method	Standard	Sample
Jones *et al.* (1982)	2.4 ± 0.4	Microdensitometry	Internal: rat lymphocytes	Root tip
Bennett & Smith (1991)	2.00	Microdensitometry	-	Root tip
Rival *et al.* (1997)	3.786 ± 0.125	Flow cytometry	External: *Petunia hybrida*	Leaf
Röser *et al.* (1997)	2.40	Microdensitometry	-	Root tip
This analysis	3.86 ± 0.26	Flow cytometry	External: *Glycine max* cv. Polanka	Leaf

Bennett, 1985). These variations provide important information on the evolution of the genome. Changes in the DNA content intraspecifically can influence the phenotypic characteristics at the cellular and organismal levels (Caceres *et al*., 1998) and can be used as an important marker to indicate that hybridization had occurred (Ellul *et al*., 2002). Variations in the oil palm DNA content may also have been caused by endopolyploidy, endoreduplication, obstruction of DNA synthesis from 2C to 4C, aneuploidy or modulation of repetitive sequences as had been observed in *Helianthus annus* (Johnston *et al*., 1996). However, variations due to stoichiometric error have not been known to occur in oil palm (Noirot, M from CIRAD, France, per. comm.).

Like in other plants, the oil palm genome contains a large number of repetitive sequences (Castilho *et al*., 2000), including retroelements, which, with transposable elements, are known to be present in all genomes so far; they have the ability to change their genomic location and/or number of copies within the genome (Sabot *et al*., 2004). Transposable elements can increase the DNA quantity in the nucleus. They, however, may not be the only contributors to any increase in the genome size (Petrov, 2001; Sabot *et al*., 2004), which can also be due to the activation of retrotransposons (Bennetzen and Kellogg, 1997), while the genome can decrease by deletion (Bennetzen *et al*., 2005).

Proposed Standard Protocol for Oil Palm DNA Content Measurement

Various genome sizes have been reported for oil palm from the different methods of estimation and reference standards used. Therefore, it is felt that a single protocol, such as the one developed in this study, be used as the standard method in order to ensure consistent results between laboratories. It has been shown that FCM is rapid, convenient and more accurate as it measures more of the nuclei than microdensitometry and image cytometry (Dolezel and Bartos, 2005). As such, it has great potential as a tool in oil palm genomic studies.

CONCLUSION

We demonstrated that laser-sourced FCM with *Glycine max* cv. Polanka as the external standard can reliably estimate the genome size of oil palm, *E. guineensis*.

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