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ANALYSIS OF OIL PALM CLONES, THEIR SUSPENSION CALLI AND REGENERANTS VIA FLOW CYTOMETRY (FCM) AND rDNA-FLUORESCENCE *in situ* HYBRIDISATION (rDNA-FISH)

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ABSTRACT

Clonal propagation of palms with good traits is desirable for the oil palm industry. In this study, flow cytometry (FCM) and 18S-25S ribosomal DNA-fluorescence in situ *hybridisation (rDNA-FISH) were utilised to analyse genetic variation in adult clonal palms, their respective suspension cultures and regenerant plantlets. The genome sizes estimated by FCM for the four adult clonal palms (using leaf samples from Frond-1) varied from 2C=2.59±0.19 pg to 2.91±0.14 pg and for their respective regenerants, the genome size varied from 2C=2.14±0.21 pg to 3.05±0.11 pg. The genome size of oil palm suspension cultures could not be analysed by FCM due to the low nuclei population, which was less than 1000. The rDNA-FISH analysis showed two hybridisation signals on interphase nuclei of suspension culture calli and regenerant plantlets, hence indicating the diploid ploidy level. Adult clonal palms with their suspension culture calli and regenerants therefore showed a similar ploidy level. However, the measurement of genome size was found to vary between the adult clonal palms and their regenerants.*

Keywords: clonal propagation, suspension culture, flow cytometry, ribosomal DNA, fluorescence *in situ* hybridisation.

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INTRODUCTION

Oil palm (*Elaies guineensis*) is an important commodity crop in Malaysia and is used for both edible and non-edible purposes. This golden crop is native to Africa and was introduced to Malaysia in 1870 as an ornamental plant. Currently, *E. guineensis* (*tenera* fruit type) is the dominant economic crop in Malaysia. According to Purseglove (1975), oil palm belongs to the family Arecaceae that contains about 225 genera and 2600 species. Oil palm is a perennial monocot plant with a long regeneration period of life cycle estimated to be more than 100 years. Oil palm is a diploid arborescent monocotyledon plant that has 2n=32 chromosomes (Madon *et al.*, 1995). Madon *et al.* (1998) categorised the oil palm chromosomes into three different groups based on the length and size where Group I contains pair No. 1 (the longest), Group II pairs No. 2-9 (intermediate length) and Group III pairs No. 10-16 (short and medium).

The species *E. guineensis* has three fruit types which can be differentiated by their shell form. They are *dura*, large fruits with thick shell; *pisifera,* with shell-less fruits and *tenera,* with smaller fruits than *dura* but with thinner shell and thicker mesocarp. *Tenera* is the commercial planting material derived

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from the hybridisation of *dura* and *pisifera* (Kushairi *et al.*, 2011). The switch from *dura* to *tenera* was based on increased oil yields of about 30%.

Efforts to further increase the oil palm yield by cloning elite palms via tissue culture are currently being pursued. In oil palm tissue culture, young leaf explants are vegetatively propagated on medium supplemented with nutrient to induce the growth of callus and eventually tissue culture plantlets. For initiation of oil palm suspension cultures, friable calli (*Figure 1*) are inoculated into a flask containing liquid medium to produce suspension cultures for the continuous production of calli for plantlet regeneration (Tarmizi, 2002; Tarmizi *et al.*, 2004; 2012). Oil palm liquid culture system is an alternative to solid culture which produces low rate of regenerants and require more space and labour. In addition, growth rate of callus is higher in liquid medium compared to the solid medium (de Touchet, 1991). Etiene and Berthouly (2002) reported the uniformity of callus growth in liquid system due to the exposed callus surface which enhances nutrient absorption for growth.

The tissue culture process in oil palm however, can give rise to abnormal palms, first reported by Corley *et al.* (1986). Tissue culture can induce somaclonal variation in regenerated plants including variation in ploidy level (Larkin and Scowroft, 1981). However, the most important abnormality observed in clonal palms, *i.e.* mantled fruit, has been associated with change in methylation (Bairu *et al.*, 2011). Somaclonal variation can be classified as inherited (genetic) or non-inherited (epigenetic) (Jain, 2001) while Kaeppler *et al.* (2000) stated that it also covered phenotypic and DNA variation. According to Karp *et al.* (1987), *in vitro* culture is able to stimulate genome size alteration owing to the change in chromosome number. Ploidy variation in micropropagation may also be caused by polysomatic tissue (composed of cells of different ploidy cells) in the original explants (D' Amato, 1985).

For oil palm, flow cytometry (FCM) has been used by Rival *et al*. (1997) and Madon *et al*. (2012) to study the genome size and determine ploidy level variation of clones and seed-derived plants using *Petunia* and *Glycine max* cv. Polanka as reference standard respectively. Madon *et al*. (2012) also utilised the ribosomal DNA fluorescence *in situ* hybridisation (rDNA-FISH) technique to determine the ploidy level of oil palm suspension calli. Utilising the rDNA FISH technique, one signal denotes a haploid, while two signals show a diploid and so on. With the establishment of these two techniques, this study was conducted to determine the genetic variation of a full set of samples consisting of clonal palms, their suspension culture calli and regenerant plantlets. This was carried out to observe changes in genome size and ploidy level throughout the tissue culture process.

MATERIALS AND METHODS

Plant Material

Four sample sets were chosen for this study and each sample set consisted of an adult *tenera* P164 clonal palm, its suspension calli and five regenerants from the particular cloned palm (*Table 1*). FCM analysis was carried out on the four P164 clonal palms (*tenera* material) using frond -1 leaf samples, their respective suspension cultures and regenerant plantlets. Frond -1 was selected due to the leaf texture which was less green and softer, thus easier to process for nuclei suspension compared to Frond 0 (Madon *et al.*, 2008). About nine samples for each frond were analysed; three samples from base, three samples from middle and three samples from the end regions. Frond-1 were collected from the adult clonal palms in MPOB Hulu Paka, Terengganu. Three flasks of suspension cultures derived from each of the P164 adult clonal palm friable calli (*Figure 1*) and five 8-month old regenerants were provided by the Tissue Culture Group, Advanced Biotechnology Breeding Centre, Malaysian Palm Oil Board Headquarters (*Figure 2*).

Flow Cytometry (FCM) Analysis

Nuclei extraction and staining. For each of the adult clonal palms and regenerants leaf samples, 1 cm x 1 cm were cut into small pieces and placed in a 50 μm Medicon tissue homogeniser containing LBO1 buffer supplemented with 50 μg PI (propidium iodide) and 50 μg RNaseA (Dolezel and Bartos, 2005). The sample was blended using Medimachine for 3 to 5 min, filtered into a 10 ml falcon tube and

TABLE 1. LIST OF P164 SAMPLE SETS CONSISTING OF ADULT CLONAL PALMS, THEIR SUSPENSION CALLI CULTURE AND REGENERANTS

No.	P164 clonal palm	Analysis method	Suspension calli culture	Analysis method	Regenerants	Analysis method
1	0.393/226	Flow	PL163	Flow cytometry	PL163	Flow
2	0.392/292	cytometry	PL168	and rDNA-	PL168	cytometry and
3	0.393/145		PL180	FISH	PL180	rDNA FISH
4	0.393/259		PL200		PL200	

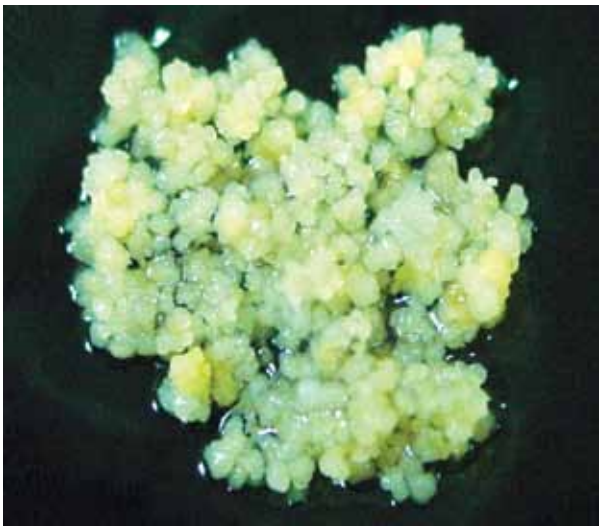
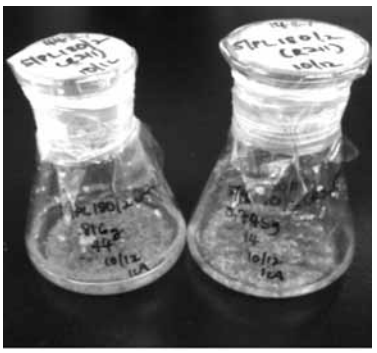


Figure 1. Friable and yellowish calli material used to initiate oil palm suspension cultures.

a)

b)



c)

Figure 2. P164 sample sets consist of: (a) adult clonal palm (Frond -1 leaflet), (b) suspension culture material and (c) regenerant plantlets.

incubated at 4ºC for 48 hr. For suspension calli, 1 ml of the suspension calli media was filtered and centrifuged to separate the pellet and supernatant fractions. The pellet containing nuclei of suspension calli was mixed with LBO1 buffer (with 50 μg PI and 50 μg RNaseA) followed by 24 hr incubation at 4°C.

Data analysis. The fluorescence intensity of each sample was measured by FACSCalibur flow cytometry (Becton Dickinson, USA) equipped with 15 mW argon ion laser at 488 nm. Histograms were collected over 1024 channels and for leaf samples 15 000 events were captured. Measurement of 2C DNA content was made using *Glycine max cv.* Polanka (2C=2.5 pg) as a reference standard. The fluorescence intensity of P164 clonal sample was compared with fluorescence intensity of soyabean to obtain the ratio, then multiplied by the genome size of soyabean, 2C=2.5 pg. Data was generated on the values of fluorescent intensity peaks and genome sizes of samples were analysed using CellQuest3 and SPSS statistical software respectively.

Ribosomal DNA-fluorescence *in situ* Hybridisation (rDNA-FISH) Analysis

Chromosome preparation. Root tips were harvested from each regenerant sample (eight months). The root tips were then pre-treated at 15°C for 5 - 6 hr in 2 mM 8-hydroxyquinoline and then fixed in Carnoy's solution (3:1, absolute ethanol:glacial acetic acid). The root tips were then transferred into 70% ethanol and stored at 4°C until needed. Root tips were then rinsed several times in enzyme buffer (0.01 M citric acid-sodium citrate, pH 4.6) and the terminal 1 mm from root tip meristem region was incubated in enzyme mixture (20% pectinase and 2% cellulase) at 37°C for 1-3 hr. Next, the softened tissue was rinsed with enzyme buffer and placed on a glass slide with a drop of 60% acetic acid. The root cap was removed with fine forceps under a dissecting microscope (Meiji Techno) and the root tip was squeezed to expel the protoplast suspension of meristematic cells. A coverslip was applied over the protoplast suspension and covered with a few layers of filter paper to remove excess acetic acid. The sample area was then tapped lightly with a steel rod followed by firm thumb pressure to help in spreading the metaphase chromosomes. The glass coverslips were then sealed at each side with rubber solution to prevent drying. Each slide was screened under phase contrast using Carl Zeiss Axioplan microscope. Slides containing more than 10 metaphase spreads were selected for *in situ* hybridisation. These slides were frozen using liquid nitrogen vapour to remove the coverslips, followed by dehydration in 70% and 100% ethanol and finally stored in minus 20°C freezer until needed.

For friable suspension calli, the samples were digested by incubation in enzyme mix (20% pectinase and 2% cellulase) overnight at 37°C prior to slide preparation. After digestion, samples were filtered through 40 μm Falcon cell strainers (Becton Dickinson) and centrifuged at 800 x *g* to pellet the nuclei. The pellet was then washed with enzyme buffer for 2 x 5 min and resuspended in 60% acetic acid. Next, 10 μl of suspension was placed on the slide and a coverslip applied. A few layers of filter papers were used to remove excess fluid before light tapping with steel rod and firm thumb pressure was applied to spread the chromosomes.

Preparation of rDNA probes (pBG35). For 18S-25S rDNA-FISH, the pBG35 probe which contains an 8.6 kb insert of rDNA from flax (*Linum usitatissimum*) was used (Goldsbrough and Cullis, 1981). DNA purification was performed using Gentra Puregene protocol kit. This probe was labelled with biotin using Bionick Labelling System (Invitrogen) according to the manufacturer's protocol.

FISH protocol. During pre-treatment, slides were cleaned and re-fixed in Carnoy fixative solution for 10 min. The slides were then washed with 96% alcohol for 2x10 min. Next, 200 μl of RNase A (100 μg ml^{-1}) was added, plastic coverslips applied on each sample slide, followed by incubation at 37°C for 1 hr in a humid chamber. Plastic coverslips were then removed and slides were washed in 2xSSC for 2x5 min. The slides were then incubated in 10 mM HCl for 5 min. After removal of HCl, 200 μl of pepsin (10 μg ml^{-1}) was added, covered by plastic coverslips and incubated at 37°C for 10-15 min in a humid chamber. The slides were then rinsed 2x5 min in 2xSSC buffer followed by incubation in 4% paraformaldehyde solution for 10 min in a fume hood. The slides were washed again with 2xSSC buffer for 5 min, and dehydrated through a series of ethanol at 70%, 90% and 96% for 2 min each.

The hybridisation mixture, consisting of 25-200 ng μl^{-1} of DNA probe, 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.15% SDS, 0.25 mM EDTA 2XSSC and 0.025 μg herring sperm DNA was denatured at 70°C for 10 min followed by immediate cooling on ice for 10 min. The 40 μl of hybridisation mixture was then added on the sample area of each slide and covered with a plastic coverslip. These slides were denatured at 80°C for 10 min followed by 37°C incubation overnight in a flatbed thermal cycler. The next day, the slides were washed in fresh 2XSSC for 3 min at 42ºC, followed by two washes of 5 min each in a 20% (v/v) formamide in 0.1XSSC, 2x5 min at 42ºC in 2XSSC and finally 2x5 min in 4XSSC/Tween (0.2%) at room temperature.

The slides were blocked with 5% BSA in detection buffer and incubated at 37°C for 30 min. Detection reagent (2 μg ml^{-1} of Cy-3 labelled Streptavidin in detection buffer) was then dropped on the slides and incubated at 37°C for 1 hr in a humid chamber. The slides were immediately washed in series of detection buffer (4xSSC, 0.% Tween 20), 2x5 min followed by 10 min at 42°C. Finally, slides were stained with 4 μg ml^{-1} of DAPI and incubated in the dark for 10 min, followed by rinsing in detection buffer again for a few min. Antifade medium (Kirkgaard and Perry Laboratories, KPL) was mounted onto the slides and glass coverslips were applied. Excess antifade media were removed and the edges of coverslips were sealed with rubber glue before screening.

The slides were screened using Carl Zeiss Axioplan epifluorescence microscope with suitable filters. The fluorescence images were captured by PAXCam 2+ camera and the images were analysed with PAX-it software for observation of hybridisation signals on metaphase chromosomes or interphase nuclei. One hybridisation signal indicates that the ploidy level is haploid, two signals indicate diploid, three signals indicate triploid and so on.

RESULTS AND DISCUSSIONS

FCM Analysis

Nuclei preparation optimisation. Each nuclei filtrate of leaf samples was incubated for 48 hr at 4ºC to allow optimum PI intercalation with the DNA strands. For suspension calli, incubation with PI was only for 24 hr at 4ºC due to the low number of nuclei. PI fluorochrome was selected for this analysis since it produced precise fluorescent intensity histogram peak with lower coefficient variation (Dolezel and Bartos, 2005). Furthermore, it did not bind preferentially to AT-GC rich regions but covered the entire DNA region (Dolezel *et al*., 2007). The nuclei suspension of the soyabean standard was immediately analysed by FCM following the preparation of the leaf samples due to the lower amount of DNA in nuclei. Soyabean was selected as an external standard because its genome is about one-third less than that of the oil palm and easy to process (Madon *et al.*, 2008).

Histogram peak analysis. A good fluorescent intensity histogram peak is defined as a symmetric sharp peak with a coefficient of variation (CV) of 3% or less. The value of CV is an important parameter in FCM technique (Prado *et al*., 2010). The best histogram peaks for the adult clonal palm was obtained between channels 240 and 320 with a CV of 1% -3% (*Figure 3*). This histogram data also showed a debris peak due to the degraded nuclei. The CV values indicate that PI was a good DNA intercalator for absolute genome size measurement (Dolezel and Bartos, 2005). Similar results were obtained for the

regenerant plantlets (*Figure 4*). Madon *et al*. (2008) also showed that PI was an efficient intercalating dye for oil palm DNA strands. For the regenerant leaf samples, the sharpest histogram peak was also obtained between channels 240-320 with CV less than 3%. However, regenerant samples contained more debris compared to the adult clonal palms (*Figure 4*, arrow) where skewed peak of the regenerant is higher than the skewed peak in leaf samples of adult clonal palm (*Figure 3*, arrow). The occurrence of debris peak may be caused by too much chopping during sample preparation (Dolezel *et al*., 2007). As for suspension calli samples, no clear peak was observed due to the low nuclei population which is less than 1000 with CV more than 5% (*Figure 5*). Due to limited sample availability, FCM is unsuitable for measuring the nuclear genome size of suspension calli. According to Dolezel and Bartos (2005), in order to determine nuclear genome size, the nuclei must be extracted in sufficient quantity followed by specific and stoichiometric DNA staining for nuclei where genome size of the reference standard is known.

2C DNA content analysis. *Table 2* lists the nuclear genome sizes of P164 clonal material. The genome size of adult clonal palm varied from 2C=2.59±0.19 pg to 2C=2.91±0.14 pg. For calli from suspension culture, histogram peak was not obtained as the nuclei population was less than 1000. As the available sample was limited, the nuclei population could not be increased. The genome size of regenerants

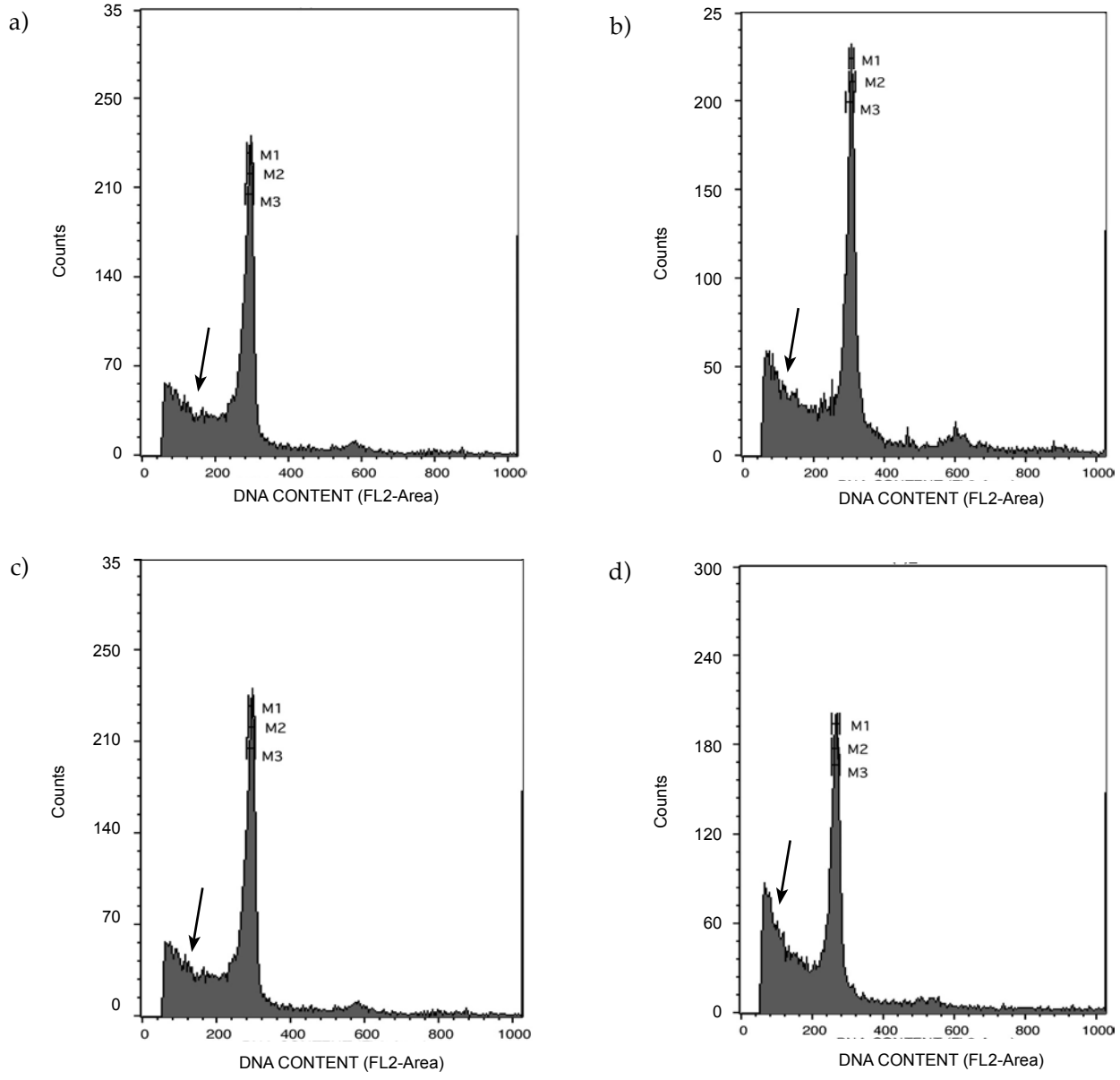


Figure 3. Fluorescent intensity histogram peaks of P164 adult clonal palms. (a) 0.393/259, (b) 0.393/226, (c) 0.393/145 and (d) 0.392/292. The arrows indicates debris peak.

TABLE 2. GENOME SIZE (2C DNA content) OF P164 CLONAL PALMS AND THEIR REGENERANTS

No.	P164 clonal palm	Genome size (pg)	Regenerants	Genome size (pg)
1.	0.393/226	2.91±0.14ab	PL163	2.14±0.21a
2.	0.393/145	2.72±0.13ab	PL180	3.05±0.11b
3.	0.393/259	2.74±0.11ab	PL200	2.79±0.14ab
4.	0.392/292	2.59±0.19ab	PL168	2.46±0.16ab

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

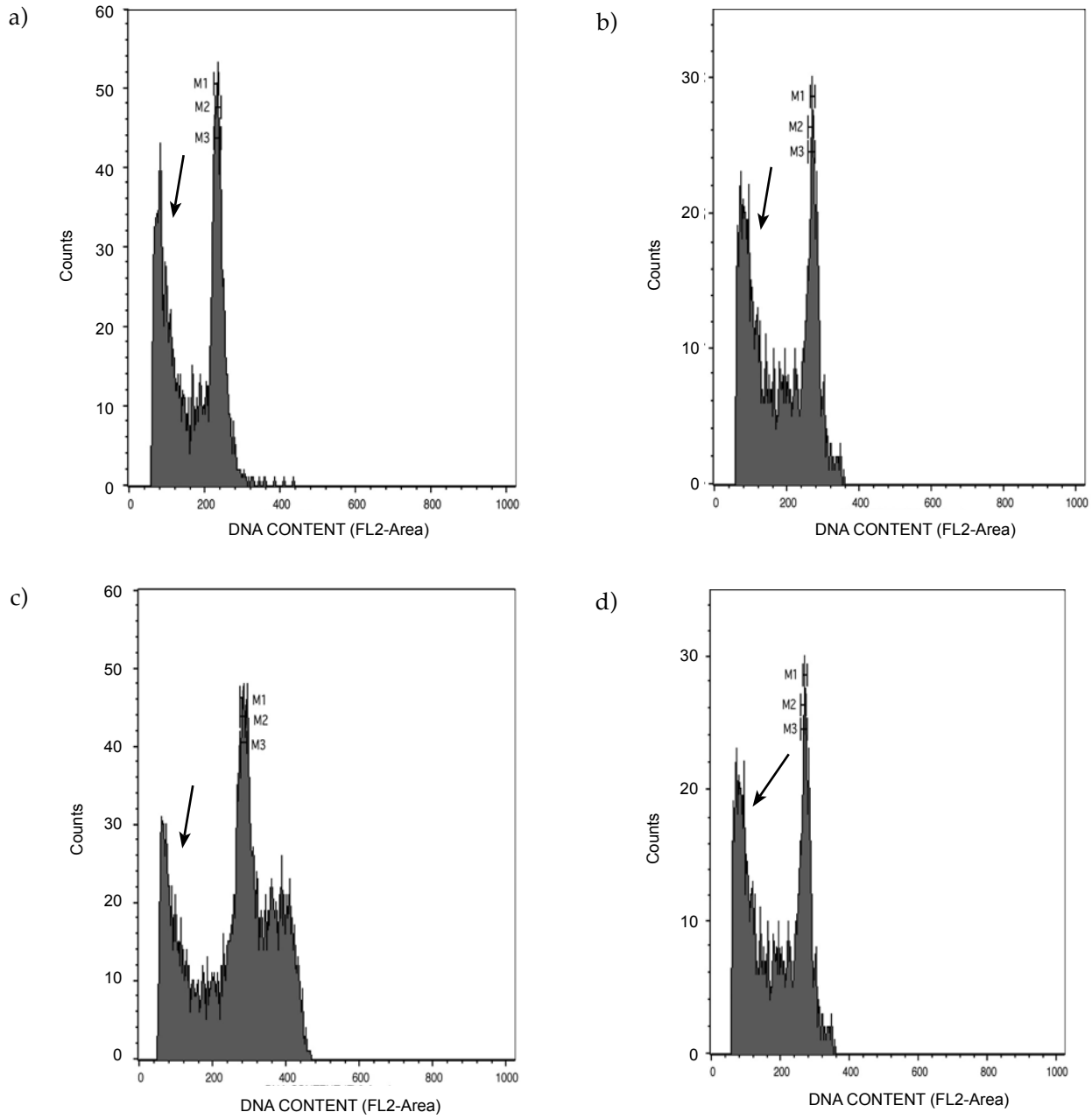


Figure 4. Fluorescent intensity histogram peaks of regenerant plantlets. (a) PL163, (b) PL168, (c) PL180, and (d) PL200. The arrows indicates debris peak

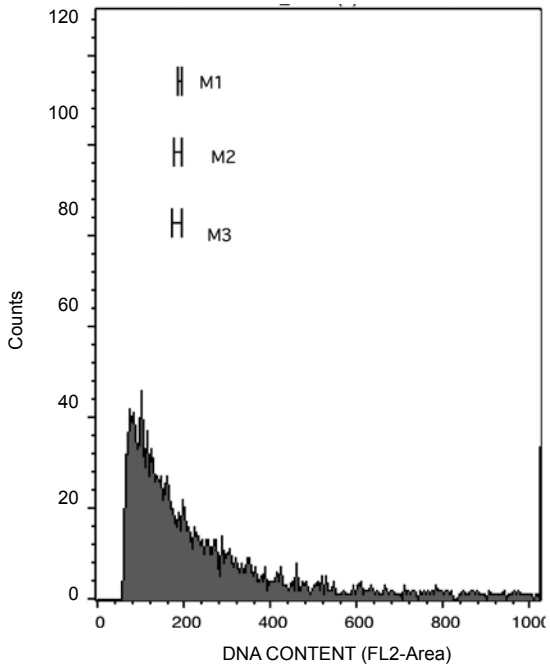


Figure 5. Fluorescent intensity histogram peaks of suspension culture calli with no clear peak observed.

varied from 2C=2.14±0.21 pg to 2C=3.05±0.11 pg. Statistical analysis showed no difference in genome size between clonal palms. No difference was also observed between clonal palm and its regenerants except for between regenerant PL163 and PL180, while all others were not different statistically.

The observed variation in genome sizes within and between adult clonal palms and regenerants may be due to the tissue culture effects which can cause changes in chromosome number, structure and sequence (Kaeppler *et al.*, 2000; Zhao *et al.*, 2005). Chromosome breakage with its consequences such as translocations, inversions, duplication and deletion (Duncan, 1997) is another factor for nuclear genome size variation. In addition, the stressful *in vitro* culture condition and plant growth regulator would also affect the nuclear DNA content of tissue culture products (Jain, 2001; Srisawat *et al*., 2005). Genome size can also vary due to the changes of ploidy state (Lee and Phillips, 1988). According to Shiiba and Mii (2005), endoreduplication that occur in explant tissues during tissue culture process will lead to changes in the nuclear genome size. The presence of transposable elements in the genome might also play an important role in genome variation (Castilho *et al.*, 2000) due to their ability to change their genomic location and/or number of copies within the genome (Sabot *et al*., 2004). Kubis *et al.* (2003) also reported that transposable genes will be activated during clonal propagation.

Ploidy level in adult clonal palms and its regenerants. Jones *et al.* (1982) reported that only diploid cells were obtained in oil palm regenerants during the tissue culture process. Rival *et al*. (1997) also reported the diploid ploidy level of oil palm calli and seed derived material analysed via FCM, where only one single peak was present in FCM histogram peak positioned around 300-380. In this study, from the fluorescent intensity histogram peak analysis, adult clonal palm samples and their regenerants showed only one single peak (G1 peak) positioned on the channel between 240-320 indicating a diploid state. Hence, it was concluded that the diploid ploidy level was maintained in the adult clonal palms and their regenerants.

Ribosomal DNA Fluorescent *in situ* Hybridisation Analysis

The hybridisation signals obtained from probes were confirmed from five experiments conducted on different days, each comprising four slides of chromosome preparation. The success rate of hybridisation was 50%. Not all interphase nuclei showed hybridisation signals but in most slides, the number of hybridised nuclei can be counted, hence ploidy level can be determined. The rDNA-FISH analysis was done on oil palm suspension culture calli derived from PL163, PL168, PL180 and PL200, where the interphase nuclei showed two hybridisation signals (*Figure 6*). These results demonstrated that the diploid ploidy level of the four suspension culture samples derived from the four P164 clonal palms. Similar results were also obtained for all regenerant plants derived from the suspension cultures of PL163, PL168, PL180 and PL200 where two hybridisation signals were also observed on the interphase nuclei, indicating their diploid ploidy level (*Figure 7*). Hybrid of *Abies* also showed the same trend in terms of ploidy level where it remained as diploid throughout callus culture and regenerant stage during clonal propagation (Libiakova *et al.*, 1995).

Ploidy variation, however, has been observed to occur during clonal propagation of several plant species namely *Pennisetum americanum* (Swedlund and Vasil, 1984), *Hordeum vulgare* (Karp *et al.*, 1987), *Triticum tauschii* (Winfield *et al.*, 1993) and *Secale cereal* L. (Puolimatka and Karp, 1993). Larkin and Scowroft (1981) have reported that regenerants will exhibit somaclonal variation when regenerated from callus due to chromosomal instability. Chromosomal instability initiated in callus stage is correlated with endoreduplication and non-disjunction during anaphase of mitosis (Lavania and Srivastava, 1988). Disturbance in cell cycle activity during tissue culture may also cause chromosome level changes and affect synchronisation in chromosome replication during cell division (Bairu *et al*., 2011) leading to chromosome breakages (Lee and Philip, 1988). Chromosome breakages were also reported as a prevalent phenomenon that triggered changes

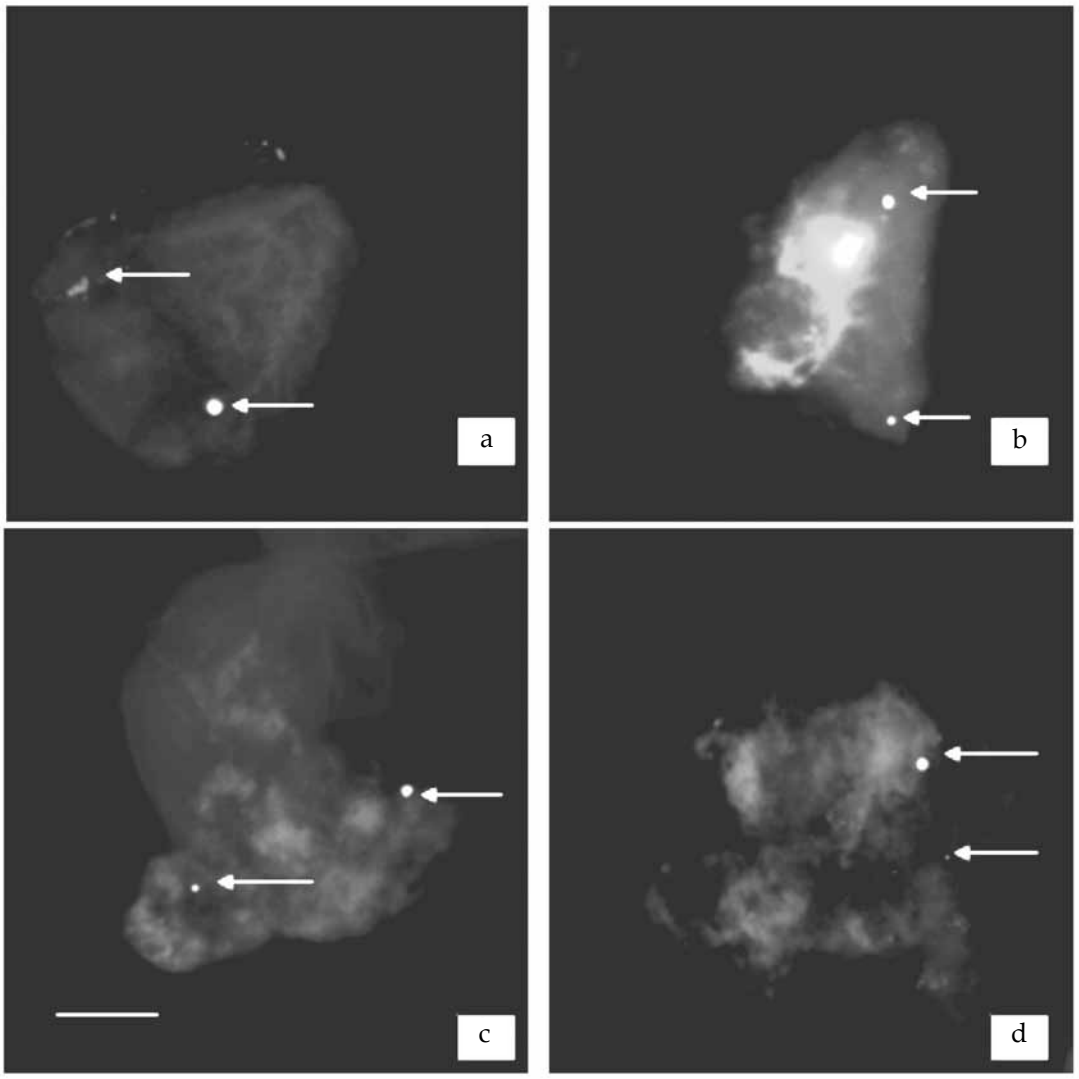


Figure 6. Hybridisation sites of 18S-25S rDNA probe on suspension culture calli interphase nuclei (a) PL163, (b) PL168, (c) PL180 and (d) PL200. The arrows indicate rDNA hybridisation sites detected by Cy3-streptavidin. The bar indicate 10 µm.

in ploidy level of barley regenerants (Hang and Bregitzer, 1993). McClintock (1984) reported that chromosome breakage will turn on genome shock, thus being able to activate transposable elements in the genome and hence increase genome size.

An unbalanced concentration of hormones such as auxin in the nutrient media can also cause polyploidisation such as in strawberry (Nehra *et al*., 1992). Fras and Maluszynka (2003) also reported on the high degree of polyploidisation during callogenenesis. Polyploidisation was also observed for potato, maize and wheat (Pijnacker, 1989; Kaeppler, 2000; Fluminhan *et al*., 1996). According to Pijnacker *et al.* (1989), a variation of ploidy level in regenerants was also due to chromosome and genome alteration, including polysomaty where somatic cells with a different ploidy level developed from the explants.

CONCLUSION

This study was carried out to determine whether any gross ploidy changes occurred during the tissue culture process and utilised the FCM method to determine nuclear genome size and the ploidy level of adult clonal palms and their regenerants. However, the genome size of suspension calli culture material could not be estimated due to the low nuclei population and limited sample availability. As such, image cytometry should be developed in future to address this issue. The rDNA-FISH method demonstrated its effectiveness in determining the ploidy level of suspension calli nuclei. From this study, it was found that the genome sizes of regenerants were inconsistent compared to the genome sizes of adult clonal palms and there was no specific trend in the genome sizes of the regenerated

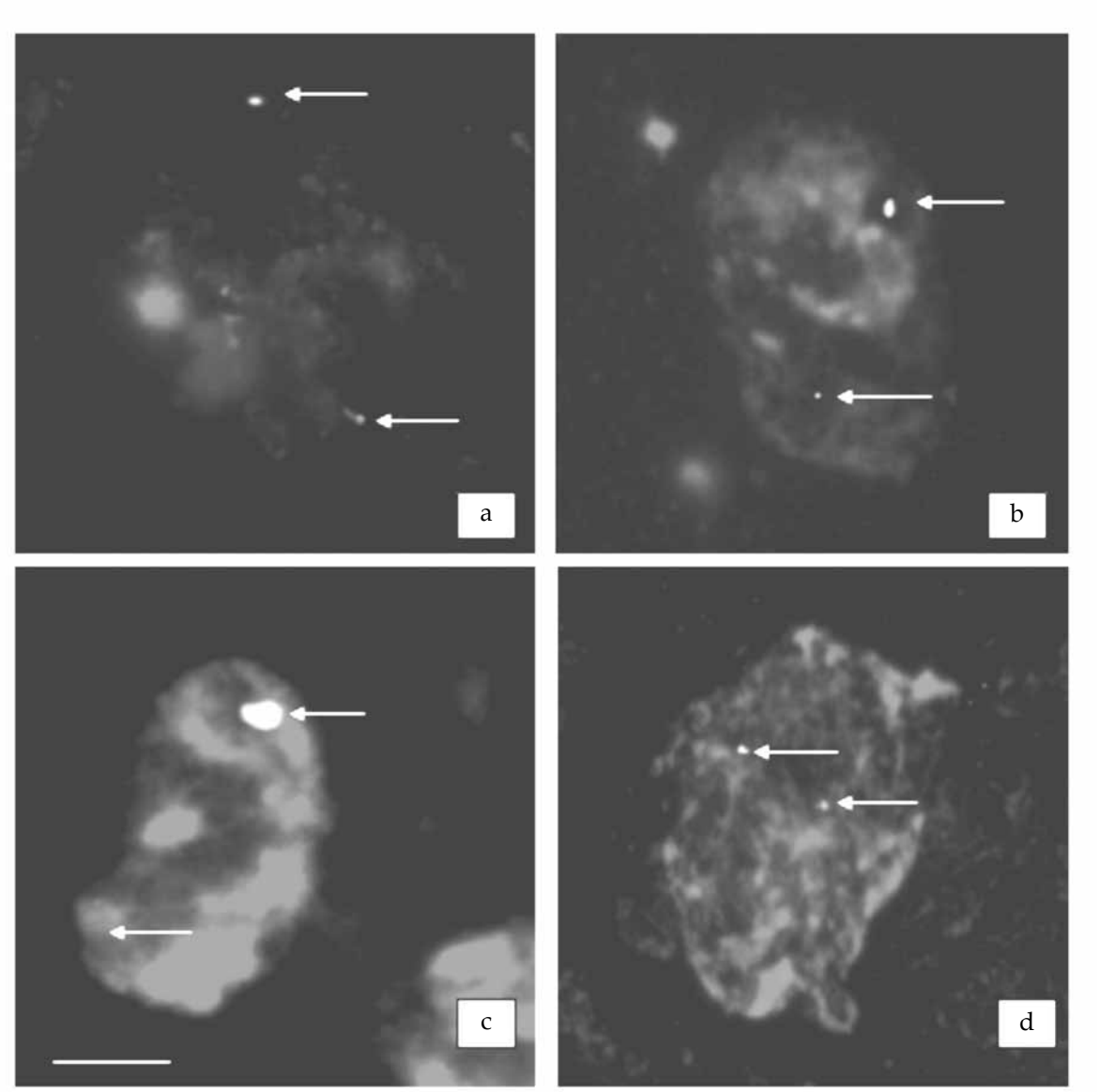


Figure 7. Hybridisation sites of 18S-25S rDNA probe on regenerant root tips meristematic interphase nuclei (a) PL163, (b) PL168, (c) PL180 and (d) PL200. The arrows indicate rDNA hybridisation site detected by Cy3-streptavidin. The bar indicate 10 µm.

plants compared to their adult clonal palms. In conclusion, it was observed that the diploid ploidy level was maintained in the adult clonal palms, its suspension calli cultures and regenerated plantlets determined via FCM and rDNA-FISH analysis.

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