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Full Length Research Paper

Flow cytometric classification of oil palm cultivars

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Efficiencie in each nuclear lysis buffer on flow cytometric analysis (FCM) of oil palm were compared for cultivar classification. Embryos and unopened leaves of one to twelve months-old seedlings were used as initial materials with five nuclear lysis buffers, including LB01, WPB, Otto's, Tris.$MgCl_2$ and Galbraith. Although these buffers showed distinct peaks of propidium idodide (PI) fluorescence for oil palm embryos, the buffers differed considerably in fluorescence intensity (FL), coefficient of variance (%CV), debris factor (%DF) and yield factor (YF) values when analyzed with their seedlings. The results show that LB01 was the most suitable buffer (lowest %CV and high FL value) for oil palm embryos resulting in 3.7 pg of 2C-DNA. For all the seedlings, only WPB gave the highest value of FL and lowest value of %CV resulting approximately in 3.8 pg of 2C-DNA, whereas the highest value of YF and lowest value of %DF were mostly found in LB01. Of interest is the fact that only WPB showed consistency position of PI fluorescence histograms when analyzed with all seedlings. WPB was therefore used to classify the cultivars of hybrid Tenera and its parents (Dura and Pisifera) by means of DNA contents. DNA contents of Dura and Pisifera ranged from 6.3 to 7.6 and 5.3 to 6.1 pg and their genome size (1C DNA contents) is therefore greater than 3,000 and 2,000 Mbp, respectively. The results confirmed that Dura, Pisifera and Tenera cultivars could be classified using FCM-derived DNA contents.

Key words: 2C-DNA value, cultivars, flow cytometry, nuclear lysis buffers, oil palm.

INTRODUCTION

Information of ploidy levels and DNA content is important in breeding program of plant varieties. Based on fruit structure, oil palm (Elaeis guineensis Jacq.) has been systematically classified into 1) Dura (thick shell; less mesocarp), 2) Pisifera (shelless; embryo rarely formed) and 3) Tenera, the Dura × Pisifera hybrid (thin shell; more mesocarp: 60 to 95%), with high oil content. Tenera is a valuable economically important source of vegetable oil and is increasingly used to power vehicle as biodiesel. Since there is high potential of conventional cross between Dura and Pisifera cultivars in the first filial hybrid trait, the DNA content of the hybrid is probably variable. However, intra-specific identification of these species remains difficulties, since they have very similar morphological features at vegetative phase. The only possible way to identify them is the presence or absence of endocarp or shell of the fruits (Sathish and Mohankumar, 2007). Investigating the variability within the species is therefore important to determine the oil yield representation and to identify unique fruit types that would help in developing improved cultivars (Narasimhamoorthy et al., 2008). Although there are many potential DNA markers to investigate cultivated clone of oil palm (Mayes et al., 2000; Barcelos et al .,

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Abbreviations: FCM, Flow cytometric analysis; **PI,** propidium idodide; **FL,** fluorescence intensity; **%CV,** coefficient of variance; **%DF,** debris factor; **YF,** yield factor.

2002; Zehdi et al., 2004), difficulties have been encountered when using these procedures on a large scale (Rival et al., 1997).

Flow cytometry (FCM) is the method offering a simple, rapid, accurate and convenient analysis for determining ploidy levels of DNA, assessment and analysis of the cell cycle of large cell populations (Dolezel, 1991; Winkelmann et al., 1998). The extension of FCM analysis has recently been supported by the relative complexity of nuclear lysis buffer compositions, which involves peak position, quality and values of DNA content in various species. A systematic comparison of nuclear lysis buffers has been investigated by Loureiro et al. (2006a, b, 2007), who compared six of the most common buffers differing in chemical composition: Galbraith, LB01, Otto's, Tris.$MgCl_2$, General Purpose Buffer (GPB) and Woody Plant Buffer (WPB). The buffers have been considered by multivariate parameters including higher fluorescent intensity (FL) and yield factor (YF), and lower % coefficient of variance (CV) and debris factor (DF) (Greihuber et al., 2007), and it was found that the different chemical composition and distinction of plant tissues could perform differently in each buffer (Loureiro et al., 2006b). Therefore, it is unacceptable to apply only single buffer with wide range of plant species (Dolezel and Bartos, 2005).

There are only three previous FCM analyses of oil palm reported by Rival et al. (1997), Srisawat et al. (2005) and Madon et al. (2008). The results of DNA content in oil palm have been reported as 3.7 pg (Rival et al., 1997; Srisawat et al., 2005) and slightly differed to 3.8 pg (Madon et al., 2008). Discrepancies of oil palm DNA content are not only affected by the differences of the techniques and types of standard used, but also affected by the types of nuclear lysis buffer used (Loureiro et al., 2006b, 2007). Hence, an appropriate nuclear lysis buffer for oil palm FCM analysis should be investigated more extensively for any further breeding and production programs of oil palm. The chosen buffer used to classify oil palm cultivars was usually characterized by giving higher FL and YF and lower %CV and DF (Loureiro et al., 2007).

In this study, our attention was focused on the discrimination of the effective nuclear lysis buffers from FCM analysis of oil palm embryos and seedlings replicated into three unopened-leaf positions (leaf-apices, leaf-middles and leaf-bases) and establishing the effective marker for classification of oil palm cultivars by using DNA content values.

MATERIALS AND METHODS

Breaking dormancy-derived seeds, one to twelve months-old seedlings of oil palm cv. Tenera and young leaves of more than ten years-old tree of Deli Dura: (D109, D067, D064, D069 and D068"), Pisifera: (Calabar; P109, LA ME ; P106, DAMI; P116, Nigeria; P110 and EKONA; P105) and hybrid Tenera (Suratthani 1, 2, 3 and 5) cultivars were prepared by the Suratthani Oil Palm Research Center, Suratthani province, Thailand. Seeds of soybean (*Glycine max* cv. Polanka), reference plant, were kindly provided by Dr Jaroslav Dolezel (Institute of Experimental Botany, Olomouc, Czech Republic).

Comparison of five flow cytometric nuclear lysis buffers

The flow cytometer was a FACSCalibur (Becton Dickinson Biosciences {BDB}, San Jose, CA) working with CellQuest software (BDB) equipped with a 488 nm argon iron laser. Propidium iodide (PI) was measured at 585 nm to read 2C nuclei histograms of 5,000 nuclei per sample. During analysis, after every three samples, the reference plant was used as a control to check the calibration of the flow cytometer in each buffer by adjusting the gain of *Glycine* to channel 200. All experiments were carried out with 3 replicates (n = 3) per treatment.

Soybean was used as external reference plants (2C=2.50 pg; Dolezel et al., 1994). Unopened leaves (replicating to leaf-apices, leaf-middles and leaf-bases) of one to twelve months-old seedlings and embryos of oil palm and the 2nd - 3rd leaves from the shoot apex of reference plants, approximately 50 mg, were finely chopped with a razor blade in 1.0 ml Tris-$MgCl_2$ (Pfosser et al., 1995), WPB (Loureiro et al., 2007), LB01 (Dolezel et al., 1989), Galbraith (Galbraith et al., 1983) and Otto's (Otto, 1990) extraction buffers containing: [200 mM Tris, 4 mM $MgCl_2$, 0.5%(w/v) Triton X-100 and 3.0%(w/v) polyvinylpyrrolidone (PVP)], [200 mM Tris.HCl, 4mM $MgCl_2$, 2mM Na_2EDTA, 86 mM NaCl, 10 mM sodium metabisulfite, 1% PVP-10, 1%(v/v) Triton X-100, pH 7.5], [15 mM Tris, 2mM Na_2EDTA, 0.5 mM spermine.4HCl, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, pH 8.0], [45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0] and [(Otto I: 100 mM citric acid, 0.5% (v/v) Tween 20 pH 2 to 3), (Otto II: 400 mM Na_2HPO_4, pH 8-9)], respectively. After extraction, fifty microliters of RNase and PI were added immediately prior to filtering through a 42 µM nylon mesh (Pfosser et al., 1995). FCM parameters and DNA contents were recorded and analyzed for selecting the chosen buffer and plant material for classifying oil palm cultivars: Forward scatter as a rough measure of particle's size (FS), side scatter as a measure of particles optical complexity (SS), fluorescence intensity of PI-stained nuclei (FL), G_0/G_1 peaks as a measure of nuclear intensity and variation in DNA staining (%CV), debris background factor as a measure of sample quality (%DF) and nuclear yield factor (YF) in order to compare the quantity of nuclei in suspension. The following equations were used:

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

$$YF = \frac{\dfrac{\text{Total number of intact nuclei}}{\text{Numberof second run (s)}}}{\text{Weight of tissue (mg)}}$$

Estimation of DNA contents for cultivars classification

Young leaves of Deli Dura, Pisifera and hybrid Tenera trees were prepared following the procedures mentioned above. FCM analysis was carried out by using the chosen buffer and subsequently determined DNA content in each cultivar using the equation:

$$2C\text{DNA content} = \frac{\text{Sample G1 mean FL}}{\text{Reference standard G1 mean FL}} \times \text{DNA content of reference standard}$$

Laboratory trial design and data analysis

The experiment was designed in accordance with the completely randomized design (CRD) with two factors, namely: five types of lysis buffer and five growth stages of oil palm seedlings with three replicates by means of three positions of unopened leaves.
The fluorescence histograms were resolved into G_0/G_1 (2C), S and G_2/M (4C) cell-cycle compartments with a peak-reflect algorithm using two Gaussian curves (WinMDI version 2.9). The FCM parameters and DNA contents of oil palm were statistically analyzed by analysis of variance (ANOVA) and the significant differences between the contents in each parameter and DNA value were tested against the *F*-distribution at $P \leq 0.05$. Tukey's testing was performed for routine multiple mean comparison.

RESULTS

Comparison of five nuclear lysis buffers for parameters estimation

Testing the five nuclear lysis buffers with embryos and one to twelve months-old seedlings of oil palm revealed significant distinctions in all parameters. The use of each buffer resulted in acceptable parameters with oil palm embryos. The PI-fluorescence peak histograms of oil palm embryos analyzed with all buffers gave a good reading in peak qualities and positions, indicating that each buffer can be used to investigate 2-C DNA contents of oil palm embryo's nuclei, considerably (Figure 1). FS, SS, FL, %CV, %DF and YF values were demonstrated. Discriminating of suitable buffer for FCM analysis of oil palm was done by deciding the following criteria: first and second highest of FL and YF and first and second lowest of %CV and DF values (Table 1).

Nuclei isolated from embryos and seedlings of oil palm with all five buffers had no significant differences in FS and SS values. Interestingly, both of these values revealed their event out of scale on FS and SS logarithm density plots, corresponding to high values of FS and SS (Table 1). In most leaf tissues, an effect similar to the phenolic compound effect was observed which involved the occurrence of two populations, higher %CV and DF. Correspondingly, two populations of particles on cytograms of FS *vs*. SS, high values of %CV and DF were found in this study. Leaf tissues treated with all buffers maintained a high level of FS, SS. %CV and %DF which might result from "the tannic acid effect" reaction.
The LB01 gave the second highest of FL (299.73) and first lowest of %CV of DNA peak (4.57%) of oil palm embryos, unfortunately the YF value showed unexpected yields in the lowest (0.68 nuclei s^{-1} mg^{-1}). Otto's buffer yielded acceptable histograms with the first highest of FL value for 385.55 and second lowest of %CV (4.93%), whereas the first lowest of debris factor (98.90%) and second highest YF value (0.96 nuclei s^{-1} mg^{-1}) were found when using Galbraith. The highest value of yield factor was found to be 0.99 nuclei s^{-1} mg^{-1} for WPB buffer.

Although, no significant difference among the buffers was obtained in FS, SS, %DF and YF values, there are significant difference in FL and %CV, implying that LB01 and Otto's had more significant efficiencies than the others (Figure 1A and Table 1). Moreover, no detectable tannic acid or phenolic compound in the solutions prepared from embryo tissues.

With the oil palm seedlings, the differences were due to different yields, debris, fluorescence intensity, %CV and peak positions observed while analyzing the buffers with one to twelve months-old seedlings. WPB buffer yielded approximately 314 for the first highest FL value and lowest of %CV (8.75%) of one month-old seedlings. The second lowest and highest of %DF and YF were also found with this buffer for 99.74% and 0.27 nuclei s^{-1} mg^{-1}, respectively. Using LB01, the sufficient amount of yields and debris factors were revealed with the first highest and lowest for 0.52 nuclei s^{-1} mg^{-1} and 98.61%, respectively. Using WPB, the peak position of PI-fluorescence intensity from young leaves of one month-old seedling resembled sustainability with those obtained from analysis of embryos using LB01 (Figure 1A and B). The most suitable buffer for one month-old seedling of oil palm is therefore WPB buffer by means of the highest of FL, lowest of %CV values (Table 1) and maintainable of histogram position.

Furthermore, the PI-fluorescence histograms of three months-old seedlings of oil palm estimated with the five buffers were demonstrated (Figure 1C). Only LB01 and WPB buffers affected the sufficient amount of FL and YF and against %CV and DF for this stage of oil palm seedlings. The buffers yielded sufficiently several parameters; the first highest of FL and lowest of %CV were detected in the nuclei solution of WPB (281.17 and 6.45%), whereas %DF and YF were compared to the first lowest (93.41%) and highest (1.81 nuclei s^{-1} mg^{-1}) values in the solution of LB01 (Table 1). In addition, the second levels of these parameters were also found correspondingly in those buffers. The chosen buffers for FCM of three month-old seedlings of oil palm are LB01 and WPB buffers. It is interesting to note that peak position of WPB-derived nuclei revealed the same position compared to the position of one month-old seedlings-derived nuclei isolated by WPB.

For six months-old seedlings, although LB01 produced the first lowest of %DF (96.44%) and the second highest of YF (1.38 nuclei s^{-1} mg^{-1}), the result showed that WPB buffer is the suitable isolation for nuclei extraction by means of the first highest FL (362.28) and lowest %CV(4.47%) values (Figure 1D and Table 1). This result resembled the analysis of twelve months-old seedlings which showed the same suitable buffer, revealing the first highest FL (285.49) and lowest %CV (6.91%) (Figure 1E and Table 1). Consequently, there are two suitable buffers (WPB and LB01) for analysis of FCM in oil palm by means of the highest of FL and YF and lowest of %CV

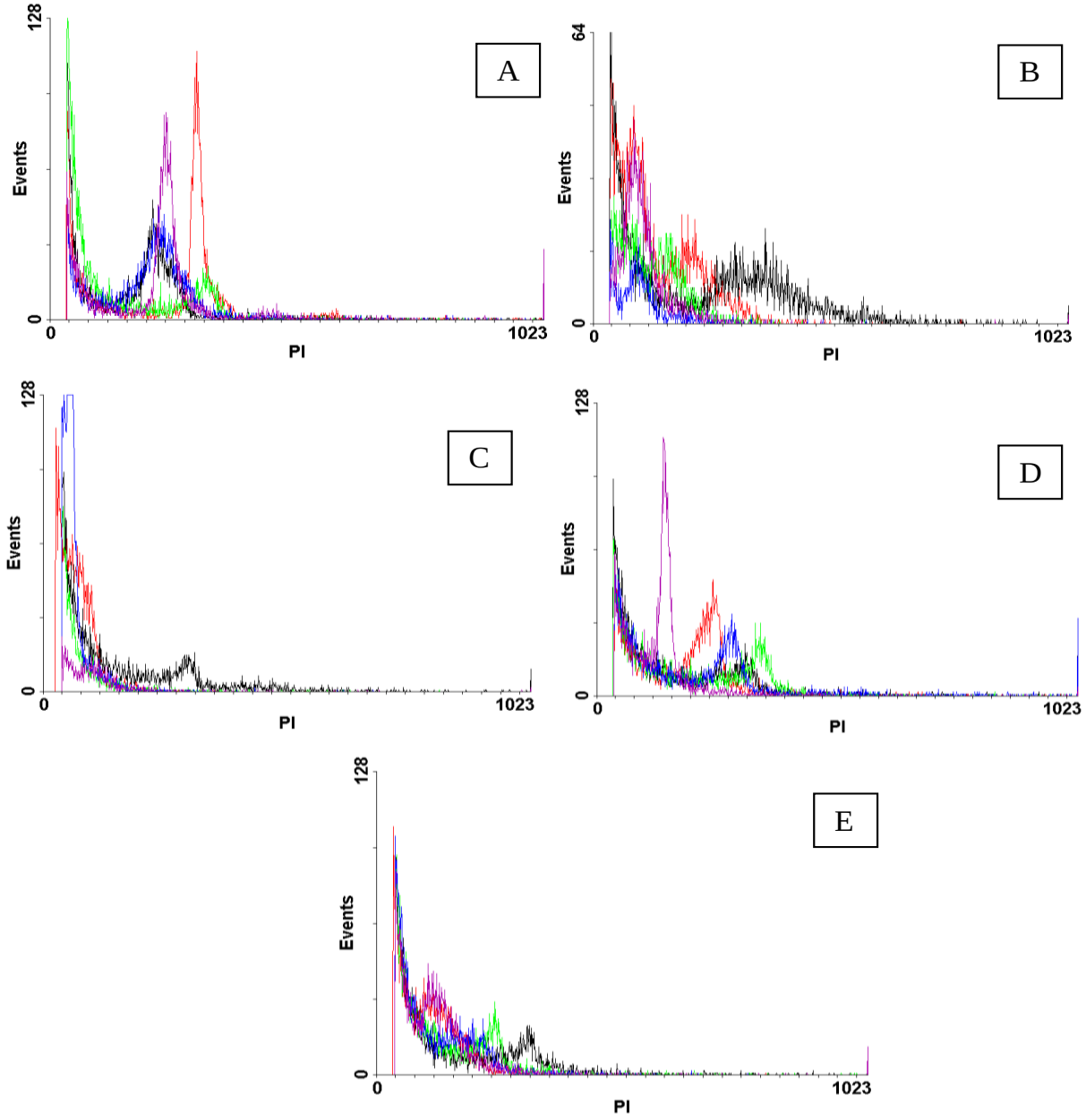

Figure 1. Histograms of relative fluorescence intensities (PI fluorescence) of oil palm embryo nuclei (A), one month-old seedling (B), three months-old seedling (C), six months-old seedling (D) and twelve months-old seedling (E) isolated with five nuclear lysis buffers. (LB01 buffer, red; WPB buffer, black; Tris.$MgCl_2$ buffer, blue; Otto's buffer; green; and Galbraith buffer: purple).

and DF. However, the position of histogram peaks could be considered significantly because only WPB gave the same position of PI histogram. These seem to be a high efficiency of WPB for maintaining quality of nuclei solution. It is interesting to note that some leaf positions of oil palm seedlings were found to be undetectable tissues for their nuclei isolation when analyzed with specific buffer. As above mentioned, the WPB buffer is accepted to estimate 2C-DNA value of oil palm cultivars.

Estimation of nuclear genome size (C-DNA values) of oil palm embryos and seedlings

Table 2 lists the average C-values of 5 growth stages of oil palm determined by using *Glycine* as external reference plant. Using LB01, the 2C-DNA contents from oil palm embryos were regularly lessen in width between 3.7 to 3.8 pg, whereas ranges from 2.7 to 3.0, 4.2 to 5.2, 2.7 to 2.8 and 1.8 to 3.1 pg were revealed when using

Table 1. Flow cytometric parameters assessed of oil palm embryos and unopened-leaves of one to twelve months-old seedling of oil palm in various isolation buffers.

Growth stage	Buffer	FCM parameter (mean ± SD)					
		FS (channel unit)	SS (channel unit)	FL (channel unit)	CV (%)	DF (%)	YF (nuclei $s^{-1}mg^{-1}$)
Embryos	**LB01**	4149.60 ± 2950.15^{a}	862.30 ± 504.36^{a}	**299.73** ± 4.98^{ab}	**4.57** ± 0.38^{a}	99.22 ± 0.15^{a}	0.68 ± 0.36^{a}
	WPB	6723.60 ± 4975.76^{a}	765.77 ± 366.31^{a}	211.36 ± 10.59^{bc}	6.59 ± 2.15^{ab}	99.13 ± 0.59^{a}	**0.99** ± 0.78^{a}
	Otto's	6854.67 ± 5155.52^{a}	2738.87 ± 2078.82^{a}	**385.55** ± 52.42^{a}	**4.93** ± 0.38^{a}	99.08 ± 0.35^{a}	0.78 ± 0.12^{a}
	Tris.$MgCl_2$	6181.40 ± 4571.84^{a}	1582.60 ± 1037.63^{a}	208.91 ± 14.46^{bc}	7.31 ± 0.30^{ab}	**98.93** ± 0.09^{a}	0.96 ± 0.26^{a}
	Galbraith	5860.37 ± 4286.90^{a}	1163.43 ± 585.23^{a}	166.33 ± 60.00^{c}	8.93 ± 2.11^{b}	**98.90** ± 0.33^{a}	**0.96** ± 0.86^{a}
One month-old seedlings	LB01	861.93 ± 136.86^{a}	496.63 ± 218.05^{a}	156.30 ± 72.84^{b}	**11.22** ± 3.73^{a}	**98.61** ± 1.09^{a}	**0.52** ± 0.26^{a}
	WPB	919.73 ± 39.66^{a}	593.13 ± 152.19^{a}	**314.65** ± 38.29^{a}	**8.75** ± 3.48^{a}	**99.74** ± 0.17^{a}	**0.27** ± 0.13^{ab}
	Otto's	944.00 ± 113.46^{a}	672.70 ± 434.13^{a}	**159.87** ± 67.45^{ab}	12.87 ± 11.39^{a}	99.89 ± 0.06^{a}	0.10 ± 0.05^{b}
	Tris.$MgCl_2$	*666.00 ± 576.86^{a}	*457.50 ± 446.86^{a}	*72.74 ± 64.19^{b}	*8.36 ± 7.26^{a}	*64.87 ± 56.25^{a}	*0.03 ± 0.03^{b}
	Galbraith	1019.40 ± 1.25^{a}	1009.533 ± 7.98^{a}	94.16 ± 37.32^{b}	18.86 ± 5.77^{a}	99.94 ± 0.01^{a}	0.07 ± 0.03^{b}
Three months-old seedlings	LB01	707.70 ± 250.07^{a}	273.27 ± 220.98^{a}	**203.72** ± 85.90^{a}	**6.52** ± 3.89^{bc}	**93.41** ± 10.08^{b}	**1.81** ± 2.46^{a}
	WPB	881.87 ± 49.47^{a}	263.80 ± 143.24^{a}	**281.17** ± 21.57^{a}	**6.45** ± 1.56^{bc}	**99.80** ± 0.06^{b}	**0.21** ±0.05^{a}
	Otto's	-	-	-	-	-	-
	Tris.$MgCl_2$	911.03 ± 56.91^{a}	233.60 ± 137.67^{a}	59.12 ± 6.17^{b}	12.86 ± 1.72^{c}	99.82 ± 0.02^{b}	0.15 ± 0.03^{a}
	Galbraith	*272.83 ± 472.56^{ab}	*141.57 ± 245.20^{a}	*14.18 ± 24.55^{b}	*1.63 ± 2.82^{ab}	*32.65 ± 56.55^{ab}	*0.41 ± 0.71^{a}
Six months-old seedlings	LB01	658.13 ± 249.40^{ab}	326.37 ± 295.42^{a}	223.24 ± 24.34^{ab}	**6.38** ± 1.01^{ab}	**96.44** ± 2.17^{a}	**1.38** ± 0.66^{ab}
	WPB	922.20 ± 24.53^{a}	365.93 ± 227.66^{a}	**362.78** ± 44.11^{a}	**4.47** ± 1.65^{ab}	99.59 ± 0.26^{a}	0.50 ± 0.30^{ab}
	Otto's	863.10 ± 49.96^{a}	306.53 ± 196.14^{a}	**228.19** ± 106.14^{ab}	7.18 ± 1.30^{ab}	99.72 ± 0.08^{a}	0.23 ± 0.10^{b}
	Tris.$MgCl_2$	*179.10 ± 310.21^{b}	*18.37 ± 31.81^{a}	*94.15 ± 163.07^{b}	*2.47 ± 4.28^{a}	*33.28 ± 57.65^{a}	*0.05 ± 0.09^{b}
	Galbraith	853.40 ± 179.79^{a}	538.93 ± 319.73^{a}	161.35 ± 12.76^{ab}	10.11 ± 1.22^{b}	**97.03** ± 1.96^{a}	**1.74** ± 0.87^{a}
Twelve months-old seedlings	LB01	466.70 ± 103.06^{a}	147.93 ± 116.39^{a}	164.34 ± 41.55^{bc}	10.28 ± 5.14^{a}	**97.57** ± 0.12^{a}	**0.83** ± 0.23^{a}
	WPB	909.67 ± 34.12^{a}	224.33 ± 57.91^{a}	**285.49** ± 28.90^{a}	**6.91** ± 2.65^{a}	99.71 ± 0.17^{a}	**0.34** ± 0.14^{a}
	Otto's	720.23 ± 63.31^{a}	142.43 ± 55.40^{a}	**238.15** ± 25.23^{ab}	**8.41** ± 3.06^{a}	**99.59** ± 0.33^{a}	0.27 ± 0.23^{a}
	Tris.$MgCl_2$	470.17 ± 98.76^{a}	141.43 ± 48.14^{a}	201.03 ± 24.71^{ab}	7.33 ± 0.55^{a}	99.70 ± 0.09^{a}	0.21 ± 0.06^{a}
	Galbraith	*425.50 ± 378.34^{a}	*395.30 ± 426.18^{a}	*83.11 ± 72.02^{c}	*8.63 ± 7.60^{a}	*64.73 ± 56.12^{a}	*1.11 ± 0.98^{a}

Values are given as mean and standard deviation of the mean (SD) of forward scatter (FS, channel units), side scatter (SS, channel units), fluorescence (FL, channel units), coefficient of variation of G0/G1 DNA peak (CV, %), debris background factor (DF, %) and yield factor (YF, %). Means for the same growth stages followed by the same letter (a, b or c) are not statistically different according to the multiple comparison Tukey's test at *P≤0.05*. The parameter's first and second highest (FL and YF) and lowest (CV and DF) values of the estimation in each essential parameter are shown in bold type. The nuclear lysis buffer chosen for the FCM of oil palm embryos and one to twelve months-old seedlings of oil palm are shown in bold type. *Buffer did not produce their nuclei from some leaf positions. Negative sign (-) indicate that no oil palm nuclei peaks were distinguishable.

Table 2. Estimation of genome size in oil palm embryos and one to twelve months-old seedlings of oil palm using *Glycine max* cv. Polanka (2C=2.5 pg) as external reference plant.

Growth stage	Buffer	C-DNA value (mean ± SD)		
		2C (pg)	1C (Mbp)	Peak CV(%)
Embryos	**LB01**	**3.75 ± 0.06**^{b}	1834	4.57 ± 0.38^{a}
	WPB	2.91 ± 0.15^{bc}	1422	6.59 ± 2.15^{ab}
	Otto's	4.88 ± 0.57^{a}	2389	4.93 ± 0.38^{a}
	Tris.$MgCl_2$	2.80 ± 0.04^{bc}	1370	7.31 ± 0.30^{ab}
	Galbraith	2.25 ± 0.71^{c}	1100	8.93 ± 2.11^{cb}
One month-old seedlings	LB01	1.95 ± 0.91^{b}	954	11.22 ± 3.73^{a}
	WPB	4.30 ± 0.54^{a}	2103	8.75 ± 3.48^{a}
	Otto's	2.08 ± 0.88^{b}	1017	12.87 ± 11.39^{a}
	Tris.$MgCl_2$	*1.38 ± 0.22^{b}	675	8.36 ± 7.26^{a}
	Galbraith	1.22 ± 0.48^{b}	597	18.86 ± 5.77^{a}
Three months-old seedlings	LB01	2.44 ± 1.09^{a}	1193	6.52 ± 3.90^{ab}
	WPB	**3.81 ± 0.30**^{a}	1863	6.45 ± 1.56^{ab}
	Otto's	-	-	-
	Tris.$MgCl_2$	0.75 ± 0.43^{b}	367	12.86 ± 1.72^{a}
	Galbraith	*0.55	269	1.63 ± 2.82^{ab}
Six months-old seedlings	LB01	2.76 ± 0.31^{b}	1350	6.38 ± 1.01^{b}
	WPB	4.91 ± 0.56^{a}	2401	4.47 ± 1.65^{b}
	Otto's	3.10 ± 1.44^{ab}	1516	7.18 ± 1.30^{ab}
	Tris.$MgCl_2$	*3.46	1692	2.47 ± 4.28^{b}
	Galbraith	1.94 ± 0.16^{b}	949	10.11 ± 1.22^{a}
Twelve months-old seedlings	LB01	2.02 ± 0.51^{bc}	988	10.28 ± 5.14^{a}
	WPB	**3.82 ± 0.39**^{a}	1868	6.91 ± 2.65^{a}
	Otto's	2.99 ± 0.31^{ab}	1462	8.41 ± 3.06^{a}
	Tris.$MgCl_2$	2.71 ± 0.33^{b}	1325	7.33 ± 0.55^{a}
	Galbraith	*1.57 ± 0.04^{c}	768	8.63 ± 7.60^{a}

Values are given as mean and standard deviation of the mean genome in mass values (2C, pg) and base pair (1C, Mbp), 1 pg = 978 Mbp (Dolezel et al., 2003). Means for the same growth stages followed by the same letter (a b or c) are not statistically different according to the multiple comparison Tukey's test at *P≤0.05*. The nuclear lysis buffer chosen for the FCM of oil palm embryos and three and twelve months-old seedlings of oil palm are shown in bold type. *Buffer did not produce their nuclei from some leaf positions. Negative sign (-) means that no oil palm nuclei peaks were distinguishable.

WPB, Otto's, Tris.$MgCl_2$ and Galbraith as nuclear lysis buffers, respectively (data not shown). Although LB01 and Tris.$MgCl_2$ were the most unchangeable buffers for a value of DNA content from FCM, only LB01 is the acceptable nuclear lysis buffer for 2C-DNA content value, followed by the first lowest value of %CV and the second highest of FL.

The 2C-DNA contents from analysis of one to twelve months-old seedlings of oil palm were significantly different in stages of oil palm seedlings and nuclear lysis buffers, with three positions of their unopened leaves. In one month-old seedlings, with the exception of WPB buffer, the lysis buffers mostly produced low 2C-DNA content (1.2 to 2.1 pg). Although leaf-bases of one month-old seedlings did not produce the highest 2-C DNA value of oil palm when using WPB buffer, leaf-base of unopened-leaves was found to be most suitable tissue for nuclei isolation. The DNA contents from this tissue were generally higher and more consistent than those obtained from leaf-apices and leaf-middles. Therefore, leaf-apices and leaf-middles of oil palm are not suitable tissues for FCM analysis.

For three and twelve months-old seedlings, the results revealed that the average 2C-DNA values from analysis with WPB were generally within the range of values obtained in the previous. Not only was the WPB buffer

found to be the suitable buffer, but also produced the highest values of 2C-DNA content. The results of 2C - DNA values from three months-old seedlings revealed that using Otto's as nuclear lysis buffer did not extract their nuclei from all leaf positions. In addition, both leaf-apices and leaf-middles were found to be less effective tissues when using Galbraith and Tris.$MgCl_2$ for three and six months-old seedlings of oil palm (Table 2). Since leaf positions used in this work were significantly different in genome size, especially 2C-DNA from leaf-bases of six and twelve months-old seedlings higher than those of leaf-apices and leaf-middles, we therefore used leaf-bases of oil palm as the plant material for FCM analysis in cultivars verifying experiment. Therefore, WPB buffer need to be used as the suitable buffer in the next research for cultivar classification in oil palm using young leaves of Dura and Pisifera cultivars as plant materials. In addition, these findings confirmed that DNA content level of oil palm (3.8 pg) could be considered as marker of Tenera for cultivar analysis by using FCM.

Estimation of nuclear genome size (C-DNA values) of Dura and Pisifera cultivars

Young leaves of five parents of Deli Dura and five parents of Pisifera (Calabar, LA ME, DAMI, Nigeria and EKONA) were investigated by using WPB as nuclear lysis buffer. FCM-derived histograms of both cultivars were differently in terms of peak position by using Tenera histograms as reference standard (2C = 3.8 pg) (Figure 2, 3 and 4). The results showed that DNA contents of Deli Dura (D109, D067, D064, D069 and D068) ranged from 6.3 to 7.6 pg, whereas the DNA content values ranging between 5.3 to 6.1 pg were found in young leaves tissues of Pisifera (Calabar; P109, LA ME; P106, DAMI; P116, Nigeria; P110 and EKONA; P105) (Table 3). Therefore, the numbers of base pairs of one haploid genome of Deli Dura and Pisifera parents had range greater than 3000 and 2000 Mbp, respectively.

DISCUSSION

Type of nuclear lysis buffer is known to be a criterion which has been less investigated (Loureiro et al., 2006a, b, 2007). Since the investigation of the various FCM
The five buffers used in this study were composed of different types and concentrations of chemical substances. Basically, Tris.$MgCl_2$ consists of pH stabilizer (Tris), inhibitor of clumping and attachment of debris (Triton X-100), substance for phenolic compounds bindings (PVP) and chromatin stabilizer ($MgCl_2$), whereas another chromatin stabilizer (spermine.4HCl) only found in LB01 buffer. WPB consists of different substances such as Na_2EDTA (chelating agent) and sodium metabisulfite (reducing agent which reduces phenolic compound reactions). Polyphenol oxidase inhibitor (catalyze the oxidation of phenolic compounds) is an acidulant such as citric acid or sodium citrate found in Otto's and Galbraith buffers, respectively. These acids are used for prevention of phenolic compound reactions. Morpholinepropanesulfonic acid (MOPS) is a pH stabilizer that is found in Galbraith buffer (Greihuber et al., 2007; Loureiro et al., 2007).

Phenolic compounds are plant secondary metabolites involved in the protection of plants against attacks of pathogens or injury, and are widely distributed in plant-derived product (Altunkaya and Gokmen, 2009). In this study, physical damage during the chopping process on leaf tissue of oil palm caused an increase of phenolic compound reaction, which often results in color degradation of the tissue or buffer solution from green to brown color. Comparing the chemical compositions on five nuclear lysis buffers, although WPB and Otto's buffers consisted of some reducing agents namely sodium metabisulfite and citric acid, respectively, only Otto's-derived nuclei solution was found to be browning. This implies that sodium metabisulfite in WPB had a positive effect for the prevention of oxidation of phenolic compounds. The effectiveness of this reducing agent could suppress some phenolic compounds release when chopping leaf tissues of oil palm in a buffer solution, resulting in consistency of their FCM 2C-DNA peak positions and their contents. As described above, the necessary substances used are an effective reducing agent namely sodium metabisulfite (in WPB) which results in high value of FL, low value of %CV and consistency of PI histogram. In addition, an effective chromatin stabilizer namely spermine 4.HCl (in LB01) results in high value of YF and low value of %DF.

After analyses of embryos and one to twelve months-old seedlings of oil palm with the five nuclear lysis buffers, the results confirmed that some phenolic compounds are regularly found in the nuclei extracting solution of seedlings than that of embryos. Because the seedlings gave the higher value of %CV than embryos, it is indicated that "the tannic acid effect" could cause effect on fluorescence histograms of oil palms seedling with higher %DF and higher %CV (Loureiro et al., 2007). Loureiro et al. (2006a) also analyzed the SS and %CV values of *Pisum sativum* nuclei in the solution of Tris.$MgCl_2$ supplemented with various concentrations of tannic acid (TA), a common phenolic compound, at a concentration of 0.75 to 1.00 mL^{-1} and showed poor reading in DNA peak positions which might be affected by their phenolic compounds. This reaction was termed "the tannic effect" by Loureiro et al. (2006a). The buffer that gave consistency of PI histograms with high FL, high YF, low %CV and low %DF should be selected for genome size analysis in cultivar verification. After analysis of five nuclear lysis buffers with embryos and

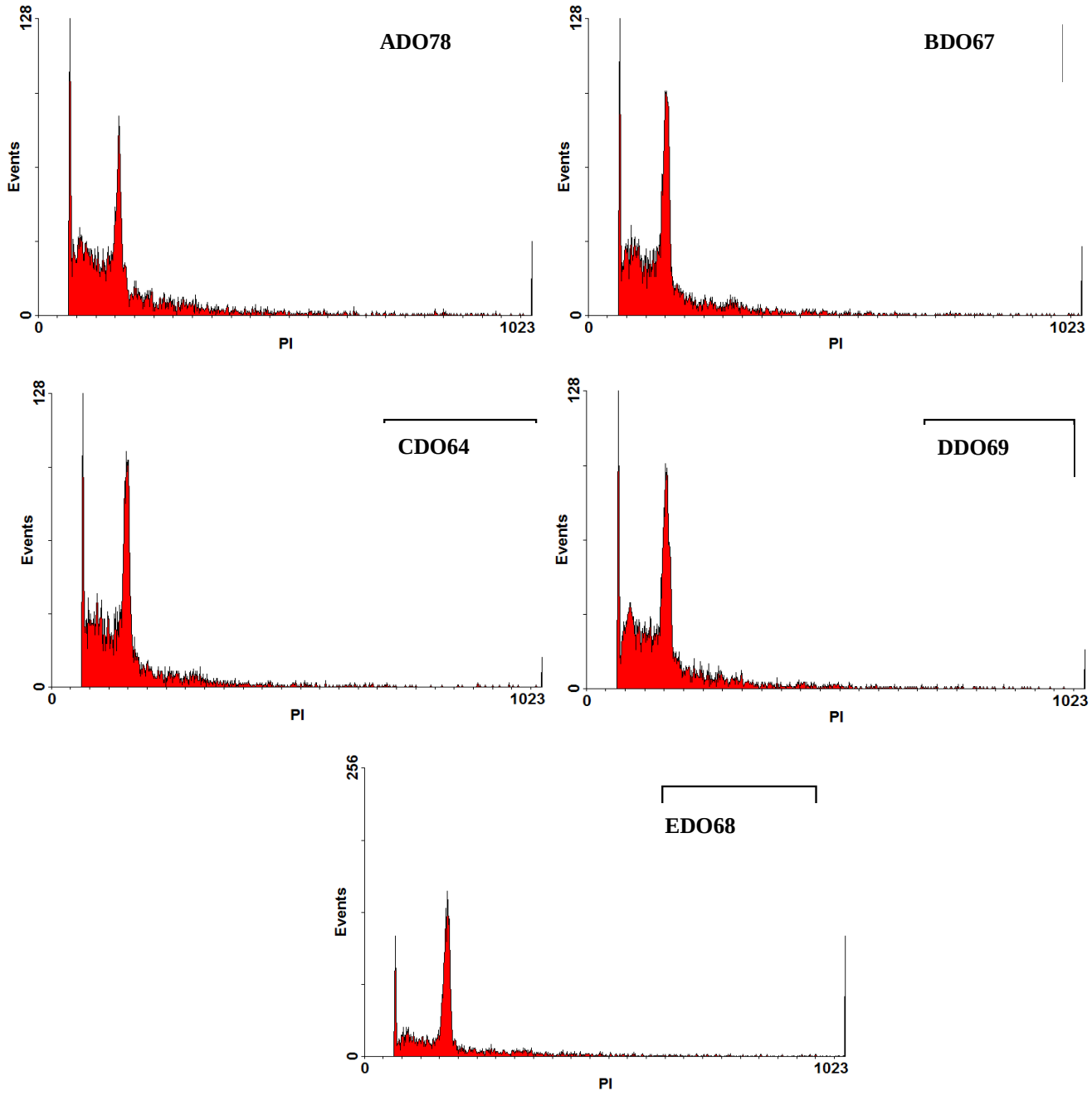

Figure 2. Histogram of relative fluorescence intensities (PI fluorescence) of five parents of Deli Dura; Deli Dura: D078 (A), Deli Dura: D067 (B), Deli Dura: D064 (C), Deli Dura: D069 (D) and Deli Dura: D068 (E), nuclei of young leaves isolated with WPB buffer.

seedlings of oil palm, the results revealed significant differences among the buffers and growth stages of oil palm used. Using LB01, 2C-DNA content of oil palm embryos was found to be 3.7 pg. This value closely resembled those obtained from Rival et al. (1997) (FACScan, Petunia hybrida, LB01), Srisawat et al. (2005) (FACSCalibur, G. max cv. Polanka, Tris.MgCl2) and Madon et al. (2008) (FACSCalibur, G. max cv. Polanka, LB01) for 3.7-3.8 pg though reference plant and type of buffer were used differently." The 2C-DNA contents of other buffers had wide range of 2C-DNA content from 2.25 to 4.88 pg, when different chemical compositions in each buffer and its concentration were used. When oil palm tissues at various growth stages were analyzed in

Table 3. Estimation of genome size (C-DNA values) in five Deli Dura and five Pisifera parents.

Hybrid Tenera	Cultivars (code)	C-DNA value (mean ± SD)		
		2C (pg)	1C (Mbp)	Peak CV (%)
Suratthani 1	Deli Dura (D078)	6.88 ± 0.67^{ab}	3362	3.84 ± 0.49^{ab}
	Calabar Pisifera (P109)	6.14 ± 0.74^{ab}	3005	4.58 ± 0.49^{ab}
Suratthani 2	Deli Dura (D067)	7.13 ± 0.27^{ab}	3484	4.17 ± 0.45^{ab}
	LA ME Pisifera (P106)	5.96 ± 0.66^{ab}	2915	6.13 ± 1.63^{a}
Suratthani 3	Deli Dura (D064)*	6.70 ± 0.23^{ab}	3278	4.52 ± 0.62^{ab}
	DAMI Pisifera (P116)**	5.37 ± 0.72^{b}	2628	6.08 ± 1.75^{ab}
Suratthani 4***	Deli Dura (D069)	6.35 ± 0.07^{ab}	3103	3.80 ± 0.34^{ab}
	EKONA Pisifera (P105)	5.44 ± 0.62^{b}	2659	5.08 ± 1.35^{ab}
Suratthani 5	Deli Dura (D064)*	6.70 ± 0.23^{ab}	3278	4.52 ± 0.62^{ab}
	Nigeria Pisifera (P110)	5.90 ± 1.09^{ab}	2884	4.81 ± 1.58^{ab}
Suratthani 6***	Deli Dura (D068)	7.62 ± 0.14^{a}	3725	3.30 ± 0.14^{b}
	DAMI Pisifera (P116)**	5.37 ± 0.72^{b}	2628	6.08 ± 1.75^{ab}

Values are given as mean and standard deviation of the mean genome in mass values (2C, pg) and base pair (1C, Mbp), 1 pg = 978 Mbp (Dolezel et al., 2003) using *Elaeis* cv. Tenera (2C = 3.8 pg) as external reference plant. Means for all cultivars followed by the same letter (a b or c) are not statistically different according to the multiple comparison Tukey's test at *P≤0.05*. * Tenera Suratthani 3 used the same Deli Dura with Hybrid Tenera Suratthani 5; **Tenera Suratthani 3 used the same DAMI Pisifera with Hybrid Tenera Suratthani 6; ***no longer available for offspring production.

different buffers, they gave different reading in DNA peak position among seedlings and its embryos, thus indicating that some interference occurred between the chopping and staining by phenolic compounds in those seedlings. Using LB01, Tris.$MgCl_2$, Otto's and Galbraith showed less 2C-DNA contents of seedlings than that of embryos, whereas higher 2C-DNA content is only found in WPB which might be affected from the preventer of phenolic compounds reaction (sodium metabisulfite) in WPB solution. For leaf position, 2C-DNA contents ranged from 1.47 to 2.90 pg for leaf-apices, whereas the DNA contents ranged from 1.09 to 5.48 pg and 0.55 to 4.75 pg when analyzed with leaf-middles and leaf-bases, respectively (data not shown). Consequently, there appears to be a close relation between the ability of nuclei isolation and unopened-leaf positions. In previous works, young leaves have been usually recommended for FCM analyses because most tissues do not contain some secondary metabolites (Jedrzejczyk and Sliwinska, 2010). In this paper, the average DNA content of leaf-bases of unopened leaves was found to be 3.89 pg (data not shown) compared to its embryos. This indicate that leaf-bases should not be avoided for FCM analysis of oil palm because of its easy nuclei isolation and freshness and its suitability for nuclei isolation in various types of isolation buffer. The youngest oil palm leaf, leaf- base, is therefore the suitable tissue for every FCM analysis of oil palm.

For cultivar verification, young leaves of five parents Deli Dura and Pisifera excised from adult tree (unopened leaves) were used as sources of nuclei. Although, the DNA contents of Deli Dura (D109, D067, D064, D069 and D068) and Pisifera (Calabar; P109, LA ME; P106, DAMI; P116, Nigeria; P110 and EKONA; P105) cultivars were not significantly different, but they should be distinctly separated into two groups. First group is the DNA contents that ranged higher than 6.3 pg (Deli Dura) and the second group with lower than 6.1 pg (Pisifera). These data were evaluated and compared to that of their hybrid. It is interesting to note that DNA contents of Tenera reported here (3.7 to 3.8 pg) were similar those obtained from Rival et al. (1997), Srisawat et al. (2005) and Madon et al. (2008) and were found to be significantly lower than that of their parents. These also resembled the results of using RAPD marker reported by Sathish and Mohankumar (2007), which revealed that Dura showed the highest amount of bands whereas the lowest was found in Tenera by using an effective marker: P15. A contradictory tendency was observed previously in the work of Madon et al. (2008) who reported DNA contents of Deli Dura and Pisifera as 4.10 and 3.64 pg, which were higher and lower than that of Tenera (3.8 pg),

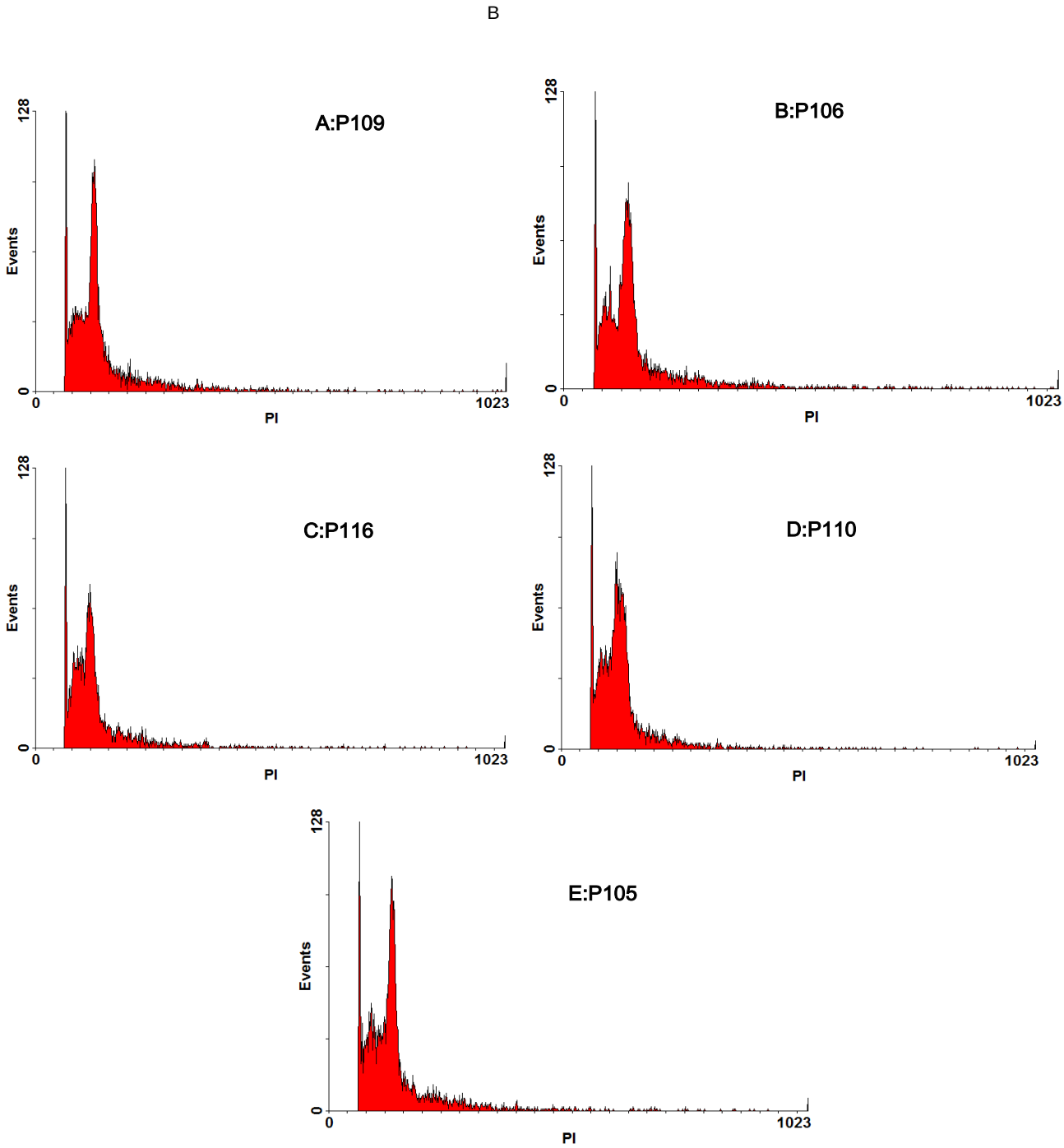

Figure 3. Histograms of relative fluorescence intensities (PI fluorescence) of five parents of Pisifera; Calabar: P109 (A), LA ME: P106 (B), DAMI: P116 (C), Nigeria: P110 (D) and EKONA: P105 (E), nuclei of young leaves isolated with WPB buffer.

respectively. Our results demonstrated that the Deli Dura and Pisifera DNA contents analyzed with WPB buffer using Tenera as external standard (3.8 pg) are first reported on using progeny DNA value to determine DNA values of their parents. The DNA content of five Deli Dura and five Pisifera parents can be used as a database in any further oil palm breeding programs and may be helpful in verifying cultivars or clone.

In conclusion, DNA contents of Deli Dura, Calabar, LA ME, DAMI, Nigeria and EKONA Pisifera can be determined by using DNA content of their hybrid as an external reference plant. The DNA contents of Tenera,

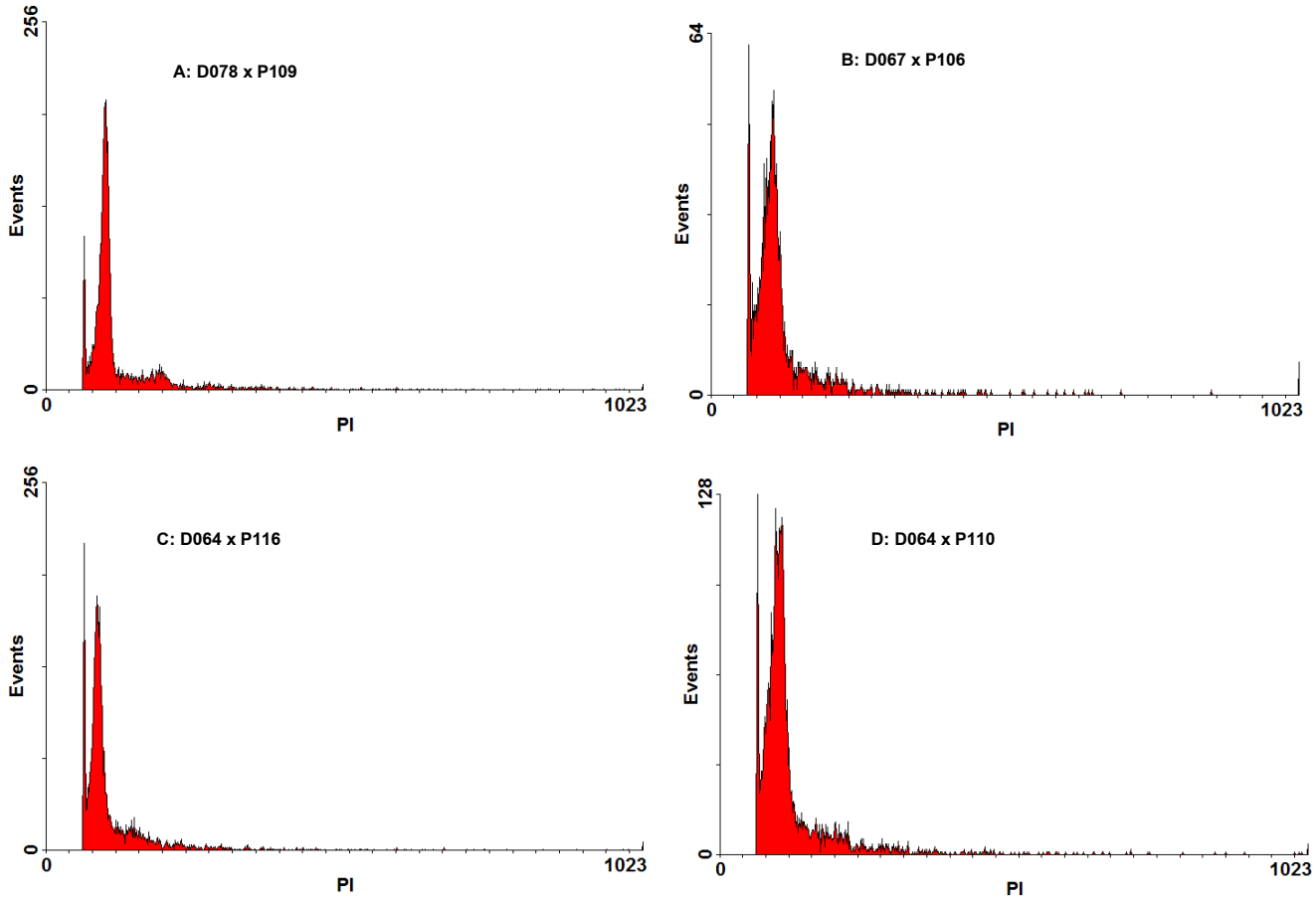

Figure 4. Histograms of relative fluorescence intensities (PI fluorescence) of four hybrid Tenera; Suratthani 1 (A), Suratthani 2 (B), Suratthani 3 (C) and Suratthani 5 (D), nuclei of young leaves isolated with WPB buffer.

Deli Dura and Pisifera are 3.8, 6.3 to 7.6 and 5.3 to 6.1 pg, respectively. These DNA contents of oil palm provide a useful database for routine breeding program and for routine assay of accepted cultivated cultivars. Further application of nuclear lysis buffer for FCM classification of oil palm cultivars should involve substances such as sodium metabisulfite and spermine.4HCl to produce higher FL and lower %CV with high YF and low %DF, respectively.

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