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GENOME SIZE ESTIMATION OF MPOB'S ELAEIS GERMPLASMS COLLECTION

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GENOME SIZE ESTIMATION OF MPOB'S *ELAEIS* GERMPLASMS COLLECTION

BY

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PENGANGGARAN SAIZ GENOM KOLEKSI MPOB *ELAEIS* GERMPLASMA

OLEH

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Laporan Projek yang dikemukakan sebagai syarat untuk memperolehi
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DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged.

30 April 2007 AINUL SHAZWIN SAHIDAN

This research project report is dedicated to my beloved family especially my grandmother, Mahaney Mohamed who had inspired me to complete this research. Grandmother was stricken with stroke during the preparation of this research project report and died on January 17, 2007. She will be greatly missed.

GENOME SIZE ESTIMATION OF MPOB'S *ELAEIS* GERMPLASMS COLLECTION

ABSTRACT

This study is focusing on genome size estimation for 11 germplasms from *Elaeis guineensis* and 5 germplasms from *Elaeis oleifera*. There are 4 stages involved in this research study which is leaf sample collections of *Elaeis* germplasms, processing of samples for nuclei preparation, followed by supplementation with propodium iodide (PI) and RNaseA prior to flow cytometry (FCM) analysis and lastly statistical analysis via MINITAB software. Flow cytometry (FCM) is a rapid and convenient technique that allows accurate determination of nuclear DNA content or genome size in plants compared to other methods. The standard reference used for the study on *Elaeis* germplasms genome size is soybean (*Glycine max cv*. Polanka, 2C=2.5 pg). According to the results, germplasm from Nigeria shows the highest genome size with 2C=4.202±0.35 pg and the germplasm from Tanzania shows the lowest genome size with 2C=3.659±0.253 pg at $p \leq 0.05$. The highest genome size from *Elaeis oleifera* is exhibited by the germplasm from Colombia with 2C=4.167±0.429 pg while the germplasm Suriname shows the lowest genome size 2C=3.392±0.534 pg at $p \leq 0.05$. The results revealed significant differences in genome size of *Elaeis guineensis* between Tanzania and Angola germplasms (2C=3.659±0.253 pg vs. 4.11±0.262 pg respectively), Tanzania and Guinea germplasms (2C=3.659±0.253 pg vs. 4.180±0.236 pg respectively), Tanzania and Madagascar germplasms (2C=3.659±0.253 pg vs. 4.137±0.131 pg respectively), Tanzania and Nigeria germplasms (2C=3.659±0.253 pg vs. 4.202±0.350 pg) where H_1 were accepted ($p \leq 0.05$). While for *Elaeis oleifera*, there are significant differences between

Suriname and Colombia germplasms (2C=3.392±0.534 pg vs. 4.168±0.429 pg respectively) as well as between Suriname and Honduras germplasms (2C=3.392±0.534 pg vs. 3.943±0.109 pg respectively) where H_1 were accepted ($p \leq 0.05$). Almost symmetrical high peak count and less than 5% coefficient variation (CV) value are the main factors considered in order to choose the best histogram peaks.

ABSTRAK

Kajian ini menjurus kepada penentuan saiz genom 11 germplasma *Elaeis guineensis* dan 5 germplasma *Elaeis oleifera.* Kajian ini mempunyai 4 peringkat iaitu pengumpulan sampel daun germplasma *Elaeis*, proses penyediaan asid nukleik sebelum penganalisian sampel daun dengan menggunakan flow cytometry (FCM) dan akhir sekali analisis statistik melalui perisian MINITAB. Flow cytometry merupakan satu kaedah yang mudah dan cepat dalam menentukan ketepatan anggaran jumlah DNA berbanding dengan kaedah-kaedah lain. Soybean (*Glycine max* cv. Polanka, 2C=2.5 pg) adalah standard piawai yang telah digunakan dalam kajian penganggaran saiz genom germplasma *Elaeis* ini. Berdasarkan keputusan yang diperolehi, germplasma daripada *Elaeis guineensis* iaitu yang berasal dari Nigeria mempunyai saiz genom yang paling tinggi (2C=4.202±0.35) pg dan Tanzania menunjukkan saiz genom yang paling rendah dengan nilai (2C=3.659±0.253) pg pada $p \leq 0.05$. Saiz genom yang paling tinggi bagi *Elaeis oleifera* pula ditunjukkan pada germplasma yang berasal dari Colombia dengan nilai 2C=4.167±0.429 pg dan nilai yang paling rendah pula adalah germplasma daripada Suriname dengan nilai 2C=3.392±0.534 pg pada $p \leq 0.05$. Daripada kajian yang dijalankan didapati terdapat perbezaan yang nyata bagi saiz genom diantara germplasma dari Tanzania dan Angola (2C=3.659±0.253 vs 4.11±0.262) pg, Tanzania dan Guinea (2C=3.659±0.253 vs 4.180±0.236) pg, Tanzania dan Nigeria (2C=3.659±0.253 vs 4.202±0.350) pg serta Tanzania dan Madagascar (2C=3.659±0.253 vs 4.137±0.131) pg daripada *Elaeis guineensis* germplasma dimana H_1 telah diterima ($p \leq 0.05$). Manakala untuk *Elaeis oleifera* pula perbezaan saiz genom yang nyata adalah diantara germplasma dari Suriname dan Colombia (2C=3.392±0.534 vs 4.168±0.429) pg serta Suriname dan Honduras (2C=3.392±0.534 vs 3.943±0.109) pg dimana H_1 telah diterima ($p \leq 0.05$). Puncak

histogram terbaik yang dipilih adalah berdasarkan nilai variasi koefisian (CV) yang rendah iaitu nilai yang kurang daripada 5% dengan puncak yang paling tinggi, runcing dan bersimetri.

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My gratitude also goes to University Putra Malaysia Bintulu Campus for giving me a chance and encouraged me to go ahead with my thesis. I also would like to thank my co-supervisor Dr. Maria Madon and all the committees of MPOB's especially En. Razuan Mahpot, En. Dzulkifly Salam and Pn. Zainab Anip who is most responsible to help me complete this thesis as well as the challenging research that lies behind it and for giving me permission to commence this thesis research.

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Lastly, I would like to express my deepest gratitude and appreciation to my beloved family members especially my mother Pn. Maskiah Abdul Manan and my grandmother Pn. Mahaney Mohamed for their endless love and support and special gratitude also goes to the only person that I love, my fiancé En. Zairul Azrin Md. Zainal whose patience love and enabled me to complete this work.

I certify that this project report entitled "Genome Size Estimation of MPOB'S *Elaeis* Germplasm*s* Collection" has been examined and approved as a partial fulfillment of the requirement for the degree of Bachelor of Bioindustry Science in the Faculty of Agriculture and Food Sciences, University Putra Malaysia Bintulu Campus.

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LIST OF ABBREVIATIONS

CV	Coefficient variation
cv	Cultivar
pg	Picogram
mM	Milimolar
mW	Miliwatt
%	Percentage
^{0}C	Degree Celsius
$\leq$	Less than or equal to
LBO1	Lysis buffer 1
DNA	Deoxyribonucleic acid
Na_2EDTA	Dinatrium ethylenediaminetetraacetic acid
KCl	Potasium cloride
NaCl	Sodium chloride
PI	Propidium iodide
ANOVA	Analysis of Variance
FACS	Fluorescence – activated cell sorter
FCM	Flow cytometry
MPOB	Malaysian Palm Oil Board
MAS	Molecular maker assisted selection
GM	Genetically modified
CCD	Charge – coupled deviced

CHAPTER I

INTRODUCTION

1.1 Background

The oil palm *Elaeis guineensis* is widely grown commercially in South East Asia, Equatorial America, Africa and South Pacific while for *Elaeis oleifera* itself was collected from Colombia, Panama, Costa Rica, Honduras, Brazil and Suriname in 1981-1982 (Rajanaidu, 1968c). Both *Elaeis guineensis* and *Elaeis oleifera* are from the subfamily Cocoineaea and belong to the family Palmae that contains 225 genera and 2600 species. The chromosome number of *Elaeis guineensis* and *Elaeis oleifera* is 2n=32 (Maria *et al*., 1998).

Elaeis guineensis can be classified according to the fruit structure that was known as *dura* (D), *tenera* (DxP) and *pisifera* (P). *Dura* is the thick-shelled fruit types with large kernels and small proportion of oil-bearing mesocarp. *Pisifera* is the shell-less fruit types with very much-reduced kernels and tenera is the commercial production of palms with the hybrid seeds *dura* (D) and *pisifera* (P). This cross breeding thin-shelled fruit type produces smaller fruits with smaller kernels but higher proportion of oil-bearing mesocarp. In Malaysian Palm Oil Board (MPOB) nursery ground, there are 11 germplasms of *Elaeis guineensis* collected from Senegal, Nigeria, Cameroon, Zaire, Tanzania, Madagascar, Angola, Gambia, Sierra Leone, Guinea and Ghana.

Another species of oil palm is *Elaeis oleifera* (HBK) Cortes, with slow stem growth, higher oleic content, high carotene but low oil yield compared to *Elaeis guineensis* (Rajanaidu *et al*., 1983). *Elaeis oleifera* and *Elaeis guineensis* hybrid (OxG) results in significantly higher oil yields and oleic content but the oil yield remains inferior to that of current *Elaeis guineensis* (*tenera*, DxP) (Hardon, 1969; Hardon and Tan, 1969). There are 6 germplasms of *Elaeis oleifera* in MPOB nursery ground, collected from Colombia, Honduras, Costa Rica, Panama and Suriname. The knowledge on genetic base is very important and useful in the efforts to study genetic diversity. Genetic diversity provides various interesting traits that exist in the germplasms collected, for example on pest and disease resistance (Maria, 1998).

The knowledge of DNA content in absolute unit (genome size) is important in many areas of research ranging from evolutionary studies to genome mapping (Galbraith *et al*., 1983). Flow cytometry (FCM) is a rapid and convenient technique that allows accurate determination of nuclear DNA content or genome size in plants (Dolezel, 1991). The analysis is based on the use of propidium iodide (PI) and on the analysis of the relative fluorescence intensity emitted by the stained nuclei. To determine genome size in absolute unit, fluorescence intensity of nuclei is compared with the fluorescence intensity of nuclei isolated from a species with known nuclear genome size. The results of the analysis are usually displayed in the form of histogram of relative fluorescence intensity. 2C is the unit used to indicate the unreplicated genome size of an organism and an example of a standard used for the study on genome size is soybean (*Glycine max cv*. Polanka, 2C=2.5 pg). Besides flow cytometry (FCM) method, there are also other methods to estimate the genome size of plants, such as microdensitometry and image cytometry method.

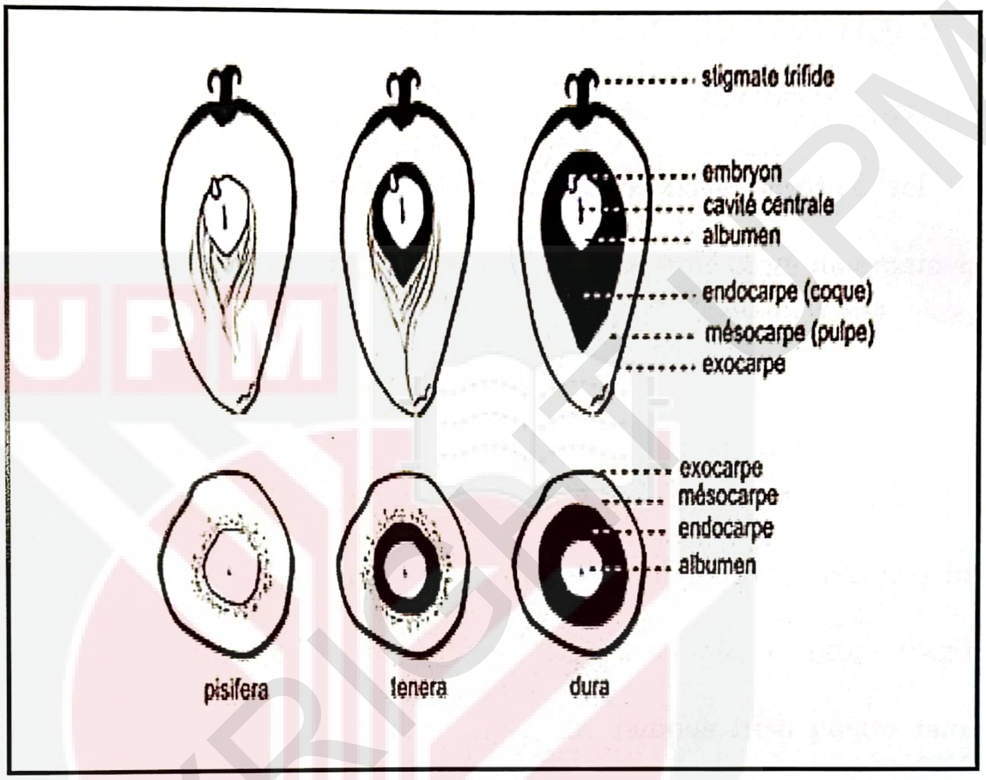


Figure 1.1 Oil palm fruit types

Source: http//www.yahoo.search/fruit/type/oil palm/htm.

1.2 Problem Statement

Both *Elaeis guineensis* and *Elaeis oleifera* are perennial monocot plants hence they have long generation time that is approximately 7-10 years and starts to bear fruit as early as three years after field planting and continues to do so for up to 25 years (Touchet (de) *et al.,* 1991). Even though conventional plant breeding has produced significant results in improving oil palm yield and oil quality but the progress is slow.

The long before fruit bearing year timeframe of the oil palm, its controlled pollination behavior, the narrow gene pool, the requirement for the large tracts of land and planting materials in breeding the oil palm contributed to the slowness of the conventional, more traditional genetic improvement in oil palm (Rajanaidu *et al.,* 1993). This limitation supported by the high demand of palm oil, make oil palm an ideal crop to use biotechnological approaches in its quest to improvement. The major approaches include tissue culture propagations, the use of molecular maker assisted selection (MAS), the production of genetically modified (GM) or transgenic crops and molecular cytogenetics.

The oil palm in Malaysia consists of very narrow genetic base hence an effort has been initiated by MPOB to collect oil palm germplasms of both *oleifera* and *guineensis* from South America and Africa continents. History has shown examples of crops fragility due to the very narrow genetic base. For example, the famous Irish potato famine where all the crops were damaged by the fungus *Phytophtora infestans*. At that time, Irish farmers grew limited number of potato clones derived from only two samples brought from South America (Crist, 1971).

Therefore using interesting traits from the various germplasms, palm oil genetic base can be improved using both conventional and biotechnological approaches. Using basic genetic information such as genome size, applied studies such as genetic mapping can be carried out before other more novel tools and techniques used.

1.3 Objective

I. To estimate and differentiate the genome size of 11 MPOB's germplasms collection of *Elaeis guineensis* (from Senegal, Gambia, Ivory Coast, Ghana, Nigeria, Cameroon, Zaire, Tanzania, Angola, Sierre Leone and Guinea).

II. To estimate and differentiate the genome size of 6 MPOB's germplasms collection of *Elaeis oleifera* (from Honduras, Costa Rica, Panama, Colombia, Brazil and Suriname).

III. To observe any variation of *Elaeis* germplasms genome size and to establish a database in genome size of *Elaeis* germplasms.

CHAPTER II

LITERATURE REVIEW

2.1 *Elaeis guineensis* and *Elaeis oleifera*

Elaeis guineensis and *Elaeis oleifera* (subfamily Cocoineae) belong to the family Palmae that contains over 225 genera and 2600 species (Purseglove, 1972). *Elaeis guineensis* are widely grown commercially in South-east Asia, Equatorial America, Africa and South Pacific. The name of the genus *Elaeis*, derived from the Greek word *elaion*, which means oil. It has become one of the most important economic crops for Malaysia and Indonesia .In 1763, Jacquin described the first species of the genus, *Elaeis guineensis* (Whitmore, 1973).

Elaeis guineensis Jacq., *tenera* (DxP) fruit type with thick mesocarp and thin shell is the commercial oil palm (2n=32) planted in Malaysia. It is a hybrid obtained by crossing the thick-shelled *dura* (D) with the shell-less female sterile *pisifera* (P). This perennial monocot grows to a height of 12 m. It starts to bear fruit as early as three years after field planting and continues to do so for up to 25 years (Touchet (de) *et al.,* 1991). The oil palm yields 4-6 t/ha/yr oil when at full yield. Currently, major DxP seed programmes are based on very restricted breeding populations derived from a few specific breeding materials ie. Deli *dura* and AVROS *pisifera* (Kushairi and Rajanaidu, 2000).

Elaeis oleifera, has slow stem growth, higher oleic content, high carotene content but low oil yield compared to *Elaeis guineensis* (Rajanaidu *et al*., 1983). Interest in *Elaeis oleifera* has also increased by the discovery of its resistance to Lethal Bud Rot in Colombia (Hartley, 1988). Crossing *Elaeis oleifera* with *Elaeis guineensis* (OxG hybrid) results in significantly higher oil yield and oleic content but the oil yield remains inferior to that of the current *Elaeis guineensis* (*tenera*, DxP) (Hardon 1969; Hardon and Tan 1969). Realizing this, MPOB (Malaysian Palm Oil Board) initiated and established a sizeable oil palm gene bank with materials collected from Africa and South America since 1975 for conservation as well as to broaden the genetic base of the breeding populations (Rajanaidu, 1994).

2.2 *Elaeis* Germplasm

Germplasm is a term used to describe the genetic resources, or more precisely the DNA of an organism and collections of that material from exotic varieties or wild species into agronomically acceptable backgrounds (Jones *et al*., 1983) Oil palm germplasms collected from different parts of the world are maintained in the form of field genebank or living collection. There are 3 ways to conserve all the germplasm material; field genebank, in-vitro and in-situ conservation. Germplasm collection is important to gather the maximum amount of genetic variability (Marshall and Brown, 1975).

There are 11 germplasms of *Elaeis guineensis* originated from Nigeria, Cameroon, Zaire, Tanzania, Madagascar, Angola, Senegal, Gambia, Sierra Leone, Guinea and Ghana. For Nigeria germplasm, the different characters of *dura* and *tenera* were studied. For *duras*, the fruit weight varied from 2 g to 23 g and the coefficient of variation (C.V) was 34%. For *tenera*, it was 1.2 g to 15.4 g and the C.V was 30%. There are 15% to 79% of the mesocarp for *duras* and 16% to 97% for teneras (Rajanaidu *et al.,* 1979; Obasola *et al.,* 1983; Ooi and Rajanaidu, 1979; Rajanaidu 1994).

Cameroon germplasms were collected at 32 different sites distributed throughout Cameroon. They were collected randomly by covering the whole country as far as possible. The fruit characteristic study showed that the *duras* weight is 10.3 g per single fruit and the mesocarp of the fruit is about 40% while for the *teneras*, it was 8.6 g per single fruit and 62.4% of the fruit is the mesocarp (Blaak, 1967).

Zaire germplasms were collected at 56 different sites distributed throughout Zaire. Zaire *duras* has 14.2 g weight of single fruit and 43.9% of the fruie is the mesocarp and the corresponding figure for the *teneras* was 12.6 g single fruit and 64.1%, the mesocarp.

Tanzania germplasms were collected at 13 sites located near Kigoma along the Lake Tanganyika. The characteristics of materials collected have been compiled. For *duras*, a single fruit weight 16.9 g and 46.7% of it is the mesocarp. The corresponding figure for *teneras* was 15.5 g of single fruit and 70.6% of mesocarp (Ricahrdson and Chavez, 1986).

Madagascar germplasms were sampled at 4 sites and 17 samples were collected. One to six samples were collected at each site and the method of data collection was similar to

Nigeria germplasms. It was difficult to identify the fruit form because they were extremely small and birds damaged the mesocarp of the fruit. Poor environment and low rainfall in the western part of Madagascar caused problems in collecting the Madagascar germplasms (Richardson and Chavez, 1986).

Angola germplasms were sampled at 8 sites. A total of 54 bunches were collected in Angola. The palm groves were dense and actively exploited for the oil at Cabinda (Rajanaidu *et al.,* 1991).

Senegal germplasms were sampled at 13 sites and 104 samples were collected in Senegal. Due to the low rainfall and differential survival rate of duras and teneras in harsh environment, only dura palms were encountered. The mean bunch weight is 5.9 kg and 35.1 % of it is the mesocarp (Rajanaidu and Jalani, 1994a).

For *Elaeis oleifera*, there are 6 germplasms originated from Colombia, Honduras, Costa Rica, Panama, Suriname and Brazil and they are maintained as field genebank in MPOB Kluang Research Station (Rajanaidu, 1986c).

2.3 Genetic Resource of Oil Palm

The Irish potato famine is one of the most dramatic example that illustrate the importance of having a wide genetic base for crops. The famine was caused by the fragility of crop due to a narrow genetic base where limited numbers of potato clones planted were damaged when they were attacked by the fungus *Phytophthora infestan* (Crist, 1971). For oil palm crops in Malaysia, the narrowness of gene pools has been recognized as a major obstacle to perform selection progress (Arasu and Rajanaidu, 1975; 1976; Hardon and Thomas, 1968; Hardon, 1974; Hardon *et al.,* 1985; Hartley, 1988; Jagoe, 1952, Ooi *et al.,* 1973; Ooi and Rajanaidu, 1979; Rajanaidhu and Abdul Halim Hassan, 1986; Thomas *et al.,* 1969).

In common with several plantation crops in South-East Asia (notably rubber, cocoa, coffee, tea, cinchona), the genetic origin of the commercial material is rather narrow and to some extent almost accidental. The oil-palm industry in Indonesia and Malaysia started with material descended from four palms (thick-shelled dura) introduced in the 19th Century in the botanical garden of Bogor (Indonesia). Their simultaneous introduction (probably from Mauritius) suggests that the four seeds may well had been derived from a single (open-pollinated) fruit bunch. Seeds of these palms and their descendants were widely distributed throughout Indonesia as ornamental palms. Avenue palms in Deli (North Sumatra) supplied the seeds for the first oil palm estates from 1911 onwards. In Malaysia, the first estate was established in 1917. By the early 1920s, a number of breeding and selection programmes had started, which produced improved planting material generally referred to as *Deli Dura*. Until the 1950s, *Deli Dura* was used exclusively as planting material in both Indonesia and Malaysia (Corley *et al.,* 1976).

Elucidation of single-gene inheritance of shell thickness caused interest in the *tenera* fruit type (sh + sh -) as commercial material obtained from a cross of *dura* (sh + sh +) with pisifera (sh - sh -). Material segregating for the shell-thickness gene descending from a single *tenera* palm (SP 540) in Sumatra was a major source of *pisifera* for several breeding programmes. This palm probably has a common origin with material in the breeding programme at Yangambi (Zaire), descending from nine *tenera* palms. By 1960s, major breeding programmes in Sumatra and Malaysia concentrated primarily on *Deli Dura* and 'Yangambi' *Pisifera* for production of commercial planting material. Since then, extensive new introductions have been produced from various breeding programmes in West Africa (Ivory Coast, Nigeria, Cameroon, Zaire) (Chevalier, 1943). In the late 1970s and early 1980s, the Palm Oil Research Institute of Malaysia (Porim) had started a systematic programme of prospection and collection of *Elaeis oleifera* germplasms from oil palm groves in West Africa, South and Central America, widening significantly the basis for breeding programmes (Hardon *et al.,* 1985).

The knowledge of genetic base is very important for crops. Molecular techniques are useful to study genetic diversity (Mayes *et al.,* 1995; Shah *et al.,* 1994). A number of tissue culture laboratories were established throughout the world especially in Malaysia to propagate high yielding oil palm. Tissue culture techniques are extremely useful to propagate parental *dura* and *pisifera* palms to produce biclonal seeds while advancement in genetic engineering can reduce the flower abnormality in oil palm clones (Corley *et al.,* 1986).

Fifty years ago, mutation breeding, cytogenetic, and quantitative genetic principles had been used to develop novel planting materials. It is important in diversifying current planting materials for fuel and nutritional products, food, nutraceuticals and pharmaceuticals. Breeding programmes can be accelerated with the advance of molecular makers and oil palm genetic mapping techniques. Through genetic transformation, interesting foreign genes can be introduced into the oil palm genome in order to modify the fatty acid composition of palm oil, insert disease resistance genes, fruit abscission genes etc (Maria,1998).

The relationship between genome size, gene number and the specific patterns of genes distributions among taxa has remained the subjects of intense interest for several decades, yet for the most part the questions remain unresolved. There are many other aspects of the genome (e.g. control of gene expression, genome organization, DNA interactions, genome structure) where knowledge is essential to understand how the information contained within the DNA sequences is translated into a fully functional oil palm especially in terms of their productivity. Nuclear DNA of an organism influences its phenotype in two ways:

I. By the expression of its genetic content (an area where knowing the DNA sequence is important).

II. By the physical effects of its mass and volume which impose absolute limits on the range of phenotypes that can be expressed by genetic control. This second effect of the DNA on an organism's phenotype is known as the 'nucleotype' - a term coined by Bennett to define those conditions of the nuclear DNA which affect the phenotype independently of its encoded informational content (Bennett *et al*., 1995).

2.4 Genome Size Estimation

The knowledge of DNA content in absolute unit (genome size) is important in many areas of research ranging from evolutionary studies to genome mapping (Galbraith *et al.*, 1997). The genomic genome size reported by Rival *et al.,* (1997) using flow cytometry technique for *Elaeis guineensis* (*tenera*) is 2C=3.736 ± 0.125 pg. Jones *et al.,* (1982) reported the value as 2C=2.4 ± 0.4 pg using the microdensitometric method on root tips of oil palm regenerants. Roser *et al.*, (1997) reported the genome size as 4C=4.00 and 4.8 pg respectively using also microdensitometric method. However, there is no value reported on the *Elaeis oleifera.*

Nuclear genome size is typically measured in eukaryotes using either densitometric measurement of Feulgen-stained nuclei with specialized densitometer quipments; using computerized image analysis or technique referred to as image cytometry ; or flow cytometry (Hardie *et al.*, 2002). Genome size correlates with a range of features at the cell and organism levels, including cell size, cell division rate depending on the taxon, body size, metabolic rate, developmental rate, organ complexity, geographical distribution and / or extinction risk (Bennett and Leitch 2005; Gregory 2005). When analyzing nuclear genome size, many authors have noted its variation within species (Price and Johnston. 1996a; Rayburn *et al.*, 1997, 2004; Ellul *et al.*, 2002). However, the occurrence of this phenomenon and its extent remains a matter of discussion, as contradictory results have been obtained by different authors when analyzing the same material (Greilhuber and Obermayer, 1997; and Price *et al.*, 2000; Suda, 2004).

Flow cytometry (FCM) is a powerful technique that was originally developed to count blood cells (Sharpiro, 2004). With improvement to the equipment and methodologies, FCM has been adapted to many research areas of biology including plant sciences (Dolezel, 1997). Since the introduction of FCM to plant studies, estimation of DNA ploidy level and determination of nuclear genome size have been the two most frequent applications (Bennett and Leitch, 2005). Owing to its ease, rapidity and accuracy, FCM has been an attractive alternative to traditional methods such as Fuelgen micro densitometry (Dolezel and Bartos, 2005).

In this study, the method of estimation is by using laser source flow cytometer and the standard used is soybean leaf tissue (*Glycine max cv.* Polanka, 2C=2.5 pg). Soybean was chosen as standard reference due to its complete genome sequencing compared to other plants and also due to the proximity of *Glycine max* genome size to oil palm. Flow cytometer is a rapid and convenient technique that allows accurate determination of nuclear DNA content or genome size in plants (Dolezel, 1991). The analysis is based on the use of propidium iodide (DNA intercalating fluorochrome) and the relative fluorescence intensity emitted from the particular stained nuclei displayed in the form of histogram of relative fluorescence intensity. To determine genome size in absolute unit, fluorescence intensity of nuclei is compared with the fluorescence intensity from a species with known genome size.

A reliable estimation of genome size required proportionality between the digitized fluorescence signal and genome size. It depends on several factors such as stoichiometry of dye binding to DNA, accessibility of DNA to the fluorochrome, fluorescence absorption and linearity of the instrument amplification system (Bagwell *et al.*, 1989). The accessibility of nuclear DNA to fluorochrome has recently been a topic of major concern as it was found that cytosolic compounds could interfere with fluorescent staining of nuclei in suspensions (Price *et al.*, 2000). These observations indicated that FCM could produce flawed data if the effect of cytosolic compounds is ignored.

Price *et al.* (2000) speculated that inhibitors that decrease dye fluorescence of nuclei were found commonly in plants. The authors did not point to a specific compound but suggested involvement of one or more of the numerous secondary metabolites. Noirot *et al.* (2000), working with coffee trees, revealed negative effects of cytosol on accessibility of DNA to propidium iodide (PI) and showed that cytosolic compounds could bias genome size estimated by up to 20%. More recently, Noirot *et al.*, (2003) identified two compounds that influenced PI fluorescence of petunia nuclei: caffeine and chlorogenic acid (a precursor of polyphenols). Whereas caffeine increases PI accessibility to petunia DNA, chlorogenic acid significantly decreased petunia nuclei fluorescence. However, in oil palm, stoichiometric errors were not observed (Noirot *et al.*, 2003).

2.5 Comparison of Methods In Determination of Genome Size

Several methods have been employed to quantify nuclear DNA. Some of the earliest studies involved bulk biochemical DNA extraction techniques to estimate the total genome size of a preparation, which was then divided by an estimate of the number of nuclei present. Although imprecise, these methods were sufficient to demonstrate the constancy of DNA across tissues and among conspecific individuals, and to hint at the pronounced variation of genome size in comparisons of different species. Analysis of reassociation kinetics similarly provided usable but somewhat questionable estimates of genome size. A short time after the publication of these initial exploratory studies, methodologies began to shift towards densitometric techniques. This approach remains prominent and underpins the methodology describe here (Maria, 1998).

2.5.1 Densitometric

Densitometric methods was first employed for relative nuclei acid quantifications in the 1930s, although actual genome size measurements were not made with these techniques until some time later. The landmarks surveys of both Swift (1950) and Mirsky and Ris (1951) made use of densitometric techniques to quantify nuclear DNA, as have countless studies (Rasch 1985). This process involves staining fixed (air-dried) tissue preparations on microscope slides by the Feulgen reaction, most often using Schiff leucofuchisn sulfurous acid reagent (although occasionally other chemical stains have been used, e.g. acriflavin, gallocyanin chromalum). Feulgen densitometry relies on the simple premise that the amount of stain bounded is directly proportional to the amount of DNA present. The quantity of stain is itself determined based on the amount of light it absorbs (i.e., its density). Detailed reviews of the physics of cytophotometry are available elsewhere (Fukuda *et al.,* 1987).

2.5.2 Flow Cytometry

Flow cytometry analyses microscopic particles in suspension, which are constrained to flow in single file within a fluid stream through the focus of intense light. Pulses of scattered light and fluorescence are collected and converted to electric current pulses by optical sensors and later classified. Because the particles are analysed individually and at high speed, large populations can be measured in a short time and the presence of subpopulations may be detected (Shapiro, 2004). Since there is no need to employ tissues with dividing cells, the ease of sample preparation, and the ability to measure DNA quickly in large populations of cells, made flow cytometry an attractive alternative to microspectrophotometry. Indeed, there has been a shift towards flow cytometry during the last decade (Bennett and Leitch, 1995).

2.5.3 Image Analysis Densitometry or Image Cytometry

Image analysis has occasionally been considered superior to flow cytometry (Pindur *et al.,* 1994; Yamamoto *et al.,* 1994). More recently, Feulgen image analysis has been used for quantitative DNA studies in plants (Venora *et al.,* 1995a,b; Cremonini *et al.,* 1998; Voglmayr and Greilhubber 1998; Volgmayr 2000), and its utility in the determination of plant genome sizes has been examined in detail. However, this technique has thus far been used in only a very small number of genome sizes studies. Feulgen image analysis provides a rapid, cost-effective and user friendly method for the measurement of genome sizes.

In this application, the microscope field is captured by a microscope-mounted CCD (charge-coupled device) or digital camera connected to a computer via a frame-grabber board. As with all digital images, these photos are displayed as a series of pixels, each of which is of a specific color and intensity. The different intensities of the various nuclear pixels represent ready-made point intensities that can be converted to absorbance values by the image analysis software. A color image of stained nuclei can be made into a single linear scale of pixels intensities by converting the image to grayscale, by using monochromatic incident light or by analyzing only one of the three constituent channels (red, green or blue) that make up the color pixel.

2.6 Advantages and Disadvantages of Flow Cytometry and the Importance of Propidium Iodide (PI) fluorescence stain in determining Genome size.

Flow cytometry has been known as a simple, rapid, accurate and convenient method for determining ploidy levels of DNA, assessment and analysis of the cell cycle of large cell populations (Winklemann *et al.,* 1998; Dolezel, 1991). However, FCM does not permit morphological confirmation of measured objects compared to image analysis densitometry where the image or the morphology of the cells can be captured using a microscope accompanied with digital camera. However, the disadvantage of image cytometry and micro densitometry is that only small number of cells can be analysed at any one time compared to flow cytometry where thousands of cells can be analyzed (Winklemann *et al.,* 1998; Dolezel, 1991).

Propidium iodide (PI) is one of the fluorescent stain or intercalating dye used to stain the nuclei. It is a fluorogenic compound that binds stoichiometrically to nucleic acids so that fluorescence emission is proportional to the genome size of the cell. In apoptosis studies, when apoptotic cells are stained with PI and analyzed with a flow cytometer, they display a broad hypodiploid (sub-G_1) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) genome size in the red fluorescence channels. This method appears to offer a number of advantages. It allows

(i) a rapid, reliable and reproducible estimate of apoptosis, (ii) simultaneous analysis of cell-cycle parameters of surviving cells. Another important concern in quantitative evaluation of apoptotics cells by flow cytometry is the discrimination of true apoptotic nuclei from nuclear debris.

CHAPTER III

MATERIAL AND METHODS

3.1 Material

3.1.1 Plant Samples

Leaf samples (frond -1) were collected from 11 *Elaeis guineensis* germplasms (Senegal, Gambia, Ivory Coast, Ghana, Nigeria, Cameroon, Zaire, Tanzania, Angola, Sierra Leone and Guinea) and 5 *Elaeis oleifera* germplasms (Colombia, Honduras, Costa Rica, Panama and Suriname) that was planted and maintained as field genebank in MPOB Kluang Research Station, Johor, Malaysia and MPOB Keratong Research Station, Pahang, Malaysia. Frond -1 was used because it gave consistent result, good and sharp histogram peak with lower CV value compared to frond 1 or frond 0 when checked with FCM. Frond -1 is shown in Appendix F. All samples were listed in Table 3.1 for *Elaeis guineensis* and Table 3.2 for *Elaeis oleifera*. Figure 3.1 shows the location of the samples area. Table 3.1 is a list of *Elaeis guineensis* germplasms. A trial can be defined as a plot or location for the planted *Elaeis* at a nursery ground.

Table 3.1 List of *Elaeis guineensis* germplasms

Germplasm Origin	**Sample (Trial/no.of tree)**	**Location**
Ghana	0.397/21	MPOB Kluang, Johor Bharu
	0.397/27	MPOB Kluang, Johor Bharu
	0.397/28	MPOB Kluang, Johor Bharu
Sierra Leone	0.355/139	MPOB Kluang, Johor Bharu
	0.355/208	MPOB Kluang, Johor Bharu
	0.355/10	MPOB Kluang, Johor Bharu
Nigeria	0.395/282	MPOB Kluang, Johor Bharu
	0.395/278	MPOB Kluang, Johor Bharu
	0.395/247	MPOB Kluang, Johor Bharu
Guinea	0.353/197	MPOB Kluang, Johor Bharu
	0.353/305	MPOB Kluang, Johor Bharu
	0.353/381	MPOB Kluang, Johor Bharu
Angola	0.311/713	MPOB Kluang, Johor Bharu
	0.311/729	MPOB Kluang, Johor Bharu
	0.311/745	MPOB Kluang, Johor Bharu
Tanzania	0.256/221	MPOB Kluang, Johor Bharu
	0.256/335	MPOB Kluang, Johor Bharu
	0.256/220	MPOB Kluang, Johor Bharu
Madagascar	0.240/3	MPOB Kluang, Johor Bharu
	0.240/19	MPOB Kluang, Johor Bharu
	0.240/20	MPOB Kluang, Johor Bharu
Gambia	0.398/96	MPOB Kluang, Johor Bharu
	0.398/108	MPOB Kluang, Johor Bharu
	0.398/106	MPOB Kluang, Johor Bharu
Senegal	0.396/357	MPOB Kluang, Johor Bharu

	0.396/87	MPOB Kluang, Johor Bharu
Zaire	0.349/247	MPOB Keratong, Pahang
	0.349/246	MPOB Keratong, Pahang
	0.349/241	MPOB Keratong, Pahang
Cameroon	0.349/18	MPOB Keratong, Pahang
	0.349/20	MPOB Keratong, Pahang
	0.349/21	MPOB Keratong, Pahang

Table 3.2 List of *Elaeis oleifera* germplasms

Germplasm Origin	**Sample (Trial/no. of tree)**	**Location**
Colombia	0.211/1238	MPOB Kluang, Johor Bharu
	0.211/1236	MPOB Kluang, Johor Bharu
	0.211/1230	MPOB Kluang, Johor Bharu
Honduras	0.211/1064	MPOB Kluang, Johor Bharu
	0.211/1316	MPOB Kluang, Johor Bharu
	0.211/1332	MPOB Kluang, Johor Bharu
Costa Rica	0.211/1267	MPOB Kluang, Johor Bharu
	0.211/1054	MPOB Kluang, Johor Bharu
	0.211/1228	MPOB Kluang, Johor Bharu
Panama	0.211/1233	MPOB Kluang, Johor Bharu
	0.211/1231	MPOB Kluang, Johor Bharu
	0.211/1229	MPOB Kluang, Johor Bharu
Suriname	0.177/3	MPOB Kluang, Johor Bharu
	0.177/4	MPOB Kluang, Johor Bharu
	0.177/68	MPOB Kluang, Johor Bharu

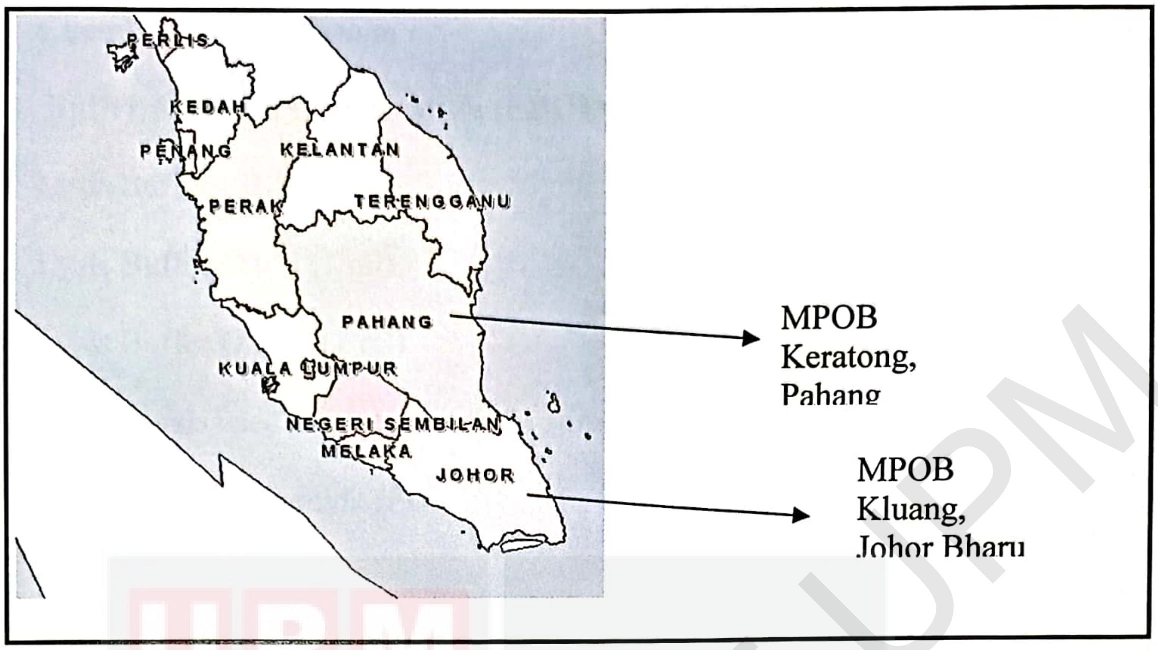


Figure 3.1 Samples location of *Elaeis guineensis* and *Elaeis oleifera*

Source: http://images.search.yahoo.com/search/images/view?back

Table 3.3 Latitude and longitude of samples locations

Location	**Latitude**	**Longitude**
Keratong,Pahang	3^0N	103^0E
Kluang, Johor Bharu	2^0N	103 ¼ ^{0}E

3.1.2 Chemicals and solutions

3.1.2.1 Buffer for Nuclei Preparation (LBOI)

1. Lysis Buffer LBO1 (1 ml)
2. Lysis Buffer Otto I (1 ml)
3. Lysis Buffer Otto II (1 ml)

   * Chemicals used in preparing these solution are listed in Appendix M.

4. 50 µl propodium iodide (PI)
5. 50 µl RNaseA

3.1.3 Tools

3.1.3.1 Tools used for Nuclei preparation

1. Surgical blades
2. 1 ml syringe
3. 10 ml falcon tube
4. Sieve 70 µm
5. Beaker 50 ml
6. Micropipetter
7. Pipette Tips

3.1.4 Equipments

1. Medicon blender - Becton Dickinson, USA
2. Medimachine - BD Medimachine™
3. Fridge
4. FACS (Fluorescence-activated cell sorter) Calibur Flow Cytometer with

argon ion laser (15mW)

5. Cell Quest Software
6. MINITAB statistic software

3.2 Methods

3.2.1 Leaf Samples Preparation

Three leaflets were taken from the base, middle and end regions of fronds -1 (refer to appendix E, F and G). The leaflets were cleaned and must be in good condition for the further steps. There are nine replicates for each leaflet from three different *Elaeis* trees of the same germplasm.

3.2.2 Nuclei preparation

For nuclei preparation, about 1cm x 1cm leaf samples were taken from the base, middle and end region of the nine different leaflets from frond -1 for both *Elaeis guineensis* and *Elaeis oleifera*. The leaf samples per leaflet were combined and sliced into smaller pieces using sharp clean blades. After that, the sliced fresh leaf materials were placed into a 50 µm Medicon blender container (Becton Dickinson, USA) containing 1 ml of lysis buffer LBO1 supplemented with 50 µg propidium iodide (PI) and 50 µg RNaseA. The mixture was blended using Medimachine for 3-5 minutes. After that, the suspension nuclei were then collected from the Medicon blender using a 1 ml syringe and filtered through a 70-µm sieve (Becton Dickkinson, USA) into a 10 ml falcon tube using a sieve. The suspension nuclei were stored at 4^0C for 48 hours prior to FCM analysis.

For *Elaeis oleifera* (Suriname) only, 1 ml Lysis Buffer Otto I was used and the leaf samples were manually chopped using sharp clean blades followed by filtering to get the suspension nuclei. Then, the suspension nuclei were centrifuged for 10 second to get the DNA pellet prior to adding 1 ml Lysis Buffer Otto II and supplemented with 50 µg PI, 50 µg RNaseA and 40 µl mercathoethanol. The mixture was immediately put into the flow cytometry for FCM analysis.

3.2.3 Determination of Genome Size

A FACS Calibur flow cytometer (Becton Dickinson, USA) equipped with an argon ion laser (15 mW) at 488 nm was used to analyze fluorescent intensity of nuclei. The histogram was collected over 1024 channels and for each samples, 25 000 events were captured. The values of oil palm and soybean histogram peak means were obtained using Cell Quest software. Using these values, 2C genome size is calculated by obtaining the ratio of oil palm to soybean fluorescent intensity and multiplied with 2.5 pg i.e genome size of soybean. A one-way ANOVA and Tukeys test were performed to detect any significant differences between the different samples.

3.2.4 Statistical Analysis

The formula that was used to calculate 2C genome size of *Elaeis* germplasm with the soybean (*Glycine max* cv, Polanka 2C=2.5 pg) as a standard external is:

$$2C\ \textit{Elaeis}\ \text{DNA} = \frac{2C\ \text{soybean DNA} \times \text{Mean of}\ \textit{Elaeis DNA}}{\text{Mean of soybean DNA}}$$

There were 27 samples (9 replicates x 3 leaflets) for each germplasm being studied and the comparison among the germplasms genome size was done. A one-way ANOVA and Tukey's test were performed to detect any significant differences between the different samples. Figure 3.2 and Figure 3.3 shows the flow chart and the diagram of the experimental procedure.

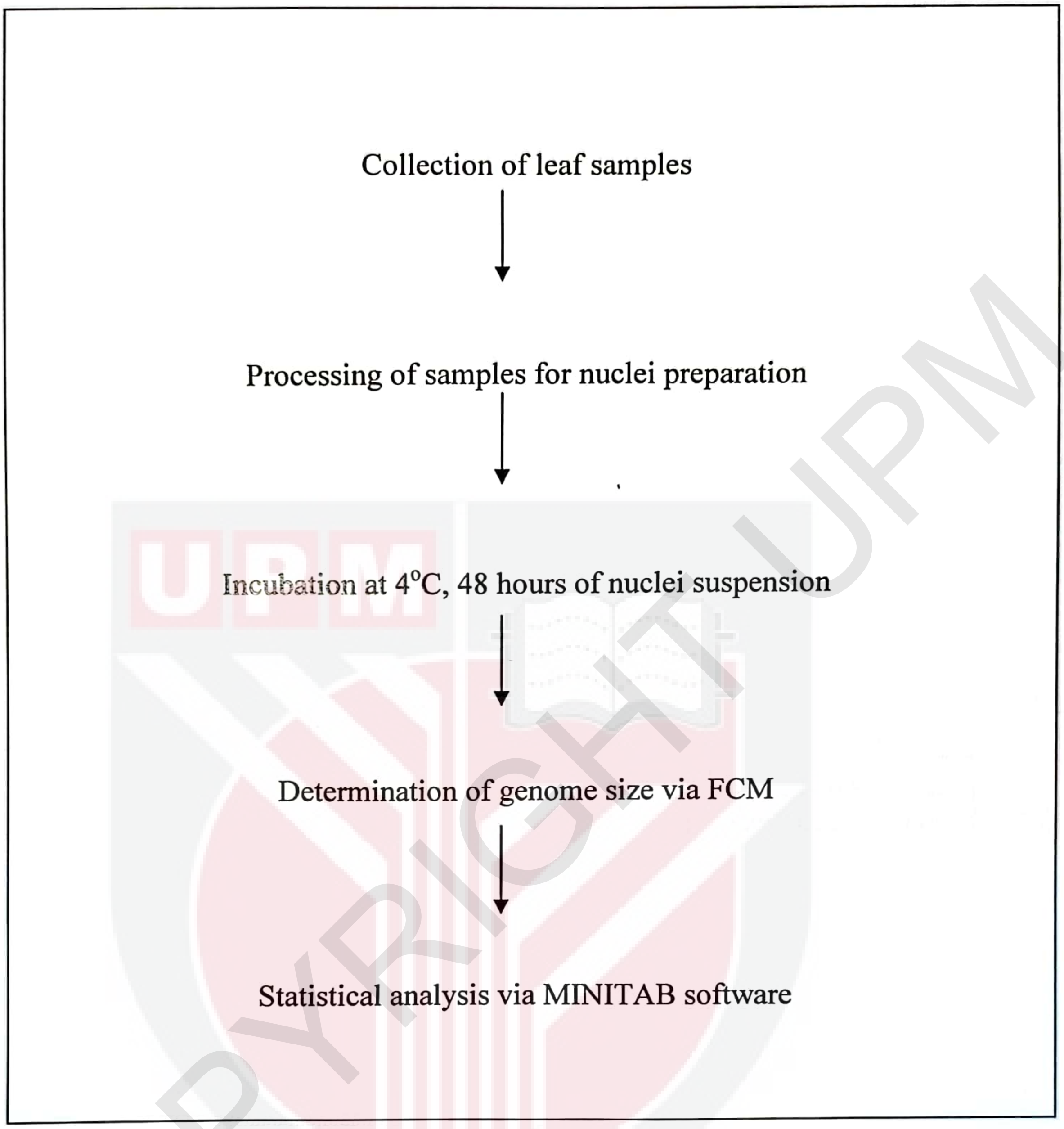


Figure 3.2 Flow Chart of Experimental Procedure

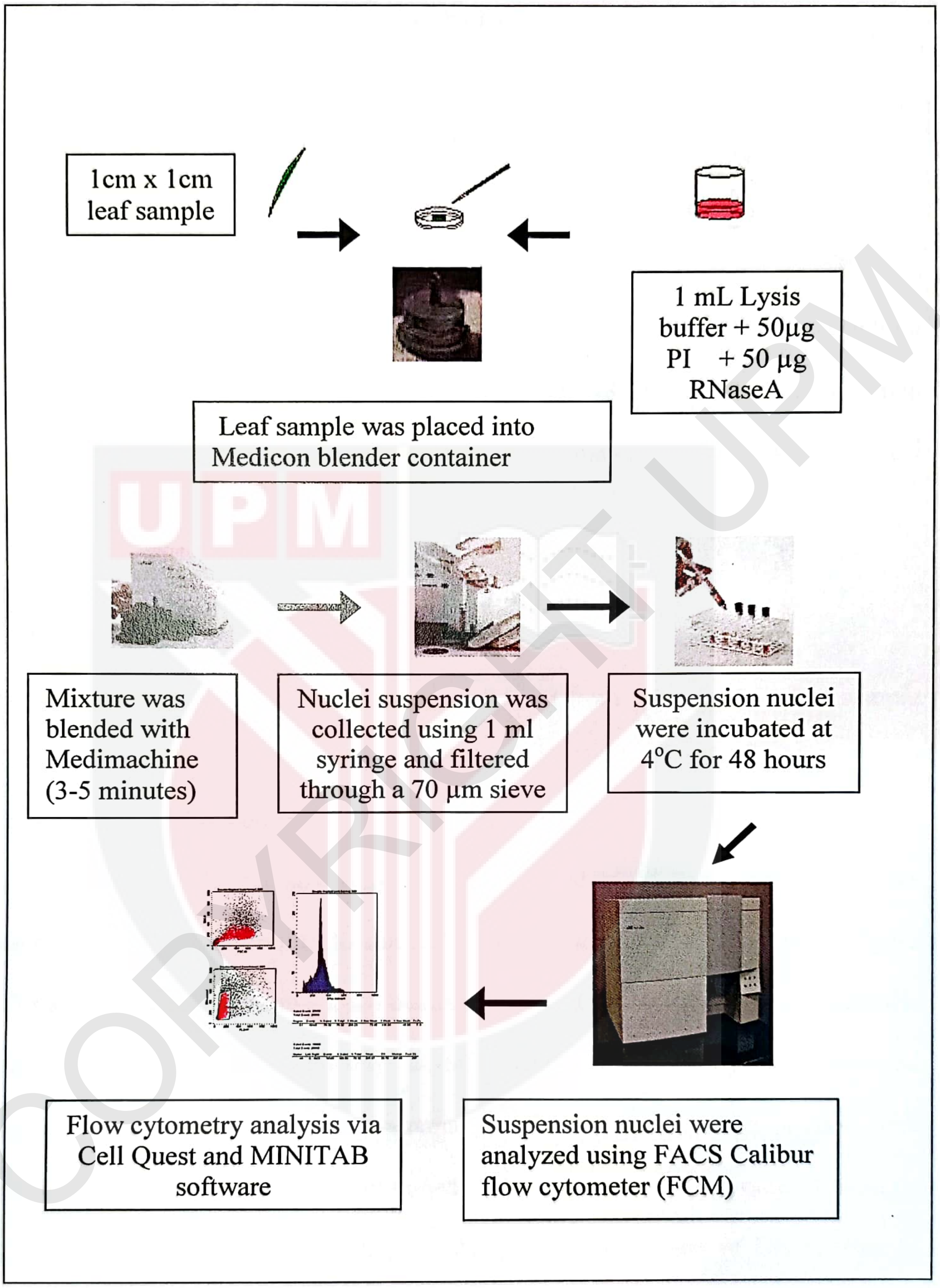


Figure 3.3 Flow Diagram of Experimental Procedure

CHAPTER IV

RESULT

4.1 Nuclei Incubation

The nuclei preparations using LBO1 lysis buffer supplemented with 50 µg propidium iodide and 50 µg RNaseA were incubated at 4°C for 48 hours to obtain optimum histogram of fluorescent intensity peak. For nuclei samples prepared using Otto I and Otto II lyses buffer, incubation step is not required.

4.2 Fluorescent Intensity Histogram Analysis

For estimation of genome size, 25,000 nuclei released from a 1cm x 1cm leaf sample were analzed using FACS Calibur flow cytometer. The high quantity of nuclei being analysed will reduce the percentage of coefficient variation (CV) for the 2C histogram peaks. The estimated genome size values were calculated based on the ratio of relative fluorescence intensity of samples to standard *Glycine max* (2C = 2.5 pg, refer Figure 4.1). For all analysis, more than 25,000 nuclei were isolated since the flow cytometer is capable of measuring approximately 20-60 nuclei per second in a low-speed configuration and well-defined histograms of fluorescent intensity peaks with acceptable CV values (<5.0%) were obtained. Most of the samples produced a single histogram peak at highest G0/G1 phase or 2C (unreplicated state of DNA, refer to Figure 4.1) while G2/M peak with 4C DNA (refer to Figure 4.1) content will occur at a higher fluorescent intensity than the G0/G1 peak and at much lower nuclei counts. This indicated existence of a small population of nuclei undergoing DNA replication prior to mitosis. The selection criteria for the 2C peaks are based on an almost symmetrical fluorescent intensity histogram peak with the highest count and less than 3% CV. This showed high quantity of 2C nuclei isolated and they were not lysed during incubation period. Figure 4.1 shows the fluorescent intensity histogram peak of *Glycine max* cv. Polanka and Figure 4.2a – 4.3d shows the impressive fluorescent intensity histogram peaks of *Elaeis guineensis* and *Elaeis oleifera* germplasms with the soybean (*Glycine max*) as the standard reference.

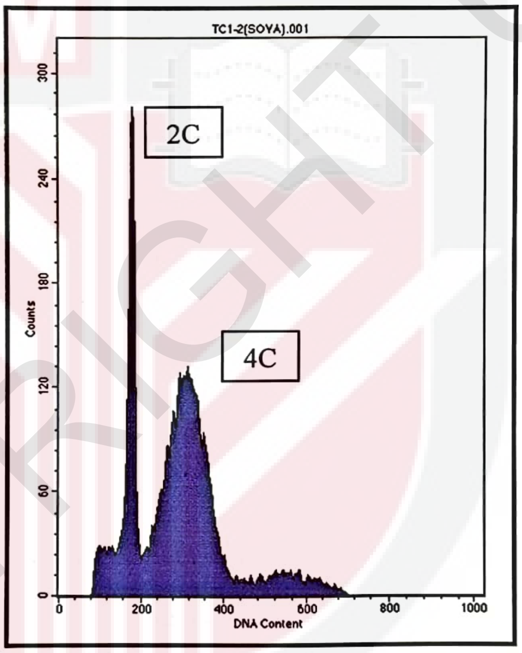


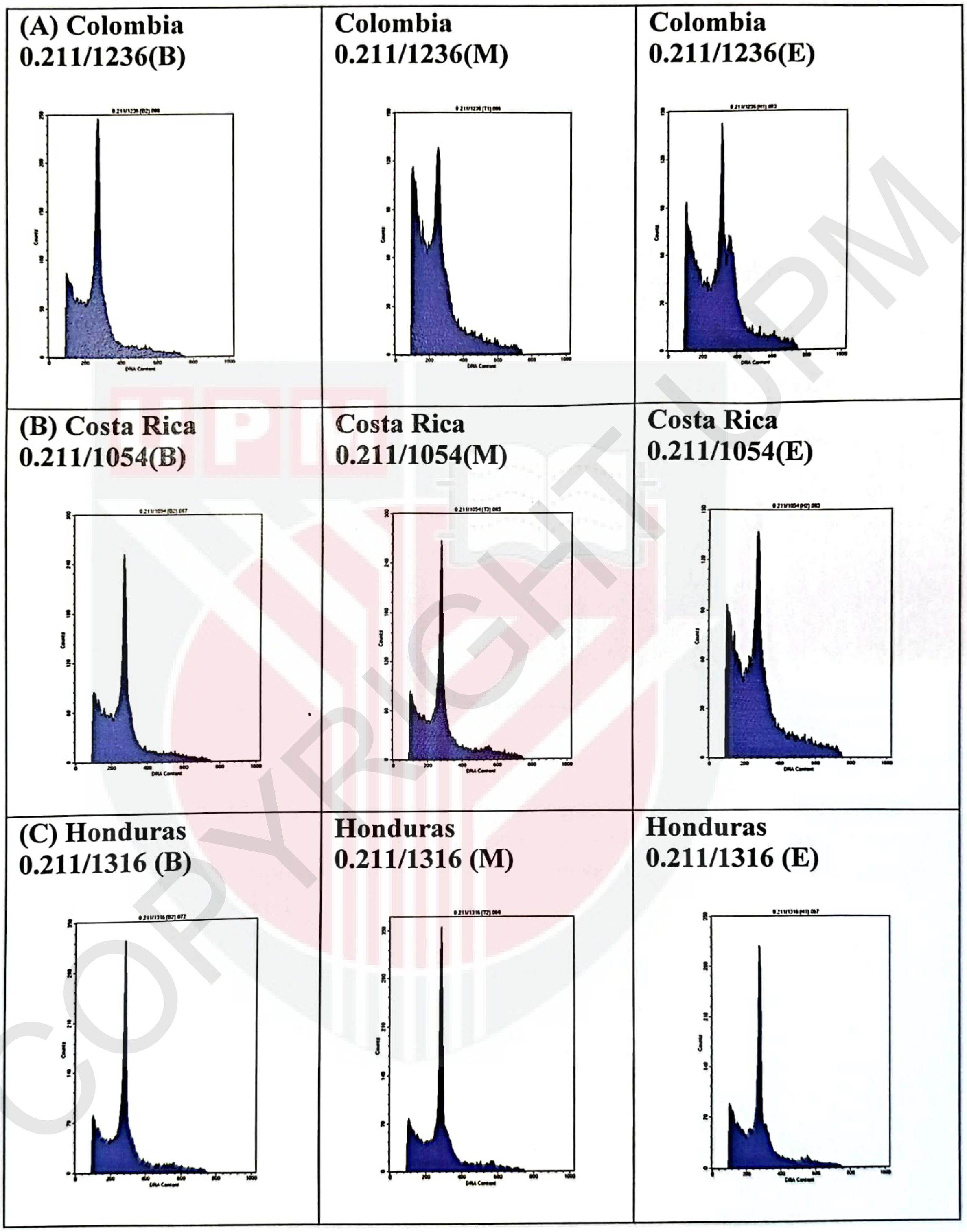
Figure 4.1 Fluorescent intensity histogram peak of *Glycine max* cv. Polanka.

2C: Unreplicated state of DNA (interphase stage)

4C: Replicated state of DNA (synthesis stage)

4.3 Fluorescent Intensity Histogram Peaks of *Elaeis* Germplasms

4.3.1 *Elaeis oleifera* Genome Size Histograms



(A) Colombia 0.211/1236(B)

Colombia 0.211/1236(M)

Colombia 0.211/1236(E)

(B) Costa Rica 0.211/1054(B)

Costa Rica 0.211/1054(M)

Costa Rica 0.211/1054(E)

(C) Honduras 0.211/1316 (B)

Honduras 0.211/1316 (M)

Honduras 0.211/1316 (E)

Figure 4.2a Fluorescence intensity histogram peaks for *Elaeis oleifera* germplasms originating from (A) Colombia (B) Costa Rica and (C) Honduras. B, M and E indicate leaf samples originating from the base (B), middle (M) and end (E) regions of Frond –1.

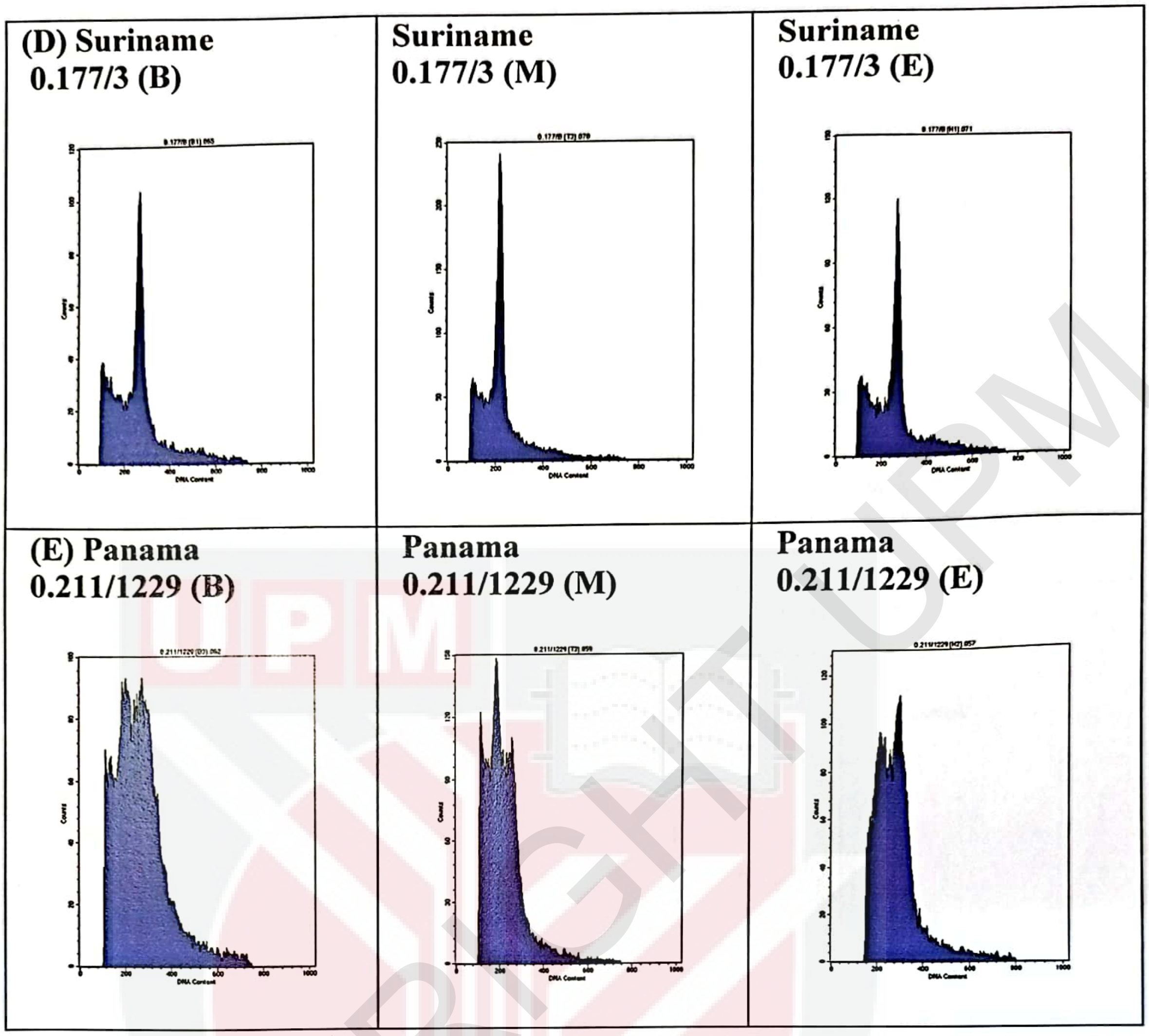
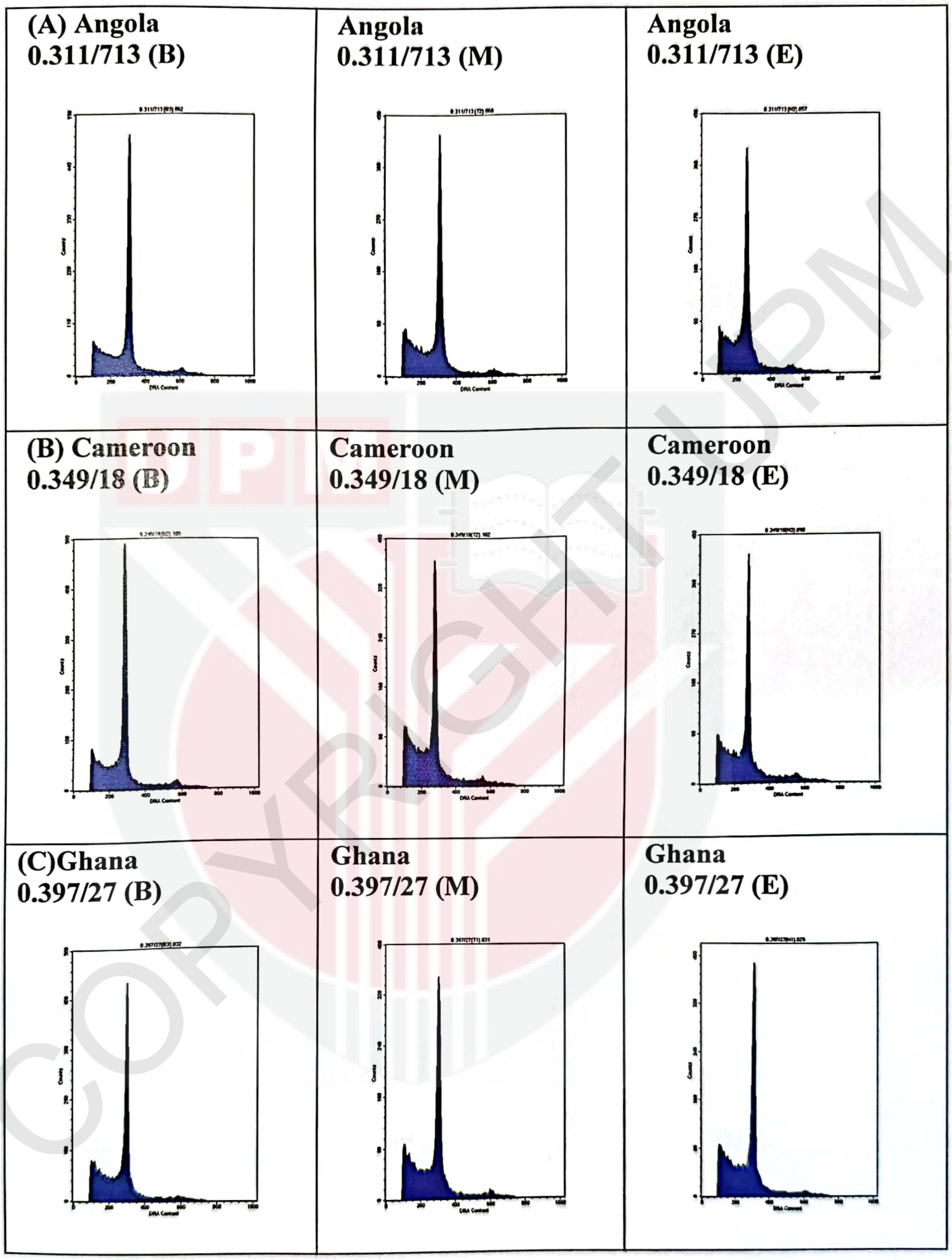


Figure 4.2b Fluorescence intensity histogram peaks for *Elaeis oleifera* germplasms originating from (D) Suriname and (E) Panama. B, M and E indicate leaf samples originating from the base (B), middle (M) and end (E) regions of Frond –1.

4.3.2 *Elaeis guineensis* Genome Size Histograms



(A) Angola 0.311/713 (B)

Angola 0.311/713 (M)

Angola 0.311/713 (E)

(B) Cameroon 0.349/18 (B)

Cameroon 0.349/18 (M)

Cameroon 0.349/18 (E)

(C)Ghana 0.397/27 (B)

Ghana 0.397/27 (M)

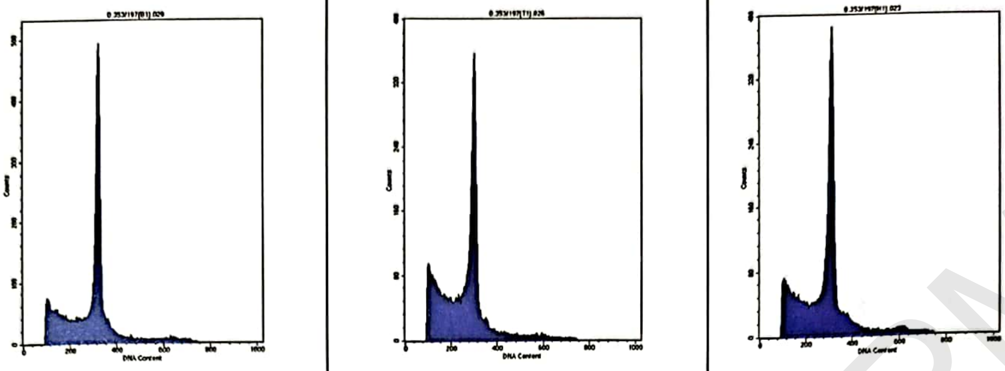
Ghana 0.397/27 (E)

Figure 4.3a Fluorescence intensity histogram peaks for *Elaeis guineensis* germplasms originating from (A) Angola (B) Cameroon and (C) Ghana. B, M and E indicate leaf samples originating from the base (B), middle (M) and end (E) regions of Frond –1.

(D) Guinea
0.353/197 (B)

Guinea
0.353/197 (M)

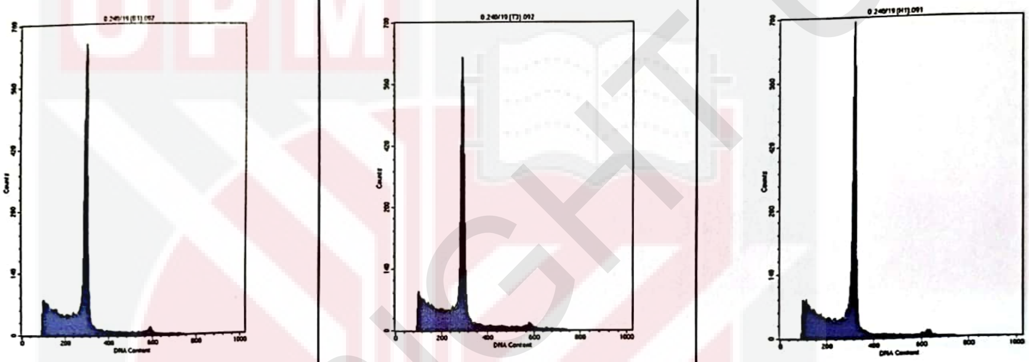
Guinea
0.353/197 (E)



(E)Madagascar
0.240/19 (B)

Madagascar
0.240/19 (M)

Madagascar
0.240/19 (E)



(F) Nigeria
0.395/282 (B)

Nigeria
0.395/282 (M)

Nigeria
0.395/282 (E)

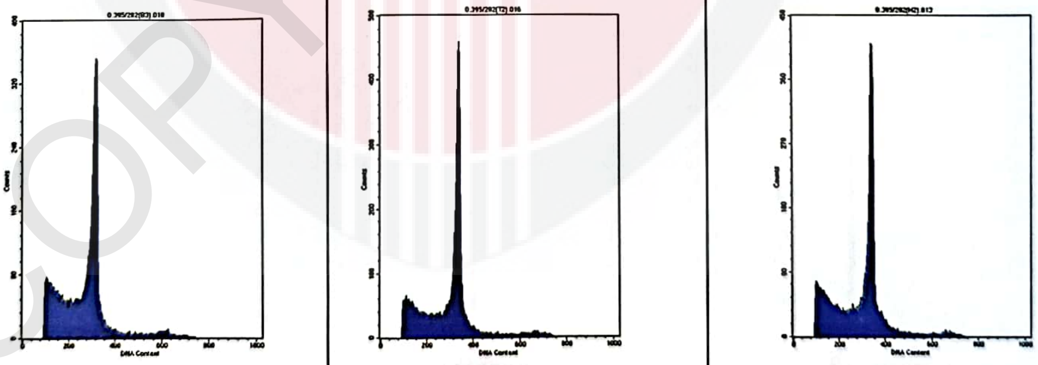
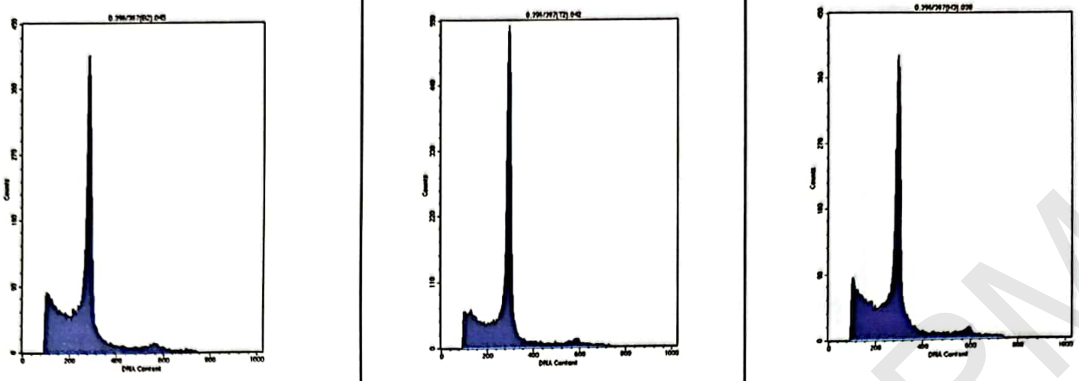
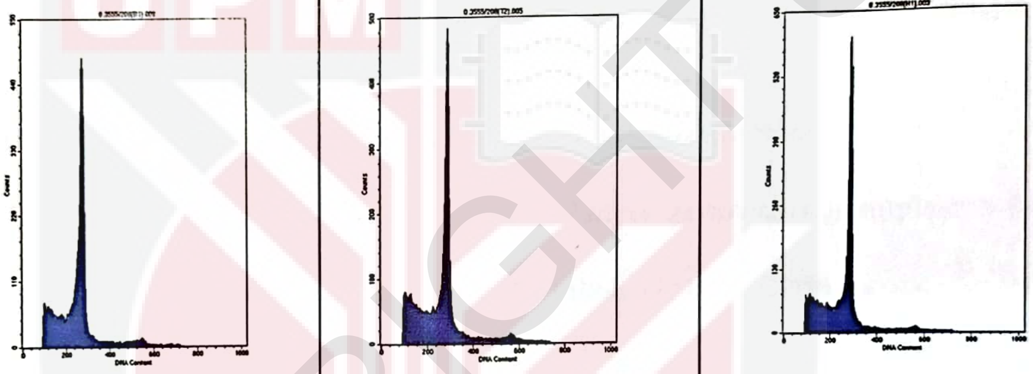


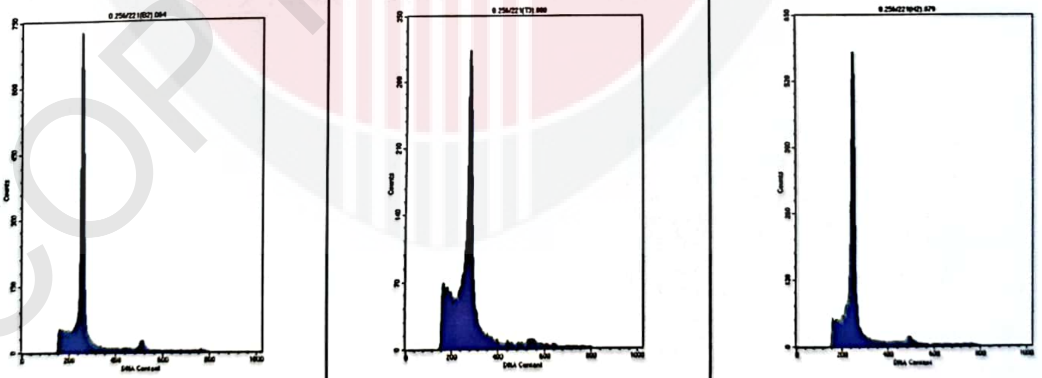
Figure 4.3b Fluorescence intensity histogram peaks for *Elaeis guineensis* germplasms originating from (D) Guinea (E) Madagascar and (F) Nigeria. B, M and E indicate leaf samples originating from the base (B), middle (M) and end (E) regions of Frond –1.



(G) Senegal 0.396/387 (B) | **Senegal 0.396/387 (M)** | **Senegal 0.396/387 (E)**

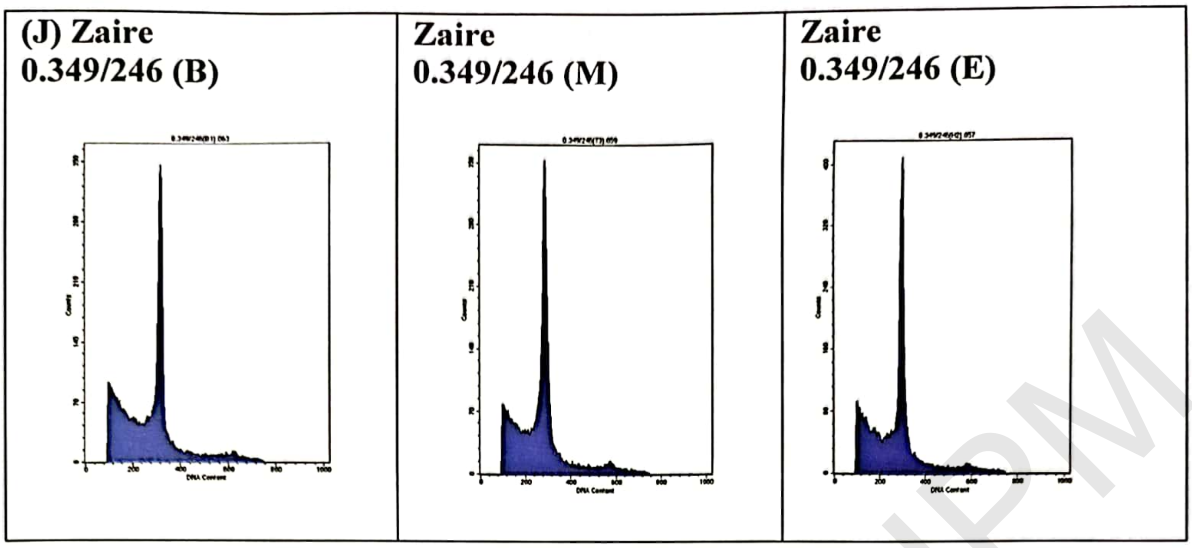


(H) Sierra Leone 0.355/208 (B) | **Sierra Leone 0.355/208 (M)** | **Sierra Leone 0.355/208 (E)**



(I) Tanzania 0.256/221 (B) | **Tanzania 0.256/221 (M)** | **Tanzania 0.256/221 (E)**

Figure 4.3c Fluorescence intensity histogram peaks for *Elaeis guineensis* germplasms originating from (G) Senegal (H) Sierra Leone and (I) Tanzania. B, M and E indicate leaf samples originating from the base (B), middle (M) and end (E) regions of Frond –1.



(J) Zaire 0.349/246 (B)

Zaire 0.349/246 (M)

Zaire 0.349/246 (E)

Figure 4.3d Fluorescence intensity histogram peaks for *Elaeis guineensis* germplasm originating from (J) Zaire. B, M and E indicate leaf samples originating from the base (B), middle (M) and end (E) regions of Frond –1.

4.4 Estimation of *Elaeis guineensis* Germplasms Genome Size

Table 4.1 lists the estimated 2C genome size of *Elaeis guineensis* germplasms from Nigeria, Guinea, Madagascar, Angola, Zaire, Gambia, Ghana, Sierra Leone, Senegal, Cameroon and Tanzania where there were 9 replicates for each germplasm being prepared (refer to appendix H and I for the calculation). Table 4.2 shows Tukey's pairwise comparison among *Elaeis guineensis* germplasms genome size.

Table 4.1 Estimated Genome Size and the CV values of *Elaeis guineensis* germplasms.

Germplasms Origin	***Mean of 2C Genome Size (pg)**	**Coefficient Variation (CV)**
Tanzania (0.256/221)	3.659 ±0.253^{a}	2.97%
Angola (0.311/713)	4.115 ±0.267^{b}	2.73%
Guinea (0.353/197)	4.180±0.236^{b}	2.52%
Madagascar (0.240/19)	4.137±0.131^{b}	2.06%
Nigeria (0.395/282)	4.202±0.350^{b}	2.45%
Zaire (0.349/246)	4.093±0.216^{abc}	3.26%
Gambia (0.398/106)	4.013±0.356^{abc}	3.33%
Ghana (0.397/27)	3.908±0.430^{abc}	2.36%
Sierra leone (0.355/208)	3.895±0.325^{abc}	2.16%
Senegal (0.390/387)	3.825±0.327^{abc}	2.74%
Cameroon (0.349/18)	3.803±0.197^{abc}	2.85%

*Means with the same alphabet (e.g: a,b and c) are not significantly different at $p \leq 0.05$.

Table 4.2 Tukey's pairwise comparison among *Elaeis guineensis* germplasms. Highlighted regions indicate there are significant differences between the germplasms.

	Angola	Cameroon	Gambia	Ghana	Guinea	Madagascar	Nigeria	Senegal	Sierra leone	Tanzania
Cameroon	-0.1421 0.7643									
Gambia	-0.3521 0.5543	-0.6632 0. 2432								
Ghana	-0.2465 0.6599	-0.5577 0.3488	-0.3477 0.5588							
Guinea	-0.5188 0.3877	-0.8299 0.0765	-0.6199 0.2865	-0.7254 0.1810						
Madagascar	-0.4754 0.4310	-0.7865 0.1199	-0.5765 0.3299	-0.6821 0.2243	-0.4099 0.4965					
Nigeria	-0.5410 0.3654	-0.8521 0.0543	-0.6421 0.2643	-0.7477 0.1588	-0.4754 0.4310	-0.5188 0.3877				
Senegal	-0.2173 0.7961	-0.5284 0.4850	-0.3184 0.6950	-0.4239 0.5895	-0.1517 0.8617	-0.1950 0.8184	-0.1295 0.8839			
Sierra leone	-0.2332 0.6732	-0.5443 0.3621	-0.3343 0.5721	-0.4399 0.4665	-0.1677 0.7388	-0.2110 0.6954	-0.1454 0.7610	-0.5761 0.4373		
Tanzania	0.0023 0.9088	-0.3088 0.5977	-0.0988 0.8077	-0.2043 0.7021	0.0679 0.9743	0.0246 0.9310	0.0901 0.9965	-0.3406 0.6728	-0.2177 0.6888	
Zaire	-0.4321 0.4743	-0.7432 0.1632	-0.5332 0.3732	-0.6388 0.2677	-0.3665 0.5399	-0.4099 0.4965	-0.3443 0.5621	-0.7750 0.2384	-0.6521 0.2543	-0.8877 0.0188

4.5 Estimation of *Elaeis oleifera* germplasms Genome Size

Table 4.3 lists the genome size and respective CV values for *Elaeis oleifera* germplasms from Colombia, Honduras, Costa Rica, Panama and Suriname. There were 9 replicates for each germplasm being used. (refer to appendix H and J for the calculation) While Table 4.4 shows Tukey's pairwise comparison among genome size of *Elaeis oleifera* germplasms.

Table 4.3 DNA content and the CV value of *Elaeis oleifera* germplasms.

Germplasms Origin	***Mean of 2C Genome Size (pg)**	**Coefficient Variation (CV)**
Suriname (0.711/3)	3.392 ± 0.534^{a}	3.67%
Colombia (0.211/1236)	4.167 ± 0.429^{b}	3.82%
Honduras (0.211/1316)	3.943 ± 0.109^{b}	2.87%
Costa Rica (0.211/1229)	3.790 ± 0.301^{abc}	3.54%
Panama (0.211/1229)	3.537 ± 0.482^{abc}	11.77%

*Means with the same alphabet (eg: a,b and c) are not significantly different at $p \leq 0.05$.

Table 4.4 Tukey's pairwise comparisons among Elaeis oleifera germplasms.Highlighted regions indicate there are significant differences between the germplasms.

	Colombia	Costa rica	Honduras	Panama
Costa rica	-0.1443 0.8999			
Honduras	-0.2977 0.7466	-0.674 0.3688		
Panama	-0.1073 1.3695	-0.4850 0.9917	-0.3317 1.1450	
Suriname	0.2534 1.2977	-0.1243 0.9199	0.0290 1.0732	-0.5939 0.8828

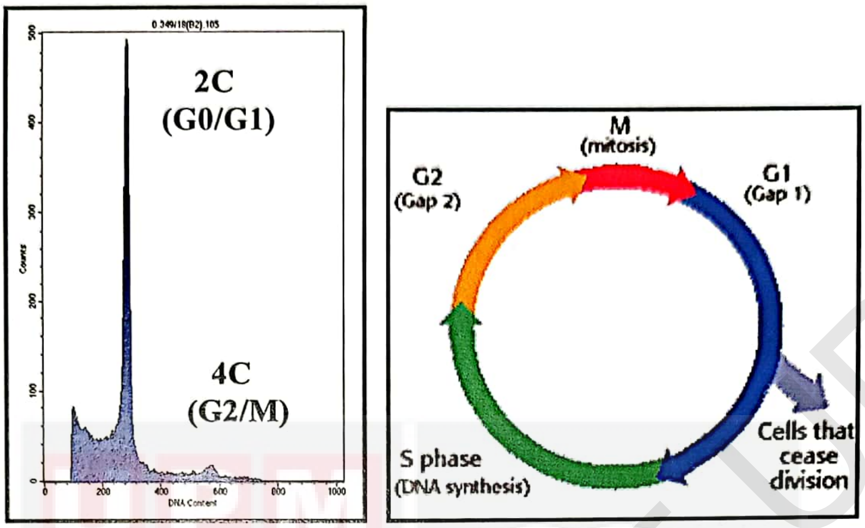
CHAPTER V

DISCUSSION

5.1 G0/G1 Histogram Peak

Flow cytometry offers a simple, rapid, accurate and convenient method for determining ploidy levels of DNA, assessment and analysis of cell cycle or large cell population (Winklemann *et al.,* 1998; Dolezel, 1991). Preparation for suspensions of intact nuclei for estimation of absolute DNA amounts is a prerequisite for a successful flow cytometry analysis. In this procedure, the nuclei are released into a nuclei isolation buffer via mechanical homogenization or manually chopping of a small amount of fresh leaf tissue. The composition of the isolation buffer is critical to facilitate the release of free nuclei in the cytoplasm and in sufficient quantities, maintain the integrity of isolated nuclei, protect their DNA against endonucleases and facilitate DNA staining.

Staining of nuclei with propidiunm iodide produced a single histogram peak at G0/G1 phase or 2C while G2/M peak with 4C genome size will occur at a higher fluorescent intensity than the G0/G1 peak with lower nuclei count. Propidium iodide is an intercalating DNA dye hence suitable for DNA absorbance detection. G0/G1 peak and G2/M peak also indicate the phases of the cell cycle (refer Figure 5.1). G0/G1 peak or 2C is the interphase stage after completion of mitosis. The chromosomes start to dissociate into DNA strands and synthesis of DNA commences during the S phase and doubles the amount from 2C to 4C at G2. This is followed by mitosis, which consists of prophase, metaphase, anaphase and telophase stages finally producing two identical daughter cells.



(A) (B)

Figure 5.1 (A) Fluorescent intensity histogram peak of *Elaeis guineensis* and (B) a diagram on stages of the cell cycle.

Source (diagram B): http//www.yahoo/search/cycle/bcell2.htm

5.2 Estimation of *Elaeis guineensis* Germplasms Genome Size

Table 4.1 listed the estimated 2C genome size of *Elaeis guineensis* germplasms from Nigeria, Guinea, Madagascar, Angola, Zaire, Gambia, Ghana, Sierra Leone, Senegal, Cameroon and Tanzania. The germplasm from Nigeria showed the highest genome size with 2C=4.202 ± 0.35 pg and the germplasm from Tanzania showed the lowest genome size with 2C=3.659±0.253 pg. According to the result, the corresponding genome size shows that the germplasms might be have very high or low genes content.

Table 4.2 showed Tukey's pairwise comparison among *Elaeis guineensis* germplasms genome size. The results revealed significant differences in genome size between Tanzania and Angola germplasms (2C = 3.659 ± 0.253 pg vs. 4.11 ± 0.262 pg respectively), Tanzania and Guinea germplasms (2C = 3.659 ± 0.253 pg vs. 4.180 ± 0.236 pg respectively), Tanzania and Madagascar germplasms (2C = 3.659 ± 0.253 pg vs. 4.137 ± 0.131 pg respectively), Tanzania and Nigeria germplasms (2C = 3.659 ± 0.253 pg vs. 4.202 ± 0.350 pg) where H_1 (significantcy) were accepted ($p \leq 0.05$). Only 7.27 % of the *Elaeis guineensis* germplasms samples from different origin were significantly different and 92.73% of the samples were not significantly different in their genome size values.

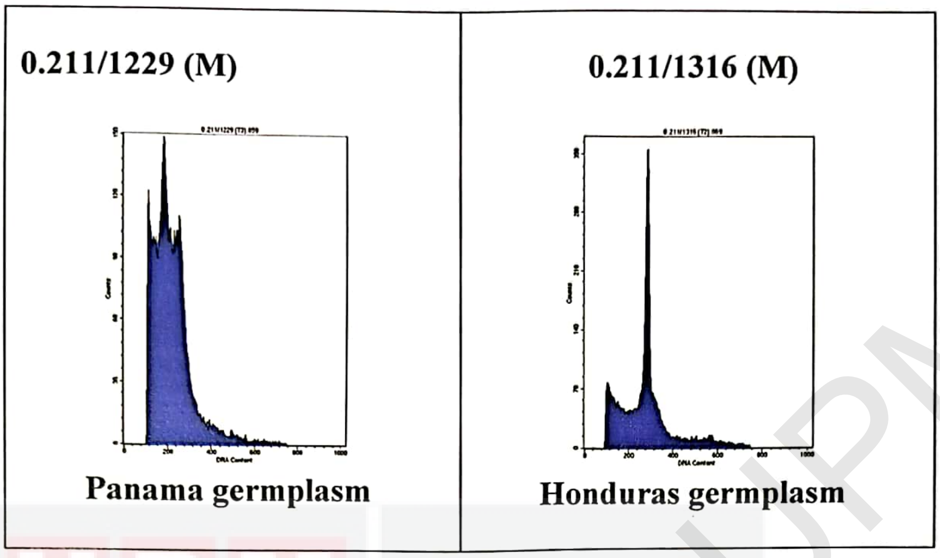
5.3 Estimation of *Elaeis oleifera* germplasms Genome Size

Table 4.3 listed the genome size and respective CV values for *Elaeis oleifera* germplasms from Colombia, Honduras, Costa Rica, Panama and Suriname. The highest genome size was exhibited by the germplasm from Colombia with 2C = 4.167 ± 0.429 pg while the germplasm from Suriname showed the lowest genome size with 2C = 3.392 ± 0.534 pg.

Table 4.4 showed Tukey's pairwise comparison among DNA contents of *Elaeis oleifera* germplasms. There are significant differences between Suriname and Colombia germplasms (2C = 3.392 ± 0.534 pg vs. 4.168 ± 0.429 pg respectively) as well as between Suriname and Honduras germplasms (2C = 3.392 ± 0.534 pg vs. 3.943 ± 0.109 pg respectively) where H_1 were accepted. From the statistical analysis, it can be observed that 20% of the *Elaeis oleifera* germplasms samples from different origin were significantly different in their DNA content while 80% were not.

5.4 Comparison of fluorescent intensity histogram peaks between Panama germplasm with other germplasms of *Elaeis oleifera*.

Almost symmetrical high peak count and less than 3-5% CV value are the main factors considered in order to choose the best histogram peaks. However, differences between fluorescence intensity peak between Panama and the other germplasms of *Elaeis oleifera* can be observed (refer to Figure 5.2). The histogram peak showed that the Panama germplasm nuclei preparation contained a lot of debris hence forming a skewed peak prior to G0/G1 peak. The CV value was 11.77% due to the high debris content. The debris is due to the presence of damaged nuclei and other organelles containing DNA. For the Panama sample, a blending technique has been used hence produced high debris while for Honduras and others germplasms, manually chopping technique with blade has been used. However, the lysis buffer used was also different, where for chopping technique they were Otto I and Otto II buffer while for blending technique LBO1 buffer was used. Chopping technique is more suitable to be used due to the properties of *Elaeis oleifera* germplasm leaves, which are thicker and greener.



0.211/1229 (M)

Panama germplasm

0.211/1316 (M)

Honduras germplasm

Figure 5.2 Fluorescence intensity histograms of Panama and Honduras germplasms showing peak of Panama with high debris compared to Honduras histogram peak.

5.5 Comparison of nuclei releasing methods, manual chopping vs. blending

The selection criteria for the 2C peaks are high counts, almost symmetrical peak and less than 3% CV of fluorescent intensity readings. These criteria indicate high quantity of 2C intact nuclei have been obtained and remained intact throughout the incubation period. For some samples, these criteria were not obtained due to blending technique. The physiology of the leaf samples, which are thick and hard, made blending technique difficult and released a lot of broken nuclei or described as debris. For example, the Panama germplasm (Figure 5.2), should be processed using manual chopping technique as Honduras germplasm (Figure 5.2). Hence depending on the physiology of the leaf samples, isolation technique need to be determined prior to an FCM analysis.

CHAPTER VI

CONCLUSION

In conclusion, estimation of genome size on *Elaeis* germplasms via flow cytometry is an efficient and fast method to be used. From this study it can be concluded that there are differences in the genome size within the *Elaeis guineensis* and *Elaeis oleifera* species and between them. The knowledge of genetic base is very important and useful to study genetic diversity. It is because genetic diversity reduces the fragility of crops due to diverse nature of interesting traits available. The information on estimated genome size can be used to setup routine assays for conventional breeding and tissue culture programmes. Besides estimation of genome size, the results also can be exploited to study cell cycle analysis on determining cell division activities. Hence for tissue culture experiments, the potential of explants used in vitro can be optimized by choosing explants with actively dividing cells. These are some of the direct applications of flow cytometry, which assist in the improvement of oil palm industry.

REFERENCES

Arasu, N.T. and N. Rajanaidu. 1975. Oil palm genetic resources, In (eds. Earp, D.A., and Newall, W.). *International Development in Oil Palm*, Kuala Lumpur, ISP. p. 16-25.

Bagwell, C.B; D. Baker; S. Whetstoe; M. Munson; S. Hitchcox; K. Ault and E.J. Lovett. 1989. A simple and rapid method for determining the linearity of a flow cytometer amplification system. *Cytometry* **10**: 689–694.

Blakk, G. 1967. Oil Palm Prospection Tour in the Bamenda Hihglands of West Cameroon. Internal Report, Unilever, London. p. 10.

Bennett, M. D. and I. Leitch. 1995. Nuclear DNA amounts in angiosperms. *Annals of Botany* **76**:113-176.

Bennet, M.D. and I.J. Leitch. 2005. Genome size evolution in plants. In *The environment of the Genome* (eds. Gregory, T.R.), Elsevier, San Diego. p. 89-162.

Chevalier. A. 1943. Taxanomie, Biogeographie et Selection des Palmiers au Genre *Elaeis. Rev. bot. appl. Agric. Trop*., **23**:295.

Cremonini, R; D. Miotto; M.A. Ngu; D. Tota; D. Pignone; S. Blangiforti and G. Venora. 1998. Cytology of Vicia species. 5. Nuclear chromatin structure, karyomorphological analysis and DNA content in newly discovered relatives of Vicia faba L. Vicia kalakhensis Khattab, Maxted et Bisby and Vicia eristalioides Maxted. Cytologia **63**: 371-379.

Crist, R.E. 1971. Migration and population change in the Irish Republic. *American Journal of Economics and Sociology*, **30**: 253-258.

Corley, R.H.V; C.H. Lee; I.H. Law and C.Y. Wong. 1986. Abnormal flower development in oil palm clones. The planter, **62**: 233-240.

Corley, R.H.V; J.J. Hardon and B.J. Wood (Editors). 1976. Oil palm research. E1-sevier Scientific Publishing Co., Amsterdam. p. 532.

Dolezel, J. 1991. Flow cytometric analysis of nuclear DNA content in higher plants. Phytocehm Anal., **2**:143-154.

Dolezel, J. 1997. Applications of flow cytometry for the study of plant genomes. *Journal of Applied Genetics* **38**: 285-302.

Dolezel, J. and J. Bartos. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany* **95**: 99-100.

Ellul, P; M. Boscaiu; O. Vicente; V. Moreno and J.A. Rossello. 2002. Intra – and interspecific variation in DNA content in *Cistus* (*Cistaceae*). *Annals of Botany* **90**: 345-351.

Fukuda, M; N. Bohm and S. Fujita. 1978. Ctophotometry and its biological application. Prog Histochem Cytochem **11**:1-119.

Galbraith, D.W; K.R. Harkins; J.M. Maddox; N.M. Ayres; D.P. Sharma and E. Firoozabady. 1983. Rapid flow cytometry analysis of the cell cycle in intact plant tissues. *Sciences* **220**: 1049-1051.

Galbraith, D.W. 1983. Flow cytometric analysis of the cell cycle in higher plants. In: Vasil, I.K. ed. *Cell culture and somatic cell genetics of plant.* New York; Academic Pres, 765-777.

Galbraith, D.W; G.M. Lambert; J. Macas and J. Dolezel. 1983. Analysis of nuclear DNA content and ploidy in higher plants. In: Robinson, J.P.; Darzynkiewicz, Z. and Dean, P.N.

Galbraith, D.W; G.M. Lambert; J. Macas and J. Dolezel. 1997. Analysis of DNA content and ploidy in higher plants. In: Robinson, J.P.; *et al.*; eds. *Current protocols in cytometry.* New York: John Wiley & Sons, Inc.

Greilhuber, J. and R. Obermayer. 1997. Genome size and maturity group in *Glycine max* (soybean). Heredity **78**: 547-561.

Greilhuber, J. 1998. Self-tanning – a new and important source of stoichiometric error in cytophotometric determination of nuclear DNA content in plants. *Plant Systematics and Evolution* **158**: 87-96.

Gregory, T.R. 2005. Genome size evolution in animals. In *The Evolution of the Genome* (eds. Gregory, T.R.), Elsivier Diego. p. 3-87.

Hardon, J. 1969. Development in oil palm breeding. In (eds. Turner, P.D). Progress in Oil Palm. Incorporated Society of Planters, Kuala Lumpur. Malaysia. p. 13-24.

Hardon, J.J. and R.L. Thomas. 1968. Breeding and selection of oil palm in Malaya. *Oleagineux*, **23(2)**: 85-90.

Hardon, J.J. and G.Y. Tan. 1969. Interspecific hybrids in the genus *Elaeis*. I and II *Euphytica*, **18**: 372-380.

Hardon, J.J. 1974. The oil palm. In (eds. Leon, J). *Handbook of Plant Introduction in Tropical Crops.* F.A.O. Agricultural Studies No. 93. Rome. p. 75-89.

Hardon, J.J; V. Rao and N. Rajanaidu. 1985. A review of oil-palm breeding. In: Russell. G.E. (Editor). Progress in plant breeding 1. Butterworths, London. p. 139-163.

Hardie, D.C; T.R. Gregory and P.D.N. Hebert. 2002. From pixels to picograms: a beginners guide to genome quantification by Feulgen mage analysis densitometry. *Journal of Histochemistry and Cytochemistery* **50**: 735-749.

Hartley, C.W.S. 1988. *The Oil Palm* (*Elaeis guineensis* Jacq.). Longman Scientific and Technical Publication. John Wiley and Sons, N.Y., p. 761.

Jagoe, R.B. 1952. Deli oil palm and early introductions of *Elaeis guineensis* to Malaya. *Agric.* J, **35**: 4-11.

Jones, L; D. Barfield; J. Barret; A. Flook; K. Pollock and P. Robinson. 1983. *Cytology of oil palm cultures and regenerants plants. Plant Tissue Culture* 1982, Japan: Maruzen, Tokyo.

Kushairi, A. and N. Rajanaidu. 2000. Breeding populations, seed production and nursery management. In: Basiron, Y.; Jalani, B.S. & Chan, K.W. (eds) Advance in Oil Palm Research Volume I. MPOB, p. 87.

Maria, M; M.M Clyde and S.C. Cheah. 1998. Cytologycal analysis of *Elaeis guineensis* and *Elaeis oleifera* chromosomes. *J. Oil Palm Res.,* **10(1)**: 68-91.

Marshall, D.R. and A.H.D. Brown. 1975. Optimum sampling strategies in genetic conservation. In (eds. Frankel, O.H. and Hawkes, J.G.). Genetic Resources for To-day and Tomorrow. Cambridge Press, Cambridge. p. 53-80.

Mayes, S; C.M. James; V. Pluhar; N.P. Batty; P.L. Jack and R.H.V. 1995. The application of biotechnology to oil palm-potential and progress. In (eds. Rao, V.; Henson, I.E. and Rajanaidu, N.). Recent Development in Oil Palm Tissue Culture and Biotechnology. Palm oil Research Institute of Malaysia, Bangi. p. 171-189.

Mirsky, A.E. and H. Ris. 1951. *The desoxyribonuclei acid content of animal cells and its evolutionary significance*. J Gen Physiol **34**: 451-462.

Noirot, M; P. Barre; C. Duperray; J. Louarn and S. Hamon. 2003. Effects of caffeine and chlorogenic acid on propidium iodide accessibility to DNA: consequences on genome size evaluation in coffee tree. *Annals of Botany* **92**: 259–264.

Noirot, M; P. Barre; J. Louarn; C. Duperray and S. Hamon. 2000. Nucleus–cytosol interactions: a source of stoichiometric error in flow cytometric estimation of nuclear DNA content in plants. *Annals of Botany* **86**: 309–316.

Obasola, C.O; N.T. Arasu and N. Rajanaidu. 1983. Collectin of oil palm genetic material in Nigeria. I. Method of collection. *MARDI Report*. p. 80.
Obermayer, R. and J. Greilhuber. 1999. Genome size in Chinese soybean accessions – stable or variable? *Annals of Botany* **84**: 259-262.

Ooi, S.C; J.J. Hardon and S. Phang. 1973. Variability in Deli dura breeding populations of the oil palm (*Elaeis guineensis* Jacq.). I. Components of branch yield. Malaya Agric. J, **49**: 112.

Ooi, S.C. and N. Rajanaidu. 1979. Establishment of oil palm genetic resources: theoretical and practical consideration. *Malay. Appl. Boil.*, p. 15-28.

Pindur, A; S. Chakraborty; D.G. Welch and T.M. Wheeler. 1994. DNA ploidy measurements in prostate cancer: differences between image analysis and flow cytometry and clinical implications. Prostate **25**: 189-198.

Price, H.J. and J.S. Johnston. Proc Natl Acad Sci USA. 1996a; **93**: 11264-11267.

Price, H.J; G. Hodnett and J.S. Johnston. 2000. Sunflower (*Helianthus annus*) leaves contain compounds that reduce nuclear propidium iodide fluorescence. *Annals of Botany* **86**: 929-934.

Purseglove, J.W. 1972. Tropical Crops. Monocotyledons. Longman Group Ltd., London. p. 479-510.

Rajanaidu, N; N.T. Arasu and C.O. Obasola. 1979. Collection of oil palm (*Elaeis guineensis* Jacq.) genetic material in Nigeria II. Phenotypic variation of natural population. MARDI *Res. Bull.*, 7(1): 1-27.

Rajanaidu, N; A.D. Kushairi; B.S. Jalani and S.C. Tang. 1983. Novel oil from exotic palms. *Malaysian Oil Sci. and Technol. J.,* **3(2)**: 22-28.

Rajanaidu, N. and H. Abdul Halim. 1986. Oil palm genetic resources programme in relation to other perennial tree crops. In (eds. Banpot Napompeth and Suranant Subhadrabandhu). *New Frontier in Breeding Researches.* Fac. Of Agric. Kasetsart University, Bangkok, Thailand. p. 505-521.

Rajanaidu, N; B.S. Jalani and Manuel, Domingos. 1991. Collection of oil palm germplasm in Angola. *ISOPB Newsletter Vol.* 8. No. 2. p. 2-3.

Rajanaidu, N; B.S. Jalani; V. Rao and A. Kushairi. 1993. Genotype environment (GE) studies in oil palm (*Elaeis guineensis*) progenies. In (eds. Rao, V., Henson, I.E., and Rajanaidu, N.). Genotype x environment Studies in Perrennial Tree Crops. Palm Oil Research Institute of Malaysia, Bangi. p. 12-32.

Rajanaidu, N. 1994. Porim Oil Palm Genebank. Pal Oil Research Institute of Malaysia. p. 19.

Rajanaidu, N. and Jalani, B.S. 1994a. Oil palm germplasm collection in Africa. 1. Senegal. *ISOPB Newsletter*, **10(1)**: 1-18.

Rajanaidu, N. 1968c. *Elaeis oleifera* collection in Central and South America. *Workshop. Proc.* palm Oil Res. Ins. Malaysia, Bangi. p. 84-94.

Rayburn, A.L; D.P. Biradar; D.G. Bullock; R.L. Nelson; C. Gourmet and J.B. Wetzel. 1997. Nuclear DNA content diversity in Chinese soybean introduction. *Annals of Botany* **80**: 321-325.

Rasch, E.M. 1985. DNA "standard" and the range of accurate DNA estimates by Feulgen absorption microspectrophotpmetry. In Cowden, R.R. and Harrison, S.H. eds. Advance in Microscopy. New York, Alan R Liss, p. 137-166.

Richardson, D.L. and Cosme Charvez 1986. Oil palm germplasm of Tanzania origin. Unpublished. p. 1.

Rival, A; T. Beule; P. Barre; S. Hamon; Y. Duval and M. Noirot. 1997. Comparative flow cytometric estimation of nuclear DNA content in oil palm (*Elaeis guineensis* Jacq) tissue cultures and seed-derived plants. *Plant Cell Reports* **16**: 884–887.

Roser, M; A. Johnson and L. Hanson. 1997. Nuclear DNA amounts in palms (*Areacaceae*). Botanica Acta **110**: 79-89.

Shah, F.H; O. Rashid; A.J. Simons and A. Dunsdon. 1994. the utility of RAPD markers for the determination of genetic variation in oil palm (*Elaeis guinneensis*). *Theor. Appli. Genet.*, 89: 713-718.

Sharpiro, H.M. 2004. *Practical flow cytometry*, 4th edn. New York: Wiley-Liss.

Suda, J. 2004. *An employment of flow cytometry into plant biosystematics*. PhD thesis, Charles University, Czech Republic. Available at http://www.ibot.cas.cz/fcm/suda/presentation/disertation.pdf

Swift 1950. The constancy of desoxyribose nuclei acid in plant nuclei. Proc Nalt Acad Sci USA **36**: 643-654.

Thomas, R.L; I. Watson and J.J Hardon. 1969. Inheritance of some components of yield in the Deli *dura* variety of oil palm. Euphytica, **18**: 92-100.

Touchet (de), B; Y. Duval and C. Pannetier. 1991. Plant regeneration from embryogenic suspension cultures of oil palm (*Elaeis guineensis* Jacq). Plant cell Rep., **10**: 529-532.

Venora, G; I. Galasso and D. Pignone. 1995a. Quantitative heterochromatin determination by means of image analysis. J Genet Breed **49**: 187-190.

Venora, G; B. Ocampo; K.B. Sing and F. Saccardo. 1995b. Karyotype of the kabuli-type chickpea (Cicer arietinum L.) by image cytometry. Ann Bot **87**: 19-728.

Voglmayr, H. and J. Greilhuber. 1998. Genome size determination in Peronosporales (Oomycota) by feulgen image analysis. Fung Genet Biol **25**: 181-195.

Volgmyar, H. 2000. Nuclear DNA amounts in mosses (Musci). Ann Bot **85**: 531-546.

Withmore, T.C. 1973. The Palms of Malaya. Longmans, Malaysia. p. 56-58.

Winkelmann, T; R.S. Sangawan and H.G. Schwenkle. 1998. Flow cytometric analysis in embryogenic and non-embryogenic callus lines of *Cyclamen persicum* Mill.: relation between ploidy level and competence for somatic emvryogenesis. Plant Cell Rep., **17**:400-404.

Yamamoto, T; H. Horiguchi; H. Kamma; M. Noro; T. Ogata; Y. Inage; E. Akaogi; K. Mitsui; M. Hori and M. Isobe. 1994. Comparative DNA analysis by image cytometry and flow cytometry in non-small cell lung cancer. Japan J Cancer Res **85**: 1171-1177.

APPENDICES

APPENDIX A

PREPARATION FOR FLOW CYTOMETRY TECHNIQUE SOLUTION

A. Preparation of lysis buffer LBO1

15 mM Tris	363.4 mg
2 mM Na_2EDTA	148.9 mg
0.5 mM spermine	34.8 mg
80 mM KCl	1.193 g
20 mM NaCl	233.8 mg
0.1% (v/v) Triton X-100	200 µl

Dissolve in 200 ml ddH_2O.
Adjust pH to 7.5 with 1N HCl.
Add with 220 µl β-mercaptoethanol (15 mM) into solution.
Filter through a 0.22 µm filter.
Store at -20 ^{0}C.

B. Propidium iodide Solution (1 mg/ml)

Propidium iodide (GIBCO BRL, USA)	50 mg

Dissolve in 50 ml ddH2O.
Filter through a 0.22 µm filter.
Store at -20 ^{0}C.

C. RNase Solution (1 mg/ml)

RNaseA	25 mg

Dissolve in 25 ml ddH2O.
Filter through a 0.22 µm filter.
Heat to 90 ^{0}C for 15 min to inactivate DNases
Store at -20 ^{0}C

D. Otto Buffer I

0.1 M citric acid monohydrate	4.2 g
0.5% (v/v) Tween 20	1 ml

Adjust volume to 200 ml
Filter through a 0.22 µm filter
Store at 4 ^{0}C

E. Otto Buffer II

0.4 M $Na_2HPO_4.12H_2O$	28.65 g

Adjust volume to 200 ml
Filter through a 0.22 µm filter
Store at room temperature

Note: A DNA fluorochrome can be added prior to final volume adjustment. In that case, the solution should be stored in darkness.

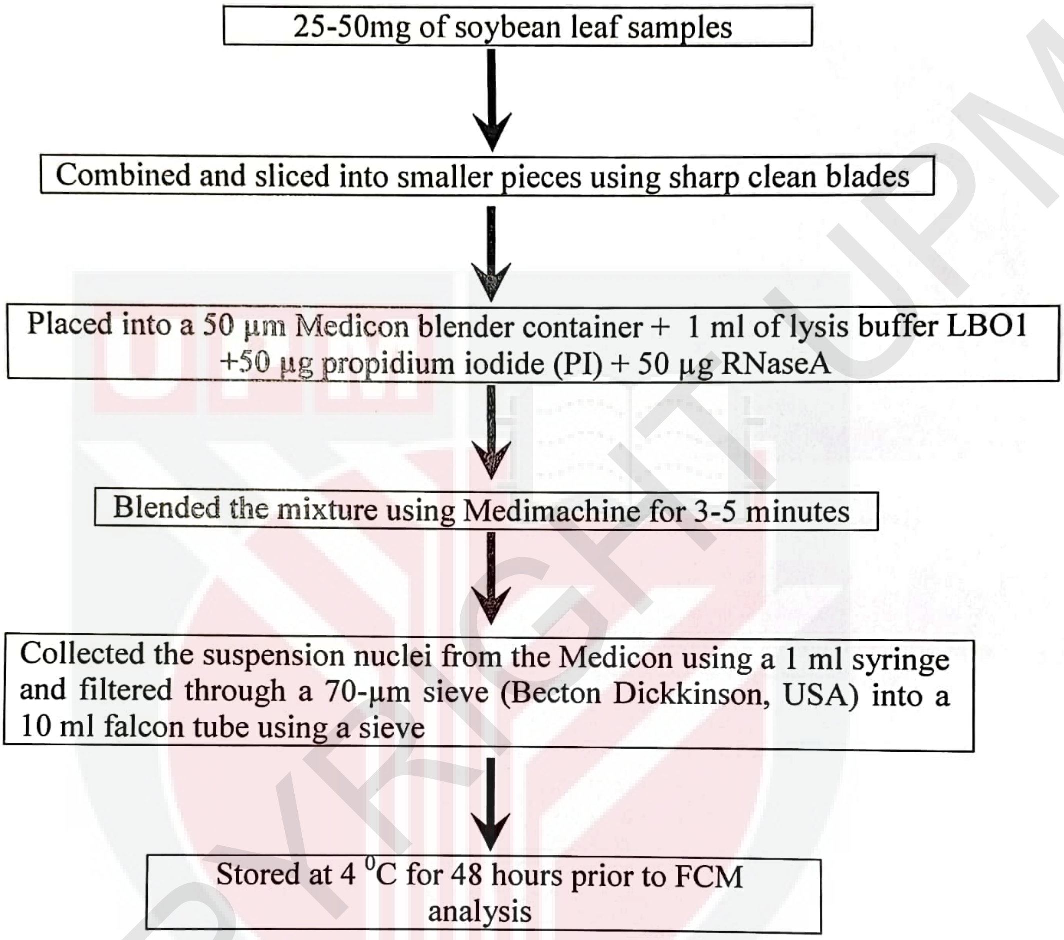
APPENDIX B

FUNCTION OF REAGENTS AND CHEMICAL COMPOUND IN DNA EXTRACTION

Component	Function
EDTA	- bind divalent cations (e.g: Mg) to prevent degradation of DNA - serve as nuclear cofactor
(KCI, NaCI)	- inorganic salt to achieve adequate ionic strength
TRIS, MOPS, HEPES	- organic buffer to stabilize the pH for DNA fluorochromes - prevent RNAse, DNAse and protease
Triton X-100, Tween 20	- non-ionic detergent to facilitate nuclear release from the cytoplasm - remove cytoplasmic remnants from the surface of isolated nuclei - disperse chloroplasts debris to aggregate
B-mecarptoethanol, dithiothreitol	- reducing agent -preserve chromatin protein and counteract the interference of phenolic compounds with DNA staining - binds phenolic compound
Spermine	- stabilize nuclear chromatin
RNAse	- to remove RNA from DNA solution
Otto Buffer I, Otto Buffer II	- improve chromatin accessibility and homogenizes chromatin structure

APPENDIX C

PREPARATION OF REFERENCE STANDARD *GLYCINE MAX* cv. POLANKA



25-50mg of soybean leaf samples

Combined and sliced into smaller pieces using sharp clean blades

Placed into a 50 µm Medicon blender container + 1 ml of lysis buffer LBO1 +50 µg propidium iodide (PI) + 50 µg RNaseA

Blended the mixture using Medimachine for 3-5 minutes

Collected the suspension nuclei from the Medicon using a 1 ml syringe and filtered through a 70-µm sieve (Becton Dickkinson, USA) into a 10 ml falcon tube using a sieve

Stored at 4 ^{0}C for 48 hours prior to FCM analysis

APPENDIX D

Differences between *Elaeis guineensis* and *Elaeis oleifera*

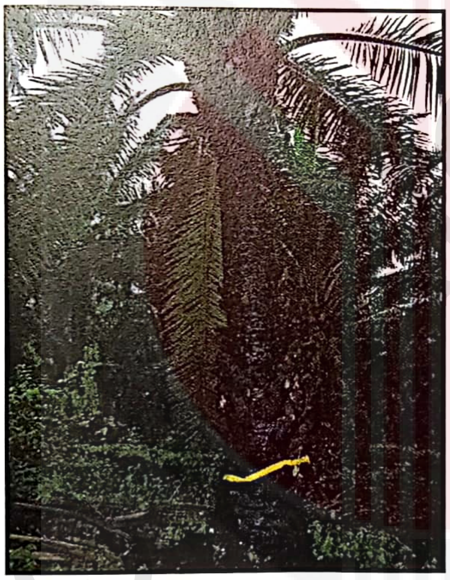
Madagascar (*Elaeis guineensis*)

Suriname (*Elaeis oleifera*)

Elaeis guineensis Germplasms

Madagascar

Tanzania



Gambia

Guinea

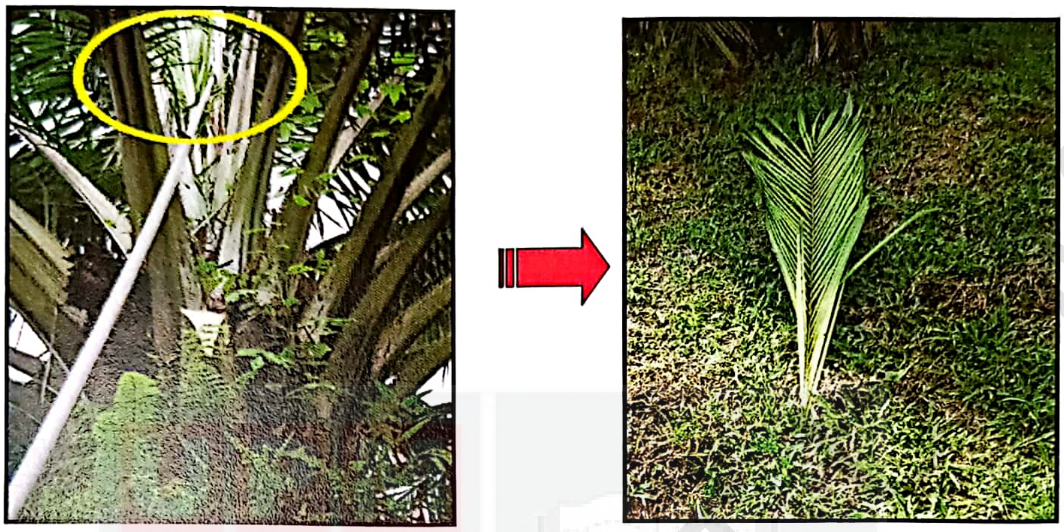
***Elaeis oleifera* germplasms**

Suriname

Colombia

APPENDIX E

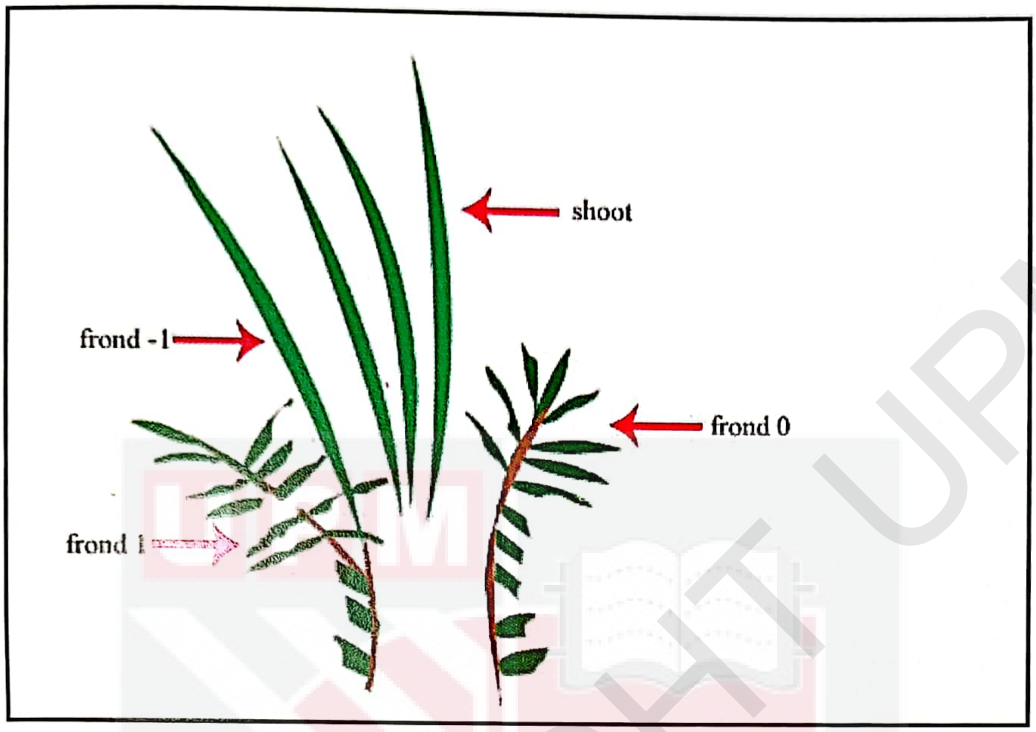
ELAEIS FROND
FROND -1



Frond 1 has to be located first before Frond –1 can be identified. Frond 1 is the youngest fully opened frond with leaflets fully detached from each other, so Fronds 0 and –1 are the next two successively younger fronds, i.e. not fully opened, or spear leaves.

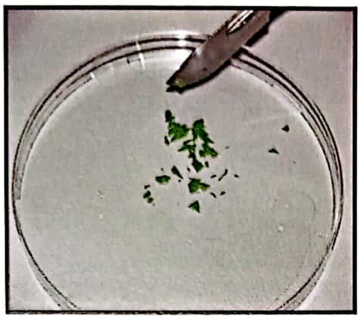
APPENDIX F

THE NUMBERING OF *ELAEIS* FROND



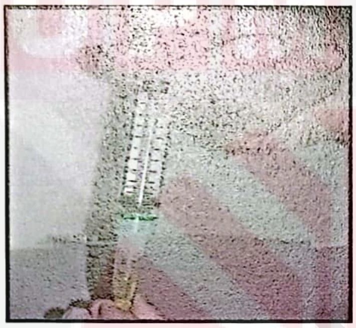
APPENDIX G

(A) TOOLS



Blade and Petri disk

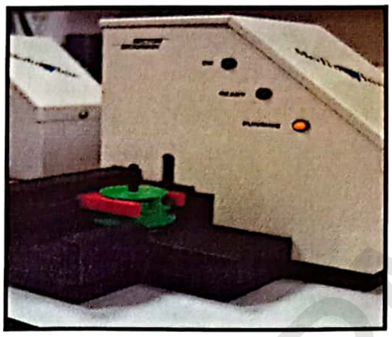
Beaker and PI solution



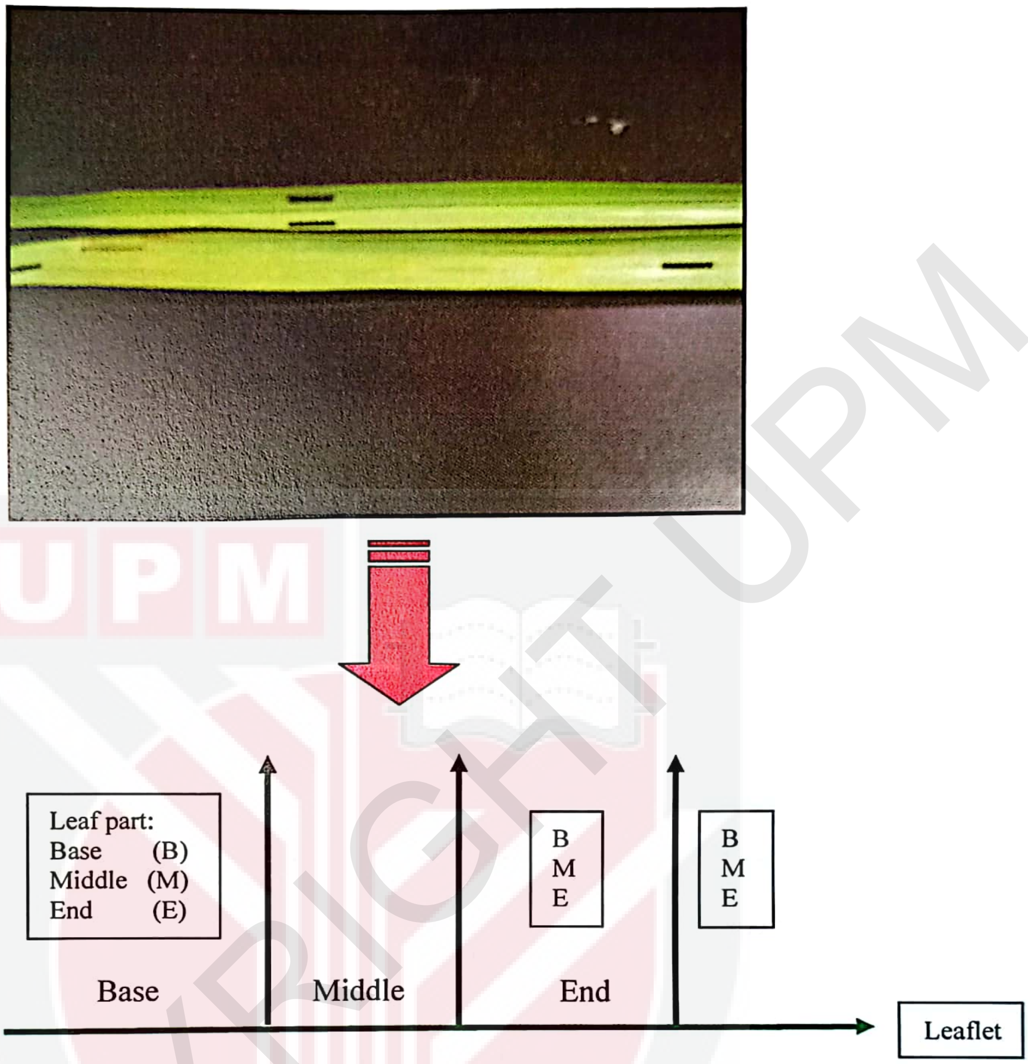
Syringe, sieve and tips

Falcon tube with suspension nuclei

(B) EQUIPMENTS

	
Medicon blender	Medimachine
	
Flow cytometry machine	Histogram figure

(C) LEAF SAMPLE



Base, middle and end region of leaf per leaflet

APPENDIX H

MEAN AND CV VALUE OF *ELAEIS* GERMPLASMS

Mean and Cv value of *Elaeis guineensis* germplasms.

		Mean			CV		
Germplasms	**Trial No.**	**B**	**M**	**E**	**B**	**M**	**E**
Gambia	**0.398/106**	273.49	260.62	318.80	2.13	2.70	3.67
		273.63	260.41	319.22	2.69	3.15	5.07
		273.72	260.46	319.83	3.15	3.62	5.70
Average		**273.61**	**260.50**	**319.28**	**2.66**	**3.16**	**4.81**
DNA		**3.86**	**3.68**	**4.51**	**-**	**-**	**-**
Tanzania	**0.256/221**	254.72	273.58	245.07	1.95	3.03	2.89
		255.14	273.08	244.54	2.47	3.54	3.08
		255.14	272.38	244.20	2.47	3.77	3.52
Average		**255.00**	**273.01**	**244.60**	**2.30**	**3.45**	**3.16**
DNA		**3.60**	**3.86**	**3.45**	**-**	**-**	**-**
Ghana	**0.397/27**	299.63	300.75	303.87	1.93	2.17	2.05
		298.49	299.58	303.67	2.23	2.46	2.41
		297.81	299.55	303.17	2.43	2.80	2.75
Average		**298.64**	**299.96**	**303.57**	**2.20**	**2.48**	**2.40**
DNA		**4.22**	**4.24**	**4.29**	**-**	**-**	**-**
Nigeria	**0.395/282**	310.90	335.06	338.53	2.24	1.83	1.81
		309.11	334.17	338.08	2.70	2.03	2.10
		308.62	332.73	336.34	3.01	2.59	2.56
Average		**309.54**	**333.99**	**337.65**	**2.65**	**2.15**	**2.16**
DNA		4.37	4.72	4.77	-	-	-
Guinea	**0.353/197**	321.76	298.31	306.55	1.89	2.37	2.54
		320.9	297.45	305.73	2.08	2.59	2.75
		320.54	296.95	305.2	2.39	2.94	3.15
Average		**321.07**	**297.57**	**305.83**	**2.12**	**2.63**	**2.81**
DNA		**4.54**	**4.20**	**4.32**	**-**	**-**	**-**
Sierra Leone	**0.355/208**	274.20	283.68	284.15	2.16	1.93	1.58
		274.20	283.07	283.75	2.16	2.37	1.92
		272.59	282.35	283.75	2.83	2.61	1.92
Average		**273.66**	**283.03**	**283.88**	**2.38**	**2.30**	**1.81**
DNA		**3.86**	**4.00**	**4.01**	**-**	**-**	**-**
Angola	**0.311/713**	292.28	302.86	259.19	2.41	1.95	3.04
		291.49	303.65	258.44	2.63	2.55	3.28
		291.17	303.33	257.34	2.96	2.97	4.05
Average		**291.65**	**303.28**	**258.32**	**2.67**	**2.49**	**3.46**
DNA		**4.28**	**4.28**	**3.65**	**-**	**-**	**-**

		Mean			CV		
Germplasms	**Trial No.**	**B**	**M**	**E**	**B**	**M**	**E**
Senegal	**0.396/387**	282.14	292.56	291.75	2.51	2.33	2.28
		280.58	292.23	291.41	2.96	2.62	2.63
		280.29	291.67	290.29	3.31	2.8	3.23
Average		**281.00**	**292.15**	**291.15**	**2.93**	**2.58**	**2.71**
DNA		**3.97**	**4.13**	**4.11**	**-**	**-**	**-**
Cameroon	**0.349/18**	284.55	277.44	278.02	2.14	2.5	2.3
		283.22	276.02	277.77	3.04	2.71	2.71
		283.01	276.14	275.73	3.32	3.2	3.72
Average		**283.59**	**276.53**	**277.17**	**2.83**	**2.80**	**2.91**
DNA		**4.01**	**3.9**	**3.91**	**-**	**-**	**-**
Madagascar	**0.240/19**	294.72	289.6	315.18	1.92	2.15	1.52
		294.75	289.6	314.66	2.27	2.15	1.85
		294.52	289.46	314.44	2.18	2.42	2.13
Average		**294.66**	**289.55**	**314.76**	**2.12**	**2.24**	**1.83**
DNA		**4.16**	**4.09**	**4.45**	**-**	**-**	**-**
Zaire	**0.349/246**	314.47	285.32	295.43	2.65	2.95	2.7
		314.11	284.65	296.18	3	3.7	2.89
		312.63	285.2	296.32	3.42	4.24	3.76
Average		**313.74**	**285.06**	**295.98**	**3.02**	**3.63**	**3.12**
DNA		**4.43**	**4.03**	**4.18**	**-**	**-**	**-**

Mean and Cv value pf *Elaeis oleifera* germplasms.

		Mean			CV		
Germplasms	**Trial No.**	**B**	**M**	**E**	**B**	**M**	**E**
Colombia	**0.211/1236**	273.46	250.68	306.12	3.32	3.85	2.55
		272.86	248.36	305.95	3.87	4.49	3.03
		271.09	248.3	305.78	4.39	5.29	3.62
Average		**272.47**	**249.11**	**305.95**	**3.86**	**4.54**	**3.07**
DNA		**3.85**	**3.52**	**4.32**	**-**	**-**	**-**
Honduras	**0.211/1316**	278.61	282.84	273.24	2.35	2.45	2.61
		278.31	282.16	272.32	2.79	2.66	2.87
		277.25	281.99	271.17	3.46	3.03	3.59
Average		**278.06**	**282.33**	**272.24**	**2.87**	**2.71**	**3.02**
DNA		**3.93**	**3.99**	**3.84**	**-**	**-**	**-**
Costa Rica	**0.211/1054**	273.37	274.37	273.67	2.71	2.74	3.47
		272.35	275.09	272.21	2.98	2.94	3.84
		271.03	273.87	271.01	3.77	3.71	5.72
Average		**272.25**	**274.44**	**272.30**	**3.15**	**3.13**	**4.34**
DNA		**3.84**	**3.88**	**3.85**	**-**	**-**	**-**
Suriname	**0.177/3**	267.22	219.11	267.51	2.99	3.49	2.96
		267.31	218.81	268.61	3.49	4.08	3.74
		267.11	217.84	267.31	4.5	4.93	4.44
Average		**267.21**	**218.59**	**267.81**	**3.66**	**4.17**	**3.71**
DNA		**3.77**	**3.78**	**3.44**	**-**	**-**	**-**
Panama	**0.211/1229**	202.09	182.94	288.95	7.37	4.12	3.18
		203.37	184.2	287.11	7.71	6.28	4.16
		202.08	183.85	287.17	8.15	7.23	4.72
Average		**202.51**	**183.66**	**287.74**	**7.74**	**5.88**	**4.02**
DNA		**3.44**	**3.11**	**4.06**	**-**	**-**	**-**

APPENDIX I

CALCULATION OF GENOME SIZE FOR *ELAEIS GUINEENSIS GERMPLASMS*

Germplasms	Trial No.	Mean x 2.5/soybean	DNA
Gambia	**0.398/106**		
	B	273.61 x 2.5 / 176.79	3.86
	M	260.50 x 2.5 / 176.79	3.68
	E	319.28 x 2.5 / 176.79	4.51
Tanzania	**0.256/221**		
	B	255.00 x 2.5 / 176.79	3.60
	M	273.01 x 2.5 / 176.79	3.86
	E	244.60 x 2.5 / 176.79	3.45
Ghana	**0.397/27**		
	B	298.64 x 2.5 / 176.79	4.22
	M	299.96 x 2.5 / 176.79	4.24
	E	303.57 x 2.5 / 176.79	4.29
Nigeria	**0.395/282**		
	B	309.54 x 2.5 / 176.79	4.37
	M	333.99 x 2.5 / 176.79	4.72
	E	337.65 x 2.5 / 176.79	4.77
Guinea	**0.353/197**		
	B	321.07 x 2.5 / 176.79	4.54
	M	297.57 x 2.5 / 176.79	4.20
	E	305.83 x 2.5 / 176.79	4.32
Sierra Leone	**0.355/208**		
	B	273.66 x 2.5 / 176.79	3.86
	M	283.03 x 2.5 / 176.79	4.00
	E	283.88 x 2.5 / 176.79	4.01
Angola	**0.311/713**		
	B	291.65 x 2.5 / 176.79	4.28
	M	303.28 x 2.5 / 176.79	4.28
	E	258.32 x 2.5 / 176.79	3.65
Senegal	**0.396/387**		
	B	281.00 x 2.5 / 176.79	3.97
	M	292.15 x 2.5 / 176.79	4.13
	E	291.15 x 2.5 / 176.79	4.11

Germplasms	Trial No.	Mean x 2.5/soybean	DNA
Cameroon	0.349/18		
	B	283.59 x 2.5 / 176.79	4.01
	M	276.53 x 2.5 / 176.79	3.90
	E	277.17 x 2.5 / 176.79	3.91
Madagascar	0.240/19		
	B	294.66 x 2.5 / 176.79	4.16
	M	289.55 x 2.5 / 176.79	4.09
	E	314.76 x 2.5 / 176.79	4.45
Zaire	0.249/246		
	B	313.74 x 2.5 / 176.79	4.43
	M	285.06 x 2.5 / 176.79	4.03
	E	295.98 x 2.5 / 176.79	4.18

APPENDIX J

CALCULATION OF GENOME SIZE FOR *ELAEIS OLEIFERA* GERMPLASMS

Germplasms	Trial No.	Mean x 2.5/soybean	DNA
Colombia	**0.211/1236**		
	B	272.25 x 2.5 / 176.79	3.85
	M	249.11 x 2.5 / 176.79	3.52
	E	305.95 x 2.5 / 176.79	4.32
Honduras	**0.211/1316**		
	B	278.06 x 2.5 / 176.79	3.93
	M	282.33 x 2.5 / 176.79	3.99
	E	272.24 x 2.5 / 176.79	3.84
Costa Rica	**0.211/1054**		
	B	272.25 x 2.5 / 176.79	3.84
	M	274.44 x 2.5 / 176.79	3.88
	E	272.29 x 2.5 / 176.79	3.85
Suriname	**0.177/3**		
	B	267.21 x 2.5 / 176.79	3.77
	M	218.59 x 2.5 / 176.79	3.78
	E	267.81 x 2.5 /176.79	3.44
Panama	**0.211/1229**		
	B	243.72 x 2.5 / 176.79	3.44
	M	220.25 x 2.5 / 176.79	3.11
	E	287.74 x 2.5 / 176.79	4.04

APPENDIX K

ONE-WAY ANALYSIS OF VARIANCE (ANOVA)

Analysis of Variance for *Elaeis guineensi* 2C

Source	DF	SS	MS	F	P > F
Germplasm	10	2.7709	0.2771	3.28	0.001
Error	85	7.1740	0.0844		
Total	95	9.9449			

```
                                    Individual 95% CIs For Mean
                                       Based on Pooled StDev
Level         N     Mean    StDev   -----+---------+---------+---------
Angola        9   4.1144   0.2616                    (-----*-----)
Cameroon      9   3.8033   0.1972         (------*-----)
Gambia        9   4.0133   0.3558                 (------*-----)
Ghana         9   3.9078   0.4300             (-----*------)
Guinea        9   4.1800   0.2355                      (-----*-----)
Madagasc      9   4.1367   0.1308                    (------*-----)
Nigeria       9   4.2022   0.3498                         (-----*-----)
Senegal       6   3.8250   0.3268          (-------*------)
Sierra L      9   3.8944   0.3254              (------*-----)
Tanzania      9   3.6589   0.2527        (-----*-----)
Zaire         9   4.0933   0.2158                         (-----*------)
                                    ------+---------+---------+--------
Pooled StDev =   0.2905                 3.60      3.90      4.20
4.50
```

APPENDIX L

ONE-WAY ANALYSIS OF VARIANCE (ANOVA)

Analysis of Variance for *Elaeis oleifera* 2C

Source	DF	SS	MS	F	P > F
Germplasm	4	3.108	0.777	5.25	0.002
Error	34	5.036	0.148		
Total	38	8.143			

```
                                     Individual 95% CIs For Mean
                                       Based on Pooled StDev
Level        N    Mean    StDev     -------+---------+---------+--------
Colombia     9  4.1678  0.4286                          (-----*-----)
Costa Ri     9  3.7900  0.3018                   (--------*------)
Honduras     9  3.9433  0.1091                      (--------*------
-)
Panama       3  3.5367  0.4823          (----------*-----------)
Suriname     9  3.3922  0.5335           (------*-----)
                                    -------+---------+---------+--------
Pooled StDev =  0.3848                     3.20      3.60      4.00
4.40
```

APPENDIX M

CHEMICALS AND SOLUTIONS

Buffer for Nuclei Preparation

1. Lysis buffer LBO1 (1 ml)
    - 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

2. Lysis Buffer Otto I (1 ml)
    - 0.1 M citric acid monohydrate, 4.2 g
    - 0.5% (v/v) Tween 20, 1 ml

3. Lysis Buffer Otto II (1ml)
    - 0.4 M $Na_2HPO4.12H_2O$, 28.65 g
    - 2 µl Mercathoethanol

PUBLICATION OF THE PROJECT UNDERTAKING

This is to certify that I have no objection to publish the project entitled "Genome Size Estimation of MPOB's *Elaeis* Germplasms Collection" by the supervisor in a joint authorship. However, it has to be evaluated by the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus and published in the form approved by the Faculty.

AINUL SHAZWIN SAHIDAN

Date: 30 April 2007