UNIVERSITI PUTRA MALAYSIA

DETECTION OF OIL PALM HAPLOIDS USING STOMATA COUNTING, FLOW CYTOMETRY, MICROSATELLITE AND GENOMIC IN SITU HYBRIDIZATION TECHNIQUES

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By

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for tthe degree of the Master of Science

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December 2015

Chairman : Rosimah binti Nulit, PhD
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Haploids are individuals with gametic chromosome (n) and valuable for breeding purposes such as to produce better hybrids and improve parental performances in hybrid combinations. Several methods were applied in order to screen haploid and doubled haploid plants such as stomata counting, flow cytometric analysis, microsatellite markers analysis and genomic *in situ* hybridization (GISH). However, the reliable method to screen for oil palm haploid and doubled haploid is yet to be reported. For ploidy level analysis, stomata counting was done by using ANNOVA but showed unreliable result due to factors such as atmosphere, water and disease. For homozygosity analysis, a hypodiploid, a mixed ploidy and two potential haploid seedlings were obtained via flow cytometry. These oil palm variants were screened with 15 microsatellite markers to determine the homozygosity. However, all samples were shown to be heterozygous. For characterization of parental genomes in intergeneric hybrids, sample used derived from wide hybridization technique which involved cross between four *dura* flowers with *Cocos nucifera* pollen. Then, GISH was carried out to characterize the parental genomes of these hybrids. The result showed the absence of signals from the hybrid chromosomes which mean that there was no *Cocos nucifera* genome introgressed into the hybrid. It may be due to the small proportion of *Cocos nucifera*. These hybrids then were further analysed using 16 specific coconut microsatellite markers. Two primers managed to exhibit the *Cocos* genome in all the samples. This proved that the microsatellite markers analysis was reliable to characterize the small proportion of *Cocos nucifera* genome in intergeneric hybrid. In conclusion, this study had showed that microsatellite analysis is the most reliable method to screen for haploid and doubled haploid oil palm.

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PENGESANAN KELAPA SAWIT HAPLOID MENGGUNAKAN PENGIRAAN STOMATA, ALIRAN SITOMETRI, PENANDA MIKROSATELIT DAN TEKNIK PENGHIBRIDAN *IN SITU* GENOMIK

Oleh

MUHAMMAD AZWAN BIN ZULKIFILI

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Haploid adalah individu yang mempunyai kandungan kromosom gametik (n) dan berharga untuk tujuan biakbaka seperti untuk menghasilkan hibrid lebih berkualiti dan memperbaiki prestasi induk di dalam kombinasi hibrid. Terdapat beberapa cara telah digunakan untuk menyaring pokok kelapa sawit haploid dan haploid berganda seperti penggiraan stomata, analisis aliran sitometri, analisis penanda mikrosatelit dan penghibridan *in situ* genomik (GISH). Walaubagaimanapun, kaedah penyaringan pokok haploid dan haploid berganda kelapa sawit masih belum ditemui sehingga kini. Untuk kajian analisis tahap ploidi, penggiraan stomata telah dilakukan menggunakan ANNOVA tetapi keputusan menunjukkan kaedah ini tidak sesuai kerana beberapa faktor seperti atmosfer, air dan penyakit. Untuk analisis homozigositi, satu anak pokok hipodiploid, satu ploidi bercampur dan 2 anak pokok berpontesi haploid telah ditemui melalui analisis aliran sitometri. Anak-anak pokok ini telah disaring dengan menggunakan 15 penanda mikrosatelit untuk menentukan tahap homozigositi. Walaubagaimanapun, semua sampel menunjukkan heterozigous. Untuk pencirian genom induk di dalam kacukan intergenerik, sampel diperolehi melalui teknik kacukan luas iaitu melibatkan penggacukan di antara empat bunga dura dengan debunga kelapa. Kemudiannya, GISH telah digunakkan untuk menentukan genom induk pada pokok-pokok hibrid ini. Keputusan menunjukkan ketiadaan signal daripada gabungan kromosom yang bermaksud tiada *Cocos nucifera* genom. Ini mungkin disebabkan oleh kandungan kecil genom *Cocos nucifera* yang berada di dalam pokok hibrid ini.Selanjutnya, pokok hibrid ini dianalisis menggunakan 16 penanda mikrosatelit spesifik kelapa. 2 primer berjaya menunjukan kehadiran genom *Cocos* di dalam semua sampel. Ini menunjukan bahawa penanda mikrosatelit adalah sesuai untuk menyaring kehadiran kecil genom *Cocos nucifera* di

dalam hibrid intergenerik.Kesimpulanya, kajian ini menunjukkan penanda mikrosatelit adalah kaedah paling sesuai untuk menyaring pokok kelapa sawit haploid dan haploid berganda.

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I certify that a Thesis Examination Committee has met on 17 December 2015 to conduct the final examination of Muhammad Azwan bin Zulkifili on his thesis entitled "Detection of Oil Palm Haploids using Stomata Counting, Flow Cytometry, Microsatellite, and Genomic *In Situ* Hybridization Techniques" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science.The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

A260	Spectrophotometer absorbance at 260nm
A280	Spectrophotometer absorbance at 280nm
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
bp	Base pairs
CV	Coefficient of variation
Cy3	Cyanine3
Cy5	Cyanine5
CTAB	Cetylmethyl- Ammonium bromide
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dUTP	2'-deoxyyuridine-5'triphosphate
RNA	Ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FCM	Flow cytometry
FITC	Fluorescent isothiocyanate
g	gram
GISH	genomic *in situ* hybridization
HCl	Hydrochloric acid
Kb	Kilo base
KCl	Potassium chlorate
M	Molarity
ml	Mililitre
mM	Milimolar
mm	Milimetre
MPOB	Malaysian Palm Oil Board
NaCl	Sodium Chloride
ng	Nanogram
nm	Nanometre
^{o}C	Degree celcius
OD	Optical density
pg	Picogram
pH	Negative logarithm of hydrogen ion concentration
PI	Propidium iodide
PCR	Polymerase chain reaction
SSR	Simple sequence repeat
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNAse	Ribonuclease
SPSS	Statistical Package for The Social Science
SSC	Saline-sodium citrate
TAE	Tri-acetate-EDTA
µl	Microlitre
µm	Micrometre
TE	Tris-EDTA buffer

LB01	Lysis buffer
EtOH	Ethanol
NaOAc	Sodium acetate

CHAPTER 1

INTRODUCTION

1.1 Introduction

The oil palm, *Elaeis,* is an important commercial crop for developing countries such as Malaysia, Indonesia and African countries. Oil World (2012) reported that Malaysia and Indonesia is the largest palm oil producer in the world, 85% of total world production. The palm oil industry has contributed 8% to gross national income, which was estimated at RM 52.99 billion. In terms of exports, palm oil is the market leader, constituting about 31.6% of the world trade in oils and fats.

Malaysia accounts for about 39 % of the world's output of palm oil and 80% of the net world export trade in palm oil. Furthermore, palm oil is the largest source of vegetable oil in the world after soybean oil and rapeseed. The largest consumer of palm oil in the world is China, followed by the EU, India and the United States. The demand for palm oil increased year by year due to the increasing palm oil consumer and awareness of the benefits of palm oil.

There are two types of oil palm planted in Malaysia, *Eleais guineensis* and *Eleais oleifera* which belong to the family Arecaceae or Palmae. The African oil palm, *E. guineensis* is native to West Africa, mostly found in Angola and Gambia, while the American oil palm, *E. oleifera* is native to tropical Central America and South America (Rajanaidu, 1986). The efforts to improve oil palm cultivation began in the 1920 and it is based on four seedlings planted in the Botanical Gardens, Bogor, Indonesia in 1848. These parental tree which is fundamental to the palm oil industry in Southeast Asia is known by *Deli dura* (Rajanaidu, 1986).

Oil palm known for its highest oil yield among seed crop. For the *E. oleifera* also commonly known as the Latin American Oil Palm, it has a unique fatty acid profile that is rich in monounsaturates (more than 60% oleic fatty acid), has more than 2000 ppm of carotenoids (mostly in the form of Beta-carotene), has over 1200 ppm of tocopherols & tocotrienols (Vitamin A) and it is extremely slow of annual height increment. Breeding programmes have hybridised the *E. guineensis* (AVROS) with *E. oleifera* (Suriname) to produce hybrid palms with inherent high oil quality.

The oil palm, *E. guineensis* were clasified into three types of shell thickness, *dura*, *tenera* and *pisifera*. Beirnert and vanderweyen (1941) discovered the inheritance of shell thickness in oil palm. This characteristic is controlled by two alleles (Sh^+) and (Sh^-). *Dura*, is homozygous dominant (sh^+sh^+) defining the characteristics of the shell which has a large kernel and mesocarp small. It produce small proportion of oil bearing mesocarp. Meanwhile, the shell less *pisifera* (P) form which is homozygous recessive ($sh^- sh^-$) has smaller fruits with a distinct fibre ring surrounding a very much reduced kernel but produce high proportion of oil bearing mesocarp. *Pisifera* serve as the main source of male parent palms and *Deli dura* the female. The cross between these two, produced high yield progeny known as *tenera.* Tenera have thin shell which is heterozygous (sh^+sh^-).

1.2 Problem statement, Justification and Objective of study

Recently, haploid technology has become an important tool in breeding oil palm. Haploid plant contain half set of gametic chromosome (n) and used for production of double haploid (2n) where chromosomes number of haploid were doubled. It can occur by *in vitro* or spontaneous in nature. There are several tehcniques to obtain haploid such as adrogenesis, parthogenesis, gynogenesis, and wide hydridization. This technique has been applied in several types of plant such as, barley, maize, rice and wheat. Haploid provide many advantages for breeder such as produced better hybrid and improved parental perfomance in hybrid combinations.

There are several ways for determine haploid plant, such as phenotypic appearance, flow cytometry and microsatellites marker. However, the reliable method to screen for oil palm haploid is yet to be reported. In this study various tools were used to find the most reliable and suitable tool for screening of putative haploid in oil palm. Generally, haploid plant is correlated with ploidy level. The number of genome sets of chromosomes is known as ploidy level. In order to estimate ploidy level of haploid plant, stomata counting has been applied to determine the ploidy level of plant. Besides that, flow cytometry also has used been to validate the ploidy level of seedlings (Madon *et al*., 2012).

Haploid or double haploid produce completely homozygous lines from donor plant. It can occur by *in vitro* or spontaneous in nature. Thus, the haploid, hypodiploid and mixed ploidy seedlings need to be further analysed using polymorphic microsatellite markers in order to determine whether these are actually haploids or spontaneous doubled haploids. The band generated should be hemizygous or homozygous (Chani *et al*., 2000).

In order to obtain haploid oil palm, intergeneric hybrids have been created *via* wide hybridization. Thus, genomic in situ hybridization (GISH), will be carried out to characterize the parental genomes introgressed into the progenies. GISH is an effective tool for parental genome analysis in both sexual and somatic hybrids. Besides that, microsatellites also able to characterize the parental genomes introgressed into the progenies. Therefore, specific microsatellite marker also has been used to determine the parental genomes introgressed into the progenies.

The objectives of study are:

1. To determine ploidy level *via* stomata counting and flow cytometry of oil palm variants (normal vs. stunted) for natural haploids.
2. To determine homozygosity level of seedlings for screening of haploids or doubled haploid.
3. To characterise parental genomes in intergeneric hybrids of *E. guineensis* x *C. nucifera.*

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