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ESTABLISHING THE TOOLS FOR ROUTINE ANAYLSIS OF CHLOROPLAST DNA DIVERSITY IN OIL PALM (ELAEIS SPP.)

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By

Ho Carl Miew

Thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chair: Professor Tan Soon Guan, PhD

Faculty: Biotechnology and Biomolecular Sciences

Oil palm (*Elaeis guineensis* Jacq.), a member of family Palmae, is one of the most efficient oilseed crops in the world. In order to meet the rising global demand on palm oil, efforts have focused on enhancing oil palm breeding, developing palms resistance towards trunk and root diseases as well as on improving stress tolerance. Molecular markers can play a vital role in improving the oil palm. To date, for oil palm, molecular markers analysis has been restricted to nuclear DNA. However, due to its non-recombinant nature and uniparental inheritance, detecting useful polymorphism at population level can be meaningful for evolutionary studies as well as to characterize the diversity of oil palm chloroplast DNA (cpDNA). Studies of chloroplast DNA diversity can reveal insights into the evolutionary and domestication history of a species. In this study, the development of an approach for isolating enriched oil palm cpDNA was a necessity in order to be able to analyze the variations present in oil palm cpDNA of selected palm germplasms. An enrichment cpDNA protocol using the combination method of sucrose gradient and cesium chloride gradient separation was adopted in order to isolate oil palm chloroplast DNA. A total of six oil palm germplasm collections (namely Angola, Ghana, Nigeria, Madagascar, Costa Rica and Suriname) were selected and the cpDNA was extracted using modified cpDNA enrichment protocol. The protocol first isolated the organelle and then using isopycnic centrifugation, cpDNA was purified. The extracted enriched oil palm cpDNA was initially verified using restriction enzyme analysis. To characterize the diversity of oil palm cpDNA, chloroplast microsatellite (cpSSR) primers were developed from chloroplast derived sequences obtained from the hypomethylated regions of oil palm as well as from the sequences reported in the published *E. guineensis* chloroplast genome in Gen Bank. Three chloroplast universal primers were also employed to analyze the diversity of oil palm cpDNA. In order to further validate the reliability of extraction protocol and cpSSR primers, PCR amplicons of a small subset of samples were sequenced. The sequencing results were then searched against public databases. Subsequently, twelve pairs of oil palm cpSSR and two chloroplast- specific universal primers were used to genotype the cpDNA diversity. The positive allele frequencies of the microsatellites locus

for the five germplasms ranged from 0.2 to 1.0. The Madagascar germplasm with positive allele frequencies ranging from 0.22 to 0.89 demonstrated the greatest diversity among the other five germplasm collections analyzed in this study. Moreover, one polymorphic oil palm cpSSR primer (*atp*1) demonstrated interspecific positive band variation between *E. guineensis* Jacq. and *E. oleifera* Cortez. The level of SSR polymorphism detected within species was low suggesting that the rate of molecular evolution in oil palm cpDNA was relatively low. However, higher levels of cpDNA polymorphisms between *Elaeis* species were detected.

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PENUBUHAN ALAT UNTUK ANALISIS RUTIN KEPELBAGAIAN DNA KELAPA SAWIT (*ELAEIS* SPP.)

Oleh

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Kelapa sawit (*Elaeis guineensis* Jacq.), adalah daripada famili Palmae, yang merupakan salah satu jenis tanaman benih minyak yang paling effisien di dunia. Demi memenuhi permintaan global yang semakin meningkat dalam hasil miyak sawit, pelbagai usaha telah dilakukan terhadap penambahbaikan prestasi sawit seperti skim biakbaka kelapa sawit, menghasilkan baka yang rintang terhadap penyakit batang dan akar serta penambahbaikan toleransi tekanan. Namun sedemikian, kebanyakan pengajian kepelbagaian DNA hanya focus pada nuclear DNA. Oleh kerana kloroplas merupakan organel yang mempunyai DNA tersendiri yang diwarisi secara unik, pengesanan polimorfisme pada peringkat populasi membolehkan kajian tersebut lebih bermakna terhadap kajian evolusi serta pencirian kepelbagaian DNA kloroplas kelapa sawit. Kajian terhadap kepelbagaian DNA kloroplas juga membolehkan kita memahami evolusi dan domestikasi sesuatu spesis tumbuhan. Dalam kajian ini, penghasilan protokol pengekstrakan yang diperkayakan dengan DNA kloroplas sawit adalah diutamakan. DNA kloroplas tersebut kemudian boleh digunakan untuk mengkaji variasi DNA kloroplas dalam sawit populasi terpilih. Protocol pengayaan kloroplas DNA tersebut mempergunakan cara mempergabungkan pemisahan kecerunan suckrose dan pemisahan sesium klorida yang dirujukkan untuk mengasingkan DNA kloroplas kelapa sawit. Enam kutipan germplasma (Angola, Gahan, Nigeria, Madagascar, Costa Rica dan Suriname) telah dipilih dan sampel DNA kloroplas sawit itu diekstrakkan dengan protocol kloroplas DNA pengayaan yang diubah suai. Protokol tersebut bertujuan mengasingkan organel kemudian menggunakan teknik pengasingan isopiknik untuk mengasingkan kloroplas DNA. Sawit DNA kloroplas yang terperkaya itu ditahkikkan dengan menjalankan analisis enzim penyekatan. Untuk mencirikan kepelbagaian DNA kloroplas sawit, primer mikrosatelit kloroplas (cpSSR) telah dihasilkan daripada kontaminasi kloroplas hasil jujukan kawasan hipomefikasi dan juga berdasarkan jujukan kloroplas *Elaeis guineensis* Jacq. Sebanyak tiga primer kloroplas sejagat (Chloroplast universal primer) juga telah digunakan untuk menganalisis kepelbagaian DNA kloroplas kelapa sawit. Untuk pengesahan lanjutan terhadap keutuhan protokol pengekstrakan dan kloroplas mikrosatelit, hasil amplifikasi primer sampel kajian yang terpilih telah diklon dan dihantar untuk penjujukan. Keputusan penjujukan dianalisis

dengan menggunakan pangkalan data umun. Kemudian, sebanyak dua belas pasang primer mikrosatelit kloroplas sawit serta dua pasang primer kloroplas sejagat telah digunakan untuk mengenotip kepelbagaian DNA kloroplas sawit. Frekuensi alel positif daripada lima germplasma (Costa Rica dikecualikan) untuk setiap lokus mikrosatelit adalah di antara 0.2 dan 1.0. gemrplasma. Germplasma Madagascar dengan frekuensi alel positif antara 0.22 dan 0.89 telah menunjukkan kepelbagainan yang terbesar antara kelima-lima germplasma yang dianalisasi dalam kajian ini. Tambahan pula, satu polimorfik cpSSR sawit (atp1) didapati menunjuk variasi interspesies jalur positif antara *E. guineensis* Jacq. and *E. oleifera* Cortez. Polimorfisme SSR yang dikesan dalam species adalah rendah, maka, ini menunjukkkan kadar molekul evolusi DNA kloroplas sawit adalah rendah. Bagaimanapun, variasi antara species *Elaeis* dalam DNA kloroplas kelapa sawit masih dapat dikesan.

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APPROVAL SHEET

I certify that a Thesis Examination Committee has met on (8-12-2014) to conduct the final examination of Ho Carl Miew on her thesis entitled "Establishing the tools for routine analysis of chloroplast DNA diversity in oil palm (*Elaeis* spp.)" 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 relevant degree. Members of the Examination committee are as follows:

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
×g	Relative centrifugal force
bp	Base-pair
BLAST	Basic Local Alignment Search Tool
BLASTn	BLAST search nucleotide databases using nucleotide query
BSA	Bovine serum albumin
cpDNA	Chloroplast DNA
cpSSR	Chloroplast simple sequence repeat/ chloroplast microsatellite
CsCl	Cesium chloride
CTAB	Cetyltrimethylammonium bromide
DIECA	Diethyldithiocarbamic Acid
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleosidetriphosphates
EDTA	Ethylenediaminetetraacetic acid disodium salt
e-value	Expected value
Gb	Gigabase
INDELs	Insertions and deletions
IR	Inverted repeat
LB	Luria broth
kbp	Kilobase-pair
M	Mole
MCA1	RNA stability factor for *Pet*A
MF	Methylation filtration
mg	Milligram
$MgCl_2$	Magnesium chloride
MISA	Microsatellite Search Tool
mM	Millimole
MPOB	Malaysia Palm Oil Board
NaCl	Sodium chloride
NaOAc	Sodium acetate
PA	Phosphatic acid
Pet	Cytochrome b_6f complex
PCR	Polymerase chain reaction
Poly A/T	Poly (deoxyadenosine: deoxythymidine)
PVP	Polyvinylpyrrolidone
SDS	Sodium dodecyl sulphate
SFR	Super fine resolution

SNP	Single nucleotide polymorphisms
SSR	Simple sequence repeat
RNA	Ribonucleic acid
rpm	Rotation per minute
RAPD	Randomly amplified polymorphic DNA
RE	Restriction enzyme
RNAase	Ribonuclease
TAE	Tris-Acetate-Ethylene Diamine Tetra Acetic Acid
TCA1	RNA translation factor 1 for *Pet*A
TE	Tric-EDTA
Trn	Transfer RNA
Uni	chloroplast specific- universal primer

CHAPTER 1

INRODUCTION

Oil palm, belongs to the genus *Elaeis* and comprises of two species; *Elaeis guineensis* Jacq. and *E. oleifera* Cortez (Hartley, 1988; Price *et al*., 2007). The commercial oil palm, *E. guineensis* is native to Africa while *E. oleifera* is found in Central South America (Hartley, 1988; Sasidharan *et al*., 2010; Zaki *et al*., 2010). Commercially grown *E. guineensis* can be further divided into three fruit forms: Tenera (hybrid of Dura and Pisifera), Dura and Pisifera. Palm oil is used both for edible (90%) and non-edible (10%) purposes.

Oil palm is a long lasting crop with a generic life of more than 200 years but an economic life span at 20 to 25 years (Hartley, 1988). In addition, intensive breeding research has been carried out to further improve the performance of the golden crop. The agronomic traits of interest to oil palm breeders are high yield, improved oil quality, slow trunk growth and disease resistance (Zaki *et al*., 2010). To assist the breeders, molecular marker technology has also been developed (Singh *et al*., 2007; Singh *et al*., 2008a; Singh *et al*., 2008b). For example, microsatellites or simple sequence repeat (SSR) markers have been applied for genetic diversity studies to develop genetic maps and have been linked to specific traits such as shell thickness (Ting *et al*., 2010). Molecular markers can help to improve the efficiency of breeding schemes and crop yield by identifying the factors such as traits that related to the crop performance (Diekmann *et al*., 2008; Singh *et al*., 2008a). However, for oil palm, molecular markers have been used mostly to analyze nuclear DNA. Chloroplast DNA has not been extensively studied. The uniparental inheritance and non- recombination nature of chloroplast DNA give advantages in analysis such as parentage and taxonomic studies. The variability of the independently transmitted genomes can be analyzed via microsatellites simultaneously in order to understand the nuclear-chloroplast interactions of oil palm as well as unravel the origin of polyploidy complexes that are common in flowering plant evolution (Powell *et al*., 1996).

The major goal of oil palm research is to help the industry elevate profit per hectare in plantations; this is of interest not only to oil palm investors but also to breeders' as well as agronomist (Price *et al.*, 2007). As know today, the use of palm oil is no longer limited to the production of edible oil; it is also being actively used as a commercially viable source of biodiesel. The tremendous progress in oil palm research has given the industry confidence to meet the challenges facing this remarkably productive crop. Despite the improvements made in the oil palm industry in the last 50 years, there remains considerable scope for further enhancing research and development activities to uplift both the upstream and downstream productivity (Basiron, 2007). Therefore, in this study, the oil palm cpSSR- based markers were used to analyze the chloroplast diversity of oil palm germplasm. Moreover, the study of oil palm chloroplast DNA is important as it provides opportunities to examine the lipid biosynthesis organelle of oil palm at the molecular level.

In this study, chloroplast DNA (cpDNA) will be the choice for analyses at the molecular level due to its potential information content (Mohanty *et al*., 2003) with three main objectives, which are as follow:

1. To establish a method for extracting enriched chloroplast DNA (cpDNA) for oil palm

2. To identify oil palm chloroplast microsatellites or Simple Sequence Repeat (SSR) markers from existing oil palm chloroplast sequences including
   a. Gene Thresher library whereby the chloroplast sequences were derived as contaminants from the genomic library constructed from the hypomethylated region of the oil palm (Low *et al*., 2014)
   b. Published *E. guineensis* Jacq. chloroplast genome in Gen Bank

3. To analyze oil palm chloroplast diversity and polymorphism in selected oil palm germplasm collections

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