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RESEARCH ARTICLE

Molecular marker based assessment of genetic diversity and morphogenetic stability in oil palm (*Elaeis guineensis* jacq.)

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Abstract

Oil palm (*Elaeis guineensis* Jacq.) is one of the most important oil crops globally. Given the rise in palm oil consumption, Thailand seeks to sustain its position, as a major global supplier of the product. Thus, development of high quality and high yielding oil palm hybrids that are suitably adapted to Thailand's climatic and environmental conditions is critical. The current research was important to investigate the clonal palms morphogenesis (leaf and frond), mantling fruiting, yield records, clonal fidelity, genetic integrity (TxT crosses legitimacy) of progenies and shell thickness (DNA band after HindIII) using SSR and CAPS markers to establish high yielding clonal palm suitable for unique environmental and climatic conditions. Superior morphogenetic characters and genetic integrity were detected from clones at the Univanich Oil Palm facility. The morphogenesis and yield records varied between clones. A normal fruiting palm (0% mantling) with better oil yield (36.97 kg/p/yr) and oil to bunch (27.73%) were recorded in T73 clone. The clonal fidelity (100%) with 8 SSR loci and progenies legitimacy (87% - 100%) with 19 SSR loci were confirmed. The CAPS marker resulted in specific DNA banding patterns that were identified in all Deli Dura palms. The current study confirms that morphogenically and genetically superior clones of the highest quality are able to produce commercial semi-clonal seeds. The results also highlight the possibility for the development of a specific marker for somaclonal variation and shell thickness.

Keywords: Breeding program, genomics markers, hybrid oil palm, semi clonal seeds, univanich

Introduction

The African oil palm, *Elaeis guineensis* is a tree-like monocotyledonous plant belonging to the Arecaceae family, native to West Africa and is widely considered the most important oil palm species (Corley and Tinker, 2015; Cosiaux *et al.,* 2018; Reddy *et al.,* 2019). Informal oil palm cultivation occurred predominantly in the western and central African coastal belt between Guinea/Liberia and Northern Angola (Murphy, 2014). High rainfall areas in equatorial regions that lay between 7° N and 7° S produced the highest yields of fresh fruit. Oil palm seeds were transported and introduced to the Dutch East Indies (Indonesia) and Malaysia during nineteenth century where systematic cultivation gradually established the species as a cash crop. Now, oil palm grows in plantations across the humid tropics of Asia, Africa and the Americas.

Southeast Asian countries contribute up to 85% of the total global production and export approximately 60% of the total crop, especially to India, Pakistan and Bangladesh (Murphy, 2014; Statista, 2020). Nowadays, oil palm plantations play major roles in the economies of many countries, including Indonesia, Malaysia, Papua New Guinea and Thailand from which higher volumes of oil and other derivatives are exported globally (Murphy, 2019). The sustained demand for the product has resulted in countries having to increase their agricultural output. For instance, Thailand's palm oil production has risen significantly, from 1.28 million metric tons in 2009 to 18.3 million tons in 2023. In Thailand, most oil palm plantations, palm oil mills and refineries are concentrated in southern regions due to favourable geographical conditions (Statista, 2023). Despite this, palm oil yield stood at approximately 2,354 kg/rai/year in 2000, prompting the development of new high-yielding oil palm varieties to achieve fresh bunch yields exceeding 4000 kg/rai/year (Khorawit and Sikun, 2005). This strategic shift has yielded measurable progress, with recent data showing a 3.52 ton/ha yield recorded in 2023-2024 (Fig 1) (IPAD, 2025). Meanwhile, sustained growth in palm oil demand and consumption rates over recent years underscores the urgency of scaling up high yielding plantations to meet market needs (Statista, 2024). However, classical plant breeding methods face significant challenges including the time required to implement monitoring and evaluation processes to improve yields, while the outcomes are dependent on multiple factors (e.g., climate, soil, genetic diversity) that complicates efforts to meet immediate demand (Cros *et al.*, 2018). For instance, frequent and severe climatic conditions lead to drought conditions and heat waves that affect oil palm yield (Feller, 2016; Bayona-Rodriguez and Romero, 2024). Furthermore, seed propagation and plant breeding systems employed for oil palm hybrid selection over several years has introduced a high degree of heterogeneity and limited genetic variability that has also negatively impacted the yield of palm oil (Wongsri and Wongsri, 2005; 2005; Martin *et al.*, 2008). General or specific combining ability programmes have achieved success with certain high performing hybrids; however, they still lack the capacity to produce varieties with consistent genotype specifications (Samphantharak, 2008; Katore and Navale, 2018; Ali and Gashaw, 2019). Therefore, new breeding programmes require the application of modern biotechnology, and molecular techniques in order to improve oil palm yield in a relatively shorter period of time to satisfy local and global demand. Rapid vegetative propagation using plant cloning techniques has been recognised as the most efficient method to propagate high-quality, high-yielding and pest-free varieties (Martin *et al.*, 2008). Significant efforts spanning several years have focused on refining cloning techniques through somatic embryogenesis or organogenesis, aiming to minimize somaclonal variation (oil palm abnormalities) linked to prolonged solid culture processes (Tisserat, 1997; Hayati *et al.*, 2004; Martin *et al.*, 2008). Somatic embryogenesis, genetic engineering and genetic transformation with callus or cell suspension cultures accelerates oil palm propagation while also enabling the introduction of ideal or novel genetic traits to enhance the quality and quantity of production (Hayati *et al.*, 2004; Martin *et al.*, 2008). Genetic variation between individuals and populations of oil palm was investigated using morphological, molecular and biochemical markers. Morphological abnormalities in oil palm increase with adverse environmental conditions and correlate with plant age. Genetic variation has been assessed using molecular markers such as simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) (Martin *et al.*, 2008). These molecular markers enable analysis of genetic stability at the DNA level (Martin *et al.*, 2008).

The present study investigates the genetic stability of hybrid seeds produced by the Univanich Oil Palm facility in Thailand, employing molecular markers to evaluate their suitability for drier conditions in Thailand. The aim of the current research was to evaluate leaf morphogenesis, frond arrangement, mantling fruiting, and genetic integrity (clonal fidelity and legitimacy) of the clonal palms and progenies using SSR makers. Shell thickness testing (DNA band after HindIII) was also evaluated using the CAPS marker to contribute to the establishment of a premium high yielding clonal palm suitable for Thailand's unique environmental and climatic conditions. Additionally, the present research supports the continuous improvement of the Univanich molecular breeding programmes for the development of molecular markers for somaclonal variation, shell thickness, drought tolerance and Ganoderma resistance.

Materials and methods

Morphogenetic characters (phenotype) of the clones

Leaf phenotypic characteristics of four- to eight-month-old Tenera clonal plantlets from the nursery were obtained from clone lines (Univanich: T140-131-10, T140-814-7 andT140-825-4) that were of differential ages based on duration of callus development, and the stage of embryo development and the shoot and rooting phases. Frond phenotype (arrangements) and 6-year yield records FFB (Fresh fruit bunches, kg/palm), BN/p (Bunch number/palm), ABW (Average bunch weight (kg)/palm, OY (Oil yield, kg/palm/year) and % O/B (Oil to bunch)] of Tenera clone lines (T74-163-1, T74-163-4, T74-163-5 and T74-267-3) and ortets were examined. The phenotype and percentage of mantling fruiting palms, (severe, mild and normal) and 10 year ortets and 4 year yield clone records [FFB, Ton/hectares (T/Ha); BN/p; ABW (kg/palm); OY (kg/p/yr); O/B (%)] from different clone lines (D58-175-1, D56-83-11, D57-1-11, D72-77-3, D81-384-1, T51-310-13, T55-131-7, T61-538-3, T73-213-4, T74-163-5 and T86-46-1) were evaluated and compared for their clonal efficiency. These clone lines are classified based on differential culture durations (months) for callus, embryos, shoot and rooting development. The ortets (parental palms) of the clonal palms were used as a controls/reference.

Legitimacy test and Shell traits analyses

Eight different breeding crosses (male and female palms: BBT T×T and DRT T×T, selfing and full siblings) produced 6-month-old seedlings (nursery stage) of the progenies (each cross containing 200 seedlings). These were used to evaluate legitimate conditions of the progenies. These crosses parental palms were planted in 1999. Seeds shell traits of seven- to twenty-one-year-old Pisifera, Dura, Tenera and commercial Tenera were evaluated to determine their fruit form.

Plant materials and Genomic DNA isolation

Fully grown leaves of selected ortets, seedlings and clones of the oil palm were collected for DNA isolation for SSR and CAPS markers genetic pattern analysis. The collected leaf samples were cleaned with 70% ethanol and ground with liquid nitrogen. Total genomic DNA was extracted using the CTAB method (Zeng *et al.*, 2002). The DNA quantity and quality was checked on a 1.0% agarose gel and Nanodrop lite spectrophotometer. Genomic DNA was stored at -20° C until PCR application.

Genotyping analysis: Simple Sequence Repeat (SSR) marker

Simple sequence repeat (SSR) genotyping was performed by PCR. The PCR was carried out with 20 µL reaction liquid containing 50 ng of template DNA.

The PCR conducted over 40 cycles. Agarose gel electrophoresis was employed to detect the presence of fragments corresponding to the expected sizes using a molecular ladder. The different alleles were identified by separating the PCR fragments on denaturing 4.5% polyacrylamide, at 75 W constant power for 1.5 - 2.5 hour. The polyacrylamide gels were stained with silver nitrate. The alleles were scored manually as multi-allelic co-dominant data. Assessment of clonal fidelity and legitimacy of progenies were conducted with 19 microsatellite loci for the SSR amplifications (Thongthawee *et al.,* 2010; Billotte *et al.,* 2005). General characteristics of SSR markers (primers) details were described in Table 7.

CAPS marker and Statistical Analyses

The genomic DNA was amplified with the use of an EgSHP primer (Babu *et al.,* 2017). The PCR products were validated for presence of fragments using agarose gel electrophoresis (550 bp on 2% agarose gel). For restriction enzyme cutting, 10 µL of PCR products were incubated with 0.5 unit of HindIII at 37 °C for 16-18 hour. The restriction fragments were visualized by 5% agarose gel electrophoresis at 75 V for 50 min. Experiments were set up with a randomized block design. Each experiment contained a minimum number of clonal palms of Tenera (16 palms per replicate × 4 replicates) and Dura (25 palms per replicate × 6 replicates) and Pisifera (20 palms per row). The data are presented as the mean value of each experiment with replicates of the study. Statistical analysis was performed with the one-way analysis of variance (ANOVA) using SPSS version 24.0 for Windows (Chicago, IL, USA). Significantly different means were separated using Duncan's multiple range test (P = 0.05).

Results and discussion

Morphogenesis, yield records and genetic fidelity

Results related to the morphogenetic analysis of leaves of the clonal plantlets in the nursery stage and fronds arrangement in field planted clonal palms are presented in Tables 1, 2 and 3 and Figures 2 and 3, respectively. Normal and abnormal (rolled) leaf phenotypic characteristics were identified in Tenera clonal lines (T140-131-10, T140-814-7 and T140-825-4) (Table 1). Each Tenera clone having minimum 16 palms per replicate and planted 4 replicates. Six years yield recorded data of ortet (T74) and the clones (T74-163-1 to T74-163-5 and T74-267-3). Means followed by same letters in each column are not significantly different (P = 0.05) using Duncan's multiple range test. The phenotype, yield records and clonal fidelity of clones were evaluated from 2018-2023. Leaf phenotype in this clonal line was influenced by the callus culture period, as well as the embryo, shoot and rooting stage (Table 1). For instance, a decrease in the length of the callus production period (<9 months) together with an increase in the embryo (>18 months) and rooting (>4 months) stages in the T140-131-10 clonal line produced plantlets that exhibited rolled leaves (Table 1 and Fig 2A). This phenotypic leaf was observed in 4-month-old plantlets (Fig 2A). However, the plantlets (8 months-old) were normal and healthy in T140-814-7 and T140-825-4 clone lines (Table 1 and Fig 2B). These plantlets were obtained from 9-month-old callus, 18-month-old embryos and 4-month-old rooting periods. The culture periods had the greatest influence on the production of normal and abnormal (somaclonal variation) clonal plantlets. An increase in culture period of embryos and rooting resulted in abnormalities in leaves of the plantlets in the nursery stage. On the other hand, genetic fidelity at the DNA level among the clonal plantlets and ortet revealed 100% genetic fidelity based on the banding pattern generated by the SSR marker (Table 1 and Fig 2C).

Table 1: Morphogenetic characteristics (phenotype) of leaves taken from 4- to 8-month-old clones in the nursery

Palm type	Clone line (#)	Culture periods (months)				Age of the clone (Months)	Leaf phenotype	Fidelity (%) (8 SSR loci)
		Callus	Embryos	Shoot	Rooting			
Ortet – Palm	T140	-	-	-	-	6 years-old	Normal	Parent palm
Clone - Ramet	T140-131-10	7	20	8	5	4	Rolled	100%
Clone - Ramet	T140-131-10	7	20	8	5	4	Rolled	100%
Clone - Ramet	T140-131-10	7	20	8	5	4	Rolled	100%
Clone - Ramet	T140-131-10	7	20	8	5	4	Rolled	100%
Clone - Ramet	T140-131-10	7	20	8	5	4	Rolled	100%
Clone - Ramet	T140-814-7	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-814-7	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-814-7	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-814-7	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-814-7	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-825-4	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-825-4	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-825-4	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-825-4	9	18	8	4	8	Normal	100%
Clone - Ramet	T140-825-4	9	18	8	4	8	Normal	100%

Table 2: Morphogenetic characteristics (phenotype) of frond arrangement, and genetic integrity of clonal oil palms

Palm type	Clone line (#)	Culture periods (months)				Trial name	Frond phenotype	Genetic integrity (8 SSR loci)
		Callus	Embryos	Shoot	Rooting			
Ortet	T74 (UPT99/2)	-	-	-	-	CLT15/1	Normal	Parent palm
Clone	T74-163-1	14	5	7	5	CLT15/1	Twisted	100%
Clone	T74-163-4	14	9	5	2	CLT15/1	Normal	100%
Clone	T74-163-4	14	9	5	5	CLT15/1	Twisted	100%
Clone	T74-163-5	14	9	5	5	CLT15/1	Twisted	100%
Clone	T74-163-5	14	9	3	2	CLT15/1	Normal	100%
Clone	T74-163-5	14	9	5	5	CLT15/1	Twisted	100%
Clone	T74-163-5	14	9	5	5	CLT15/1	Twisted	100%
Clone	T74-163-5	14	9	3	2	CLT15/1	Normal	100%
Clone	T74-267-3	14	8	6	2	CLT15/1	Normal	100%
Clone	T74-267-3	14	8	6	2	CLT15/1	Normal	100%
Clone	T74-267-3	14	8	6	2	CLT15/1	Normal	100%

Table 3: Morphogenetic characteristics (phenotype), yield records and genetic integrity of clonal oil palms

Palm type	Clone line (#)	Trial name	Frond phenotype	Yield records (6 years)					Genetic integrity (8 SSR loci)
				FFB (kg/p)	BN/p	ABW (kg/p)	OY (kg/p/yr)	O/B (%)	Fidelity (%)
Ortet	T74 (UPT99/2)	CLT15/1	Normal	154.58 a	12.73 cd	12.15 a	45.02 a	29.12 b	Parent palm
Clone	T74-163-1	CLT15/1	Twisted	114.38 g	13.67 bc	8.37 d	42.06 b	30.94 a	100%
Clone	T74-163-4	CLT15/1	Normal	153.95 ab	14.00 b	9.71 bc	21.66 cd	26.34 d	100%
Clone	T74-163-4	CLT15/1	Twisted	106.50 h	11.50 de	9.26 c	18.42 j	7.32 i	100%
Clone	T74-163-5	CLT15/1	Twisted	119.68 f	14.80 ab	8.09 de	24.51 g	26.30 de	100%
Clone	T74-163-5	CLT15/1	Normal	93.20 k	11.83 d	7.88 e	21.46 h	19.26 g	100%
Clone	T74-163-5	CLT15/1	Twisted	129.23 de	13.67 bc	9.46 c	26.87 e	26.00 e	100%
Clone	T74-163-5	CLT15/1	Twisted	103.33 ij	11.17 e	9.25 c	22.04 h	24.13 f	100%
Clone	T74-163-5	CLT15/1	Normal	87.35 l	9.50 f	9.19 c	20.06 i	17.67 h	100%
Clone	T74-267-3	CLT15/1	Normal	152.58 bc	15.17 a	9.94 b	32.53 c	28.96 bc	100%
Clone	T74-267-3	CLT15/1	Normal	130.60 d	14.50 ab	9.01 cd	16.61 k	19.65 g	100%
Clone	T74-267-3	CLT15/1	Normal	104.27 i	10.67 ef	9.78 bc	31.72 cd	26.01 e	100%

FFB, Fresh Fruit Bunches. BN, Bunch Number. ABW, Average Bunch Weight. OY, Oil Yield. O/B, Oil to Bunch. Kg, Kilo gram. P, palm. Yr, Year. CLD, Clonal Dura.

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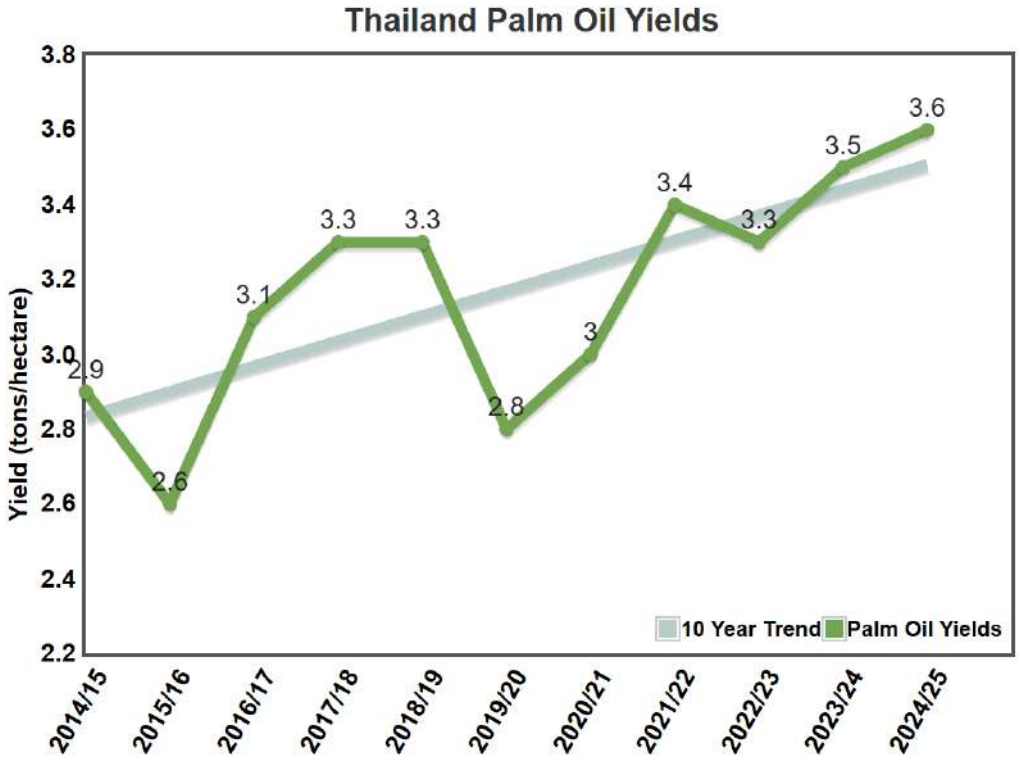
Fig. 1: The palm oil yields record data in Thailand (IPAD 2025)



In vitro regeneration of elite oil palms demonstrated marked success with its application at the different stages of growth and development (callus, embryogenesis, shoot and root regeneration and ex-vitro transplantation). Despite this, lengthy culture durations at any stage resulted in the development of abnormalities in the oil palm which can be attributed to somaclonal variation (Rohani *et al.*, 2003). Similarly, the present study revealed abnormalities during each of the growth stages when prolonged culture periods were applied. Prolonged embryogenesis (> 18 months) and root regeneration (> 4 months) stages influenced detrimental effects that manifested as rolled leaf abnormalities in the plantlets. Furthermore, shorter culture durations at the callus stage (< 9 months) also resulted in rolled leaf abnormalities in 4-month-old nursery plantlets from the T140-814-10 clone. These results indicated that careful consideration of the culture period during each stage of development is important to avoid abnormalities or somaclonal variation in these in vitro derived plantlets. Producing a large number of embryos with repetitive subculture regimes in one culture period increases the risk of somaclonal variation. Somaclonal variation may also developed in tissue culture through genetic and epigenetic mechanisms (Karim, 2021). Genetic fidelity estimated through the inspection of SSR banding patterns revealed 100% genetic fidelity in ortet and clonal plantlets. This suggests that in vitro propagation derived clonal plantlets are not influenced by genetic variation, instead, these may be temporary changes in epigenetic characters (not involving changes to the DNA sequences itself). These epigenetic changes resulted in poorly transmitted modifications to the phenotype of plantlets but are identical to the parent cells. The epigenetic changes revert to the normal phenotype but some are long lasting and even transfer during sexual propagation (Smulders and De Klerk, 2011).

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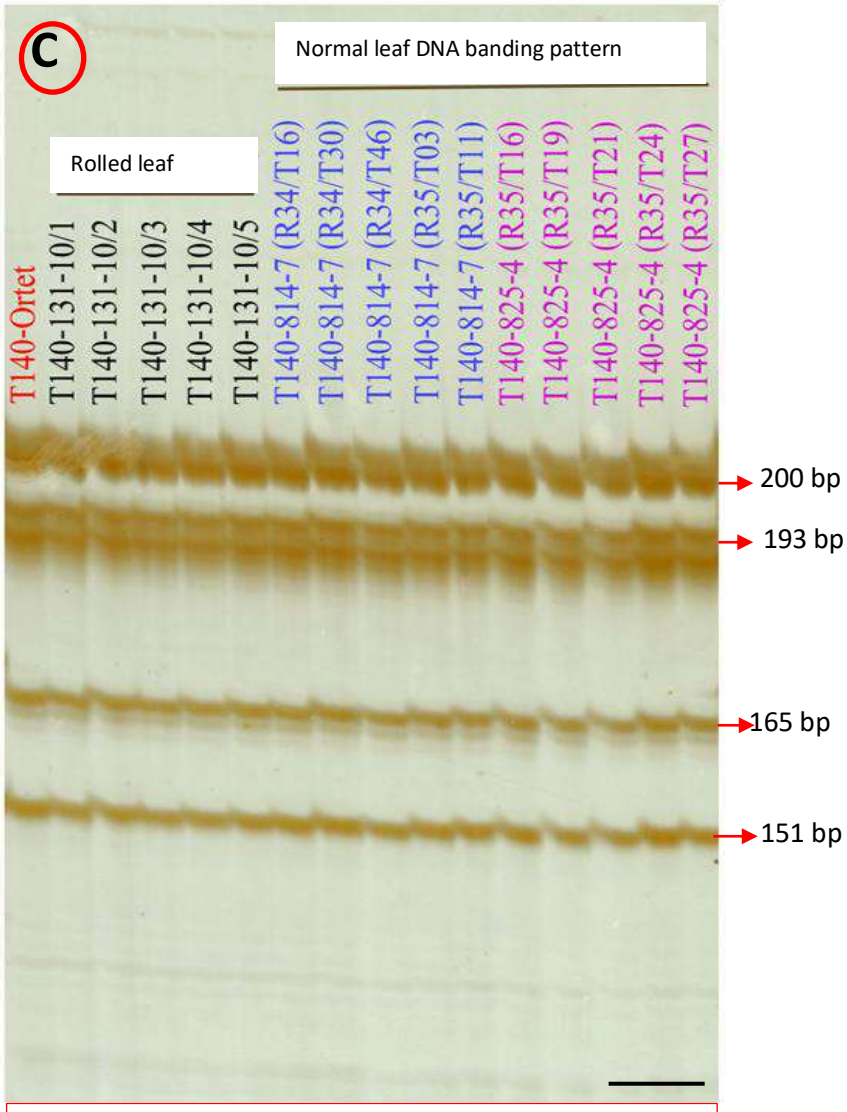
Fig. 2: Leaf phenotypic characters of 4-8 months-old clones in nursery and DNA fingerprint of clonal Tenera (T140). (A) Rolled leaf of 4 months-old ramets. (B) Normal leaf of 8 months-old ramets. (C) DNA banding pattern of rolled and normal leaf of 4 and 8 months old ramets. The bar (A-C) = 5 mm.

Rolled leaf of 4 months-old ramets

Normal leaf of 8 months-old



DNA banding pattern of rolled and normal leaf

This can be caused by DNA methylation, DNA amplification, histone modification and transposable elements (Smulders and De Klerk, 2011). The epigenetic changes that induce abnormal phenotypic characteristics in oil palm plantlets take several years to manifest in several plant traits including flower and fruit development, fresh fruit bunch, bunch number and average bunch weight and oil yield. The Tenera clones (T74-163-1, T74-163-4, T74-163-5, and T74-267-3) exhibited distinct frond phenotypes (normal vs. twisted), with developmental variation linked to the duration of embryo culture, shooting, and rooting phases (Table 2). This can be caused by DNA methylation, DNA amplification, histone modification and transposable elements (Smulders and De Klerk, 2011).

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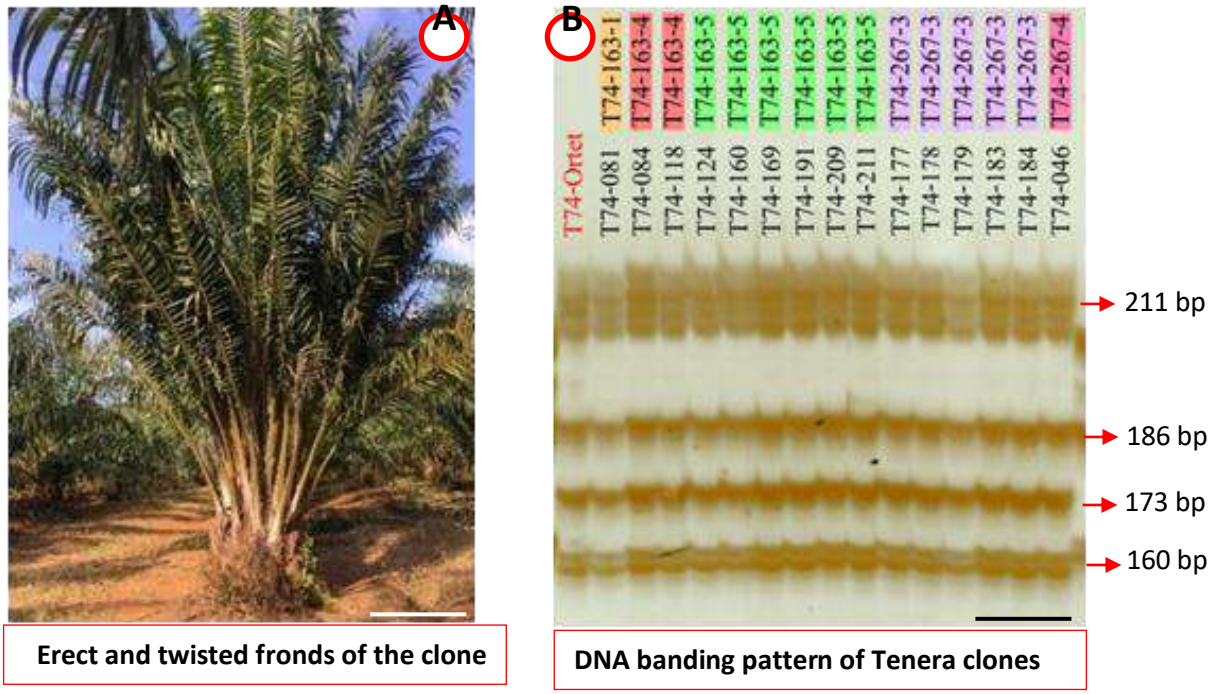
Fig. 3: Frond phenotypic characters of 7 years-old field planted clones and DNA fingerprint of clonal Tenera (T74). (A) Erect and twisted fronds of the clone from 4 years-old field planted. (B) DNA banding pattern of twisted and normal fronds of 4 years-old field planted clones. The bar (A-B) = 5 mm.



To evaluate how culture durations affect the development and production of healthy, high-quality fruiting palms, this study assessed callus induction, embryo formation, shooting, and rooting phases in both Dura and Tenera clones. Callus maintained for <8 months and embryos maintained for <8 and >12 months, produced severe and mild abnormal fruiting palms, respectively (Table 4,5 and Fig 4a-d). The Dura and Tenera ortets yield data records were 10 years. Each Tenera clone having minimum 16 palms per replicate and planted 4 replicates. Means followed by same letters in each column are not significantly different (P = 0.05) using Duncan's multiple range test. Each Dura clone having minimum 25 palms per replicate and planted 6 replicates. Four years yield recorded data. Mantling % = mantled fruits bunch palms. T, Tenera. D, Dura. Moreover, a high frequency (85.2%) of severely abnormal fruiting palms was recorded in Dura clone (D58-175-1) in response to specific genotypic characteristics (Fig 4c). However, the Tenera clones (T51 – T86) resulted in the production of normal fruiting palms (Fig 4a). Severe and mild mantled fruit of the clones was also observed in this study, whereby stamen (male) and staminodes (female) were transformed with carpels like structures (extra carpels). In mantled oil palms, flowers develop into fruit that produce a lower oil yield, while severely mantled flowers are aborted which leads to total loss of oil yield (Adam *et al.*, 2007; Mgbeze and Iserhienrhien, 2014). In this study, shorter (<8 months) and longer (>8 and <12 months) culture periods for callogenesis and embryogenesis of the Dura clones resulted in severe and mildly abnormal (mantled) fruiting palms. The percentage of mantled fruiting palms varied between these clones. There was no mantled fruiting palms observed in Tenera clone lines. The results of the study indicate that the mantled fruiting palms depended on culture processing periods either early or extended periods in callus, embryos, shoot and rooting development. This study revealed a similar banding pattern related to genetic fidelity (100%) of DNA in Dura and Tenera clones and their ortets, indicating no genetic variation between normal and mantled fruiting palms as well as ortets of the clones.

Table 4: Fruiting palm mantling, phenotypic and genetic integrity of clonal oil palms

Palm type	Clone line (#)	Culture periods (months)				Fruiting palm mantling (%)	Fruiting palm phenotype	Genetic integrity (8 SSR loci)
		Callus	Embryos	Shoot	Rooting			
Clone	D58-175-1	9	14	12	4	85.2 a	Severe abnormal	100%
Clone	D56-83-11	9	17	11	4	11.1 c	Mild abnormal	100%
Clone	D57-1-11	10	18	8	4	14.9 b	Mild abnormal	100%
Clone	D72-77-3	6	7	6	3	0.5 d	Mild abnormal	100%
Clone	D81-384-1	9	7	4	4	15.6 b	Mild abnormal	100%
Ortets	D56-D81 (Average)	-	-	-	-	0	Normal	100%
Clone	T51-310-13	9	10	21	4	0	Normal	100%
Clone	T55-131-7	13	12	9	4	0	Normal	100%
Clone	T61-538-3	8	12	14	2	0	Normal	100%
Clone	T73-213-4	13	12	5	2	0	Normal	100%
Clone	T74-163-5	14	10	5	2	0	Normal	100%
Cone	T86-46-1	9	8	2	4	0	Normal	100%
Ortets	T51- T86 (Average)	-	-	-	-	0	Normal	100%

Table 5: Fruiting palm yield records and genetic integrity of clonal oil palms

Palm type	Clone line (#)	Yield records (4 years)					Genetic integrity (8 SSR loci)
		FFB (T/Ha)	BN/p	ABW(kg/p)	OY (kg/p/yr)	O/B (%)	Fidelity (%)
Clone	D58-175-1	6.91 j	3.97 f	8.43 cd	9.87 k	22.87 cd	100%
Clone	D56-83-11	17.16 g	12.28 de	9.03 cd	24.64 i	22.98 d	100%
Clone	D57-1-11	16.99 gh	12.93 de	7.97 d	24.42 i	23.00 cd	100%
Clone	D72-77-3	24.47 bc	15.55 c	9.87 c	34.36 d	22.47 d	100%
Clone	D81-384-1	19.50 f	13.86 d	8.74 cd	27.06 g	22.20 de	100%
Ortets	D56-D81 (Average)	32.66 a	14.51 d	15.98 a	50.50 a	22.22 d	100%
Clone	T51-310-13	18.03 g	15.69 c	7.90 d	20.55 h	18.23 g	100%
Clone	T55-131-7	23.76 c	20.99 a	10.63 bc	30.95 ef	21.08 f	100%
Clone	T61-538-3	21.74 e	18.23 b	7.42 d	31.02 e	22.16 de	100%
Clone	T73-213-4	25.33 b	20.88 a	11.84 b	36.97 c	27.93 ab	100%
Clone	T74-163-5	17.85 g	13.61 d	7.84 d	26.20 h	23.49 c	100%
Cone	T86-46-1	14.99 i	12.08 de	8.47 cd	22.33 j	23.84 c	100%
Ortets	T51- T86 (Average)	22.17 d	15.13 c	10.87 bc	44.58 b	28.90 a	100%

FFB, Fresh Fruit Bunches. BN, Bunch Number. AWB, Average Bunch Weight. OY, Oil Yield. O/B, Oil to Bunch. T, Ton. Ha, hectares. D58, D56, D57, D72 and D84 clones Ortet were from CDD99 (Congo Deli Dura).

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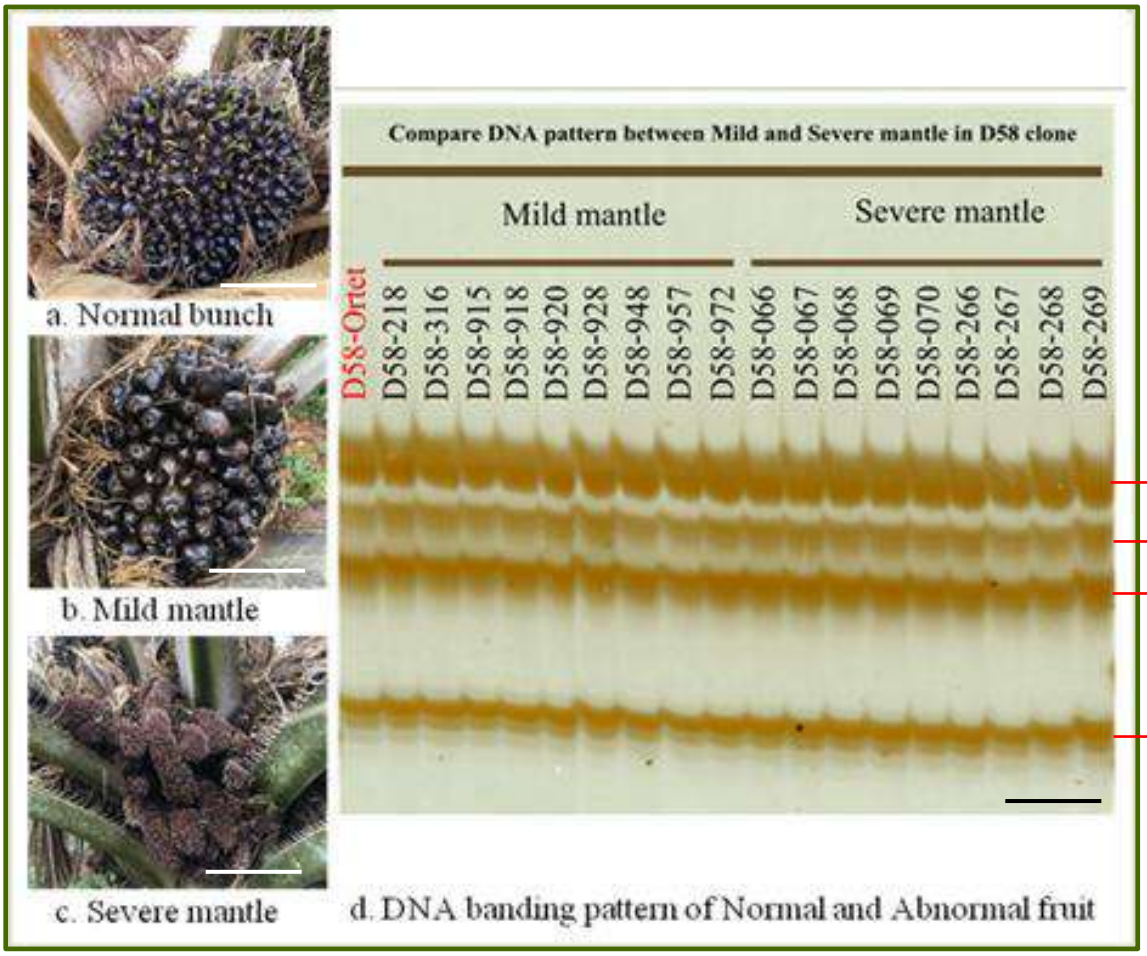
Fig. 4: Fruits bunch phenotypes and DNA banding pattern of mild and severe mantled fruits bunch of 7 years-old field planted clonal Dura palms. (a) Normal fruits bunch. (b) Mild mantled fruits bunch. (c) Severe mantled fruits bunch. (d) DNA banding patters of mantled fruits bunch Dura clones. The bar (a-d) = 5 mm.



Four-year yield records varied between abnormal fruiting palms of Dura and normal fruiting palms of Tenera. A significant improvement in FFB (24.47 T/Ha) was recorded in the D72 clone although it was significantly lower than the ortets yield records (average FFB, 32.66 T/Ha). The bunch number per palm was significantly higher in D72 clones (15.55/palm) compared to ortets (average 14.51 BN/p). However, no significant differences in O/B between clones and ortets were noted (Table 5). Although a higher FFB (25.33 T/Ha) was recorded for normal fruiting palms of the Tenera clone (T73), this was not significantly different to abnormal fruiting palms (0.5%) of the Dura clone (D72). In this study, Tenera clone (T73) produced the highest BN (20.88/p), ABW (11.84 kg/p), OY (36.97 kg/p/yr) and O/B (27.73%) compared to the clones (Table 5). This indicated that the genotype of the Tenera clone is important for producing the best yields in oil palm. Four-year yield records of clones (Dura and Tenera) revealed improved yield performance over the 10-year yield records (average) of the ortets (Table 5). We employed SSR markers to evaluate the genetic fidelity of normal and abnormal fruiting palms in both Dura and Tenera clones (Table 4 and Fig 4d).

The analysis revealed no variation in the allelic banding patterns among the clones, indicating that both normal and abnormal fruiting clonal palms are genetically identical to their respective ortets. The yield records of normal and mantled fruiting palms varied; however, the percentage O/B was not significantly differed in Dura clone lines. On the other hand, BN/p and percentage O/B was significantly better in T73-213-4 Tenera clone line. A severe bunch loss and parthenocarpic fruit set was accompanied by abnormality which exists between different clone lines of the palm (Mgbeze and Iserhienrhien, 2014; Corley and Tinker, 2003). Mechanism of epigenetic changes and identifying the genes involved in this mechanism in the flower of the oil palm is essential in improving oil yield (Jaligot *et al.,* 2011). Improved Dura and Tenera clones observed in this study were used for further analysis of semi-clonal production.

Progenies legitimacy and validation of shell traits

PCR amplification using 19 SSR (microsatellite) loci generated distinct allele banding patterns and size profiles across eight different breeding parents and their progeny. For downstream analysis, eight loci were selected based on their relatively uniform allele frequency distributions. The allelic profiles of both parental lines (T × T) in the BBT and DRT trials were validated by observing segregation patterns in their progeny (Table 6 and Fig 5). This technique demonstrated high accuracy in validating the genotypic data of the tested crosses. In the BBT full-sibling crosses (UV201/938 female × UV201/927 male and UV202/1110 female × UV202/734 male), one SSR locus mismatch was observed, corresponding to a 93% parentage legitimacy rate. In contrast, the DRT full-sibling cross (UV217/75 female × UV217/76 male) exhibited two SSR locus mismatches, resulting in an 87% legitimacy rate. These mismatched progenies were confirmed as off-types, not belonging to the respective crosses. However, other BBT crosses showed no mismatches, achieving a 100% legitimacy rate (Table 6 and Fig 5). Overall, the SSR marker analysis effectively served as a quality control tool for early selection and crossbreeding verification in oil palm nurseries.

Table 6: Progenies legitimacy test from breeding trails of 6 months-old seedlings of the oil palm planted in the nursery

Cross No.	Breeding				Progeny	Illegitimate progeny	
	Trial	Type	Female	Male	Total seedlings	Mismatch plant (Total SSR loci)	Legitimacy rate
1	BBT TxT	Selfing	UV196/871	UV196/871	200	0	100%
2	BBT TxT	Full-sibling	UV196/664	UV196/871	200	0	100%
3	BBT TxT	Full-sibling	UV196/664	UV196/662	200	0	100%
4	BBT TxT	Full-sibling	UV196/891	UV196/662	200	0	100%
5	BBT TxT	Full-sibling	UV201/938	UV201/927	200	1	93%
6	BBT TxT	Selfing	UV202/734	UV202/734	200	0	100%
7	BBT TxT	Full-sibling	UV202/1110	UV202/734	200	1	93%
8	DRT TxT	Full-sibling	UV217/75	UV217/76	200	2	87%

The cross of parental palms (Dura and Pisifera) planted in the field at 1999. Each Tenera cross having minimum 16 palms per replicate and planted 4 replicates. BBT, Binga Breeding Trial. DRT, Drought Resistant Trial. TxT, Tenera x Tenera. UV, Univanich.

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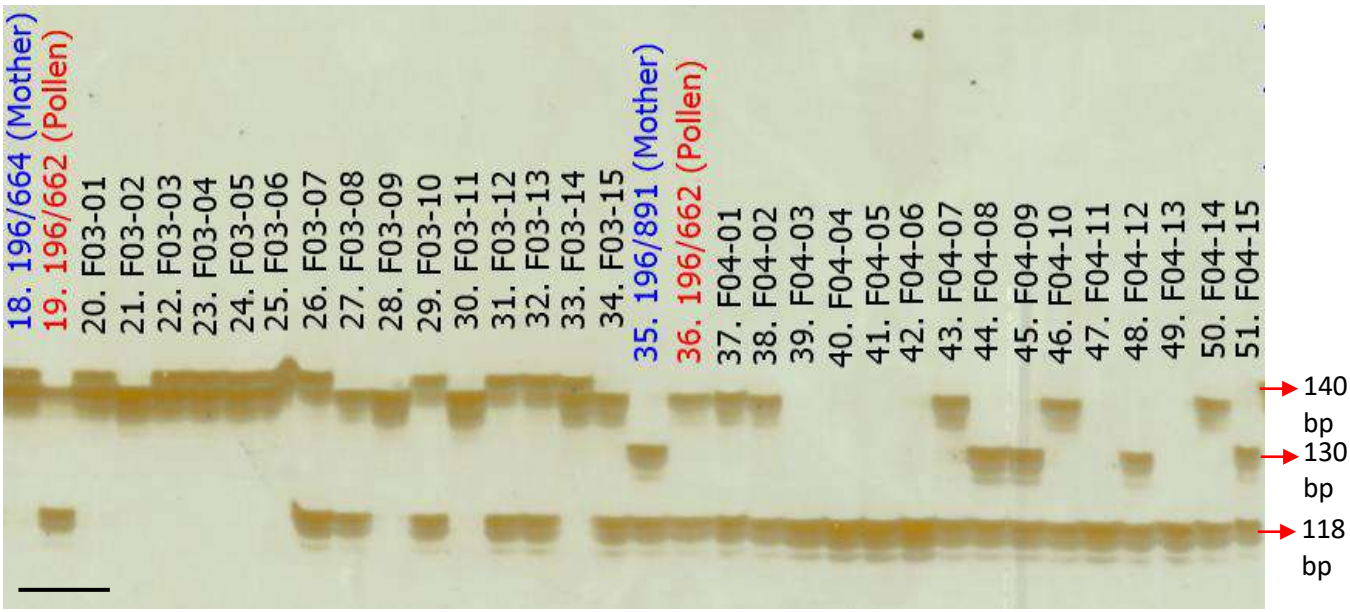
Fig. 5: DNA banding patterns of TxT progenies (8 months-old nursery planted seedlings) with primer mEgCIR0521 for legitimacy confirmation. The bar = 5 mm



Success of breeding programs depends on genetic variability and selection of genetically superior individuals that offer an excellent genetic combination to produce different traits that are of agronomic interest. The cross-breeding program suggested to produce desirable plant type (genetic variability) and high yielding varieties (Navaneetha *et al.*, 2019; Ismail *et al.*, 2020). Controlled breeding of oil palm ensures sustainable high yields (FFB, BN/p, ABW, OY and O/B) from the progenies. However, evaluating the legitimacy of progenies is essential in ensuring that the plantlets do not compromise on yield. This is traditionally conducted through detection from the fruit of the oil palms but this process requires lengthy periods to allow plantlets to set fruit. Alternatively, legitimacy can be performed either in roots or leaves of the seedlings (Zolkafli *et al.*, 2021). The SSR molecular marker, has been used for identifying legitimate progeny from the same pattern of allelic profiles as their parents (Billotte *et al.*, 2005; Thongthawee *et al.*, 2010; Zolkafli *et al.*, 2021). In this investigation, the molecular study confirmed the legitimacy of progenies (BBT T×T and DRT T×T, selfing and full-sibling) in the trials in order to select elite oil palms breeds for successive breeding cycles to meet several traits of interest to the Univanich agroindustry. In the present study, mismatching loci were observed for illegitimates using SSR markers. The SSR loci had 1 mismatch with 93% accuracy of genotype and parentage in the BBT full-sibling (UV201/938 female × UV201/927 male and UV202/1110 female × UV202/734 male) cross, while, more illegitimates (2 mismatch with 87% accuracy) were found in the DRT full-sibling (UV217/75 female × UV217/76 male) cross. These results supported the accuracy of genotype and parentage of the crosses. These profile mismatch loci between the progenies and parental palms, indicated potential contamination from pollen within pollination bags or unspecific pollen through insect interactions or by human error (tying bags too loosely, mislabeling and mix-ups). A similar conclusion was drawn in the previous breeding programs (Corley, 2005; Zolkafli *et al.*, 2021). In this study, 100% legitimacy of the progenies was confirmed in the crosses of BBT T×T both selfing and full-sibling of female and male (UV196/ 871 and 664 × UV196/871 and 662, UV196/891 × UV196/662 and UV202/734 × UV202/734)

These results clearly showed that human errors should be strictly controlled to ensure legitimacy in controlled crosses. The polymorphic nature of SSR markers confirmed the progenies were detected effectively to ensure purity of controlled crosses for improving the oil palm breeding. Three types (Pisifera, Dura and Tenera) of oil palm exist in the natural environment. These are classifieds based on fruit shell thickness which is determined by the Sh gene in the shell. The homozygous Dura genotype exhibits a thick shell with dominant Sh allele (Sh/Sh), the homozygous Pisifera genotype does not have a shell and exhibits a recessive Sh allele (sh/sh) while the genotype of hybrid Tenera (Dura × Pisifera) has an intermediate shell thickness with heterozygous Sh alleles (Sh/sh). Shell thickness of Pisifera, Dura and Tenera palms planted at the Univanich plantation (UV family) in different age (7 to 21 years-old) were validated using the EgSHP primer (cleaved amplified polymorphic site, CAPS marker) (Table 8). The CAPS marker differentiated 2 alleles (300 and 250 bp) in Dura genotype, one allele (600 bp) in the Pisifera genotype except UV264 Pisifera (3 alleles, 600, 300 and 250 bp) and three alleles (600, 300 and 250 bp) in the Tenera genotype. The sequencing of shell allele banding pattern presented SNPs; however, SNP2 contributed to the variation in shell thickness and was used to identify the fruit forms. The DNA profile after HindIII restriction enzyme digestion of DNA sequences presented as thymine in the Pisifera genotypes except the UV264 family (adenine and thymine), adenine in the Dura genotypes and adenine and thymine in the Tenera genotypes (Table 8 and Fig 6). Each Tenera having minimum 16 palms per replicate and planted 4 replicates. Each Dura having minimum 25 palms per replicate and planted 6 replicates. Each Pisifera having minimum 20 palms per row. This CAPS marker study validated the selection of oil palm families with desirable parents, Dura and Pisifera and high yielding Tenera, supporting to the farmers and commercial production. Selection of oil palm type by using DNA markers is a valuable tool that can be applied to shorten the breeding cycle and to select elite varieties of oil palm. Most recently, SSR and SNP markers were used to detect genetic diversity, develop linkage maps and conduct GWAS studies which elucidates the architecture of plant genomes and the relationship between genotype and phenotype (Billotte *et al.*, 2005; Babu *et al.*, 2017). The shell thickness, Sh gene specific primer pair was developed earlier using 300 genomic and 8 genic SSR markers, whereas the CAPS marker was applied to differentiate fruit type with specific allele profiles (Babu *et al.*, 2017). In the present study, segregation of shell thickness was identified in the homozygous genotypes of Dura and Pisifera and the heterozygous genotype of Tenera in the breeding and progeny trials. This reduced contamination of Dura, Pisifera and Tenera planting materials will greatly benefit Univanich oil palm breeding programs. The fruit type of the UV family containing Pisifera, Dura and Tenera was validated in order to confirm the segregation of the palm type. In this study, the CAPS marker was used to identify the fruit type of the oil palm. The CAPS marker differentiated oil palm fruit type, however, 3 alleles in UV264 (Pisifera) and 2 alleles in UV1002 (Tenera) were identified. These shell allele sequencing results demonstrated that SNP2 (nucleotide T/T present in Pisifera, A/A present in Dura and A/T present in Tenera) contributed to differential fruit type in these palms. In the present study, contamination of the Tenera and Dura palms in the specific progeny trials of Pisifera (UV264) and Tenera (UV1002) were highly frequent. The CAPS marker facilitated the selection and distribution of desirable high yielding palm type in the progeny trials which is a major breakthrough to the Univanich breeding programs and commercial exploitation. Our molecular breeding research findings demonstrate that the specific period (duration) at which callus, embryos, shoots and roots are cultured impacts the development of normal and healthy clones of oil palm. Unstandardized culture periods during each of these developmental stages could result in epigenetic changes due to

somaclonal variation, including abnormal vegetative growth (rolled leaf and twisted frond arrangement) and fruit mantling. Additionally, normal and healthy clones performed better in terms of yield which is useful for selection of high yielding semi-clonal production of oil palm. The present study highlights that the potential of SSR markers facilitated progeny (eight different controlled breeding crosses) confirmation while the CAPS marker ensured selection of the most appropriate type of the fruit palm in the UV family progeny trials. These markers utilized for Univanich breeding programs and commercial semi-clonal seeds production. Further studies, particularly involving specific markers for epigenetic characters and shell thickness, are necessary to identify and develop the highest quality and yielding palms. These genetically superior oil palms will support the palm oil industry and satisfy global customers demand.

Table 7: General characteristics of SSR markers (primers) selected for evaluation of genetic fidelity and progeny legitimacy in oil palm

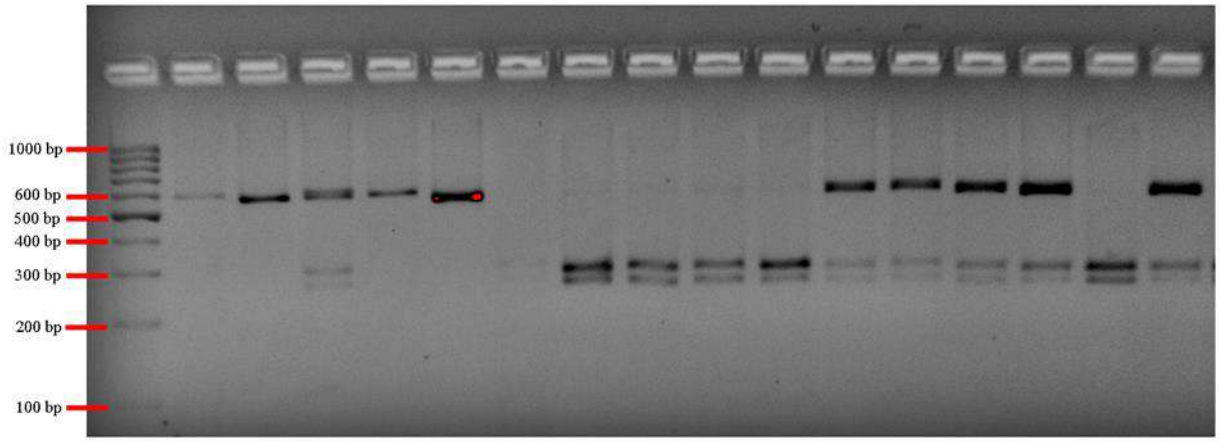
Markers (Locus name)	Sequences (5' - 3') and Annealing temperature (50)	Chromosome	PCR product size (bp)	Reference
mEgCIR3428	F: CATTTTGACAGCTCGTGATG R: TTGGCCGCTATATCTCCAAC	1	177	Thongthawee *et. al.*, 2010
mEgCIR3301	F: GTTAGCTGGCCGGTCTGTAG R: AACATTAGTCCCAGAAGGTTGG	3	185	
mEgCIR0894	F: TGCTTCTTGTCCTTGATACATAGA R: TCTCCACATGAACTCCATCG	7	153	
mEgCIR3111	F: TTTCGCAAGAGATCCTGACA R: CAGATGGCCGCTGAATTTAC	8	214	
mEgCIR3825	F: CACATTGGAGAGCACTTGGA R: TCTCTCTGTGGTTTTAATGGAAGA	12	211	
mEgCIR3546	F: CGTCGACCTCTTCATACATTAAAC R: GCATTGCACATACCAGCAAC	14	198	
mEgCIR3633	F: GCATTTCAACATCTCCCAAATC R: AGCAGTATATGATAATCTGCAAGGC	14	165	
mEgCIR0782	F: CTTTCAATGCCCTAGCGTTC R: GTCGAAAGAAGCGAGATGCT	16	227	
mEgCIR0802	F: CTCCTTTGGCGTATCCTTTA R: TACGTGCAGTGGGTTCTTTC	1	217	
mEgCIR2427	F: GAAGGGGCATTGGATTT R: TACCTATTACAGCGAGAGT	14	116	Billotte *et. al.*, 2005
mEgCIR0521	F: GTGACTTTGGGCTGAAT R: ACAGCATCTCCAACTCTAT	15	137	
mEgCIR0886	F: GATCTGCCGGTGCTCCTA R: CTCAGTTTAGTCGATCCTTCCATTG	8	157	
mEgCIR3785	F: AAGCAATATAGGTTCAGTTC R: TCATTTTCTAATTCCAAACAAG	10	284	
mEgCIR3809	F: CCTTGCATTCCACTATT R: AGTTCTCAAGCCTCACA	1	113	
mEgCIR378	F: TTTTACAACAACCCCAGAGA R: GTTACCTGAGCTTGTGTTTATC	1	249	
mEgCIR2518	F: GATCCCAATGGTAAAGACT R: AAGCCTCAAAAGAAGACC	3	277	Billotte *et al.*, 2005
mEgCIR3040	F: ATCTCTTGTGGGTGCGTTAG R: TGTGGCTGCTGCTTATTTTA	4	189	
mEgCIR2813	F: GCTTTGTTGCAGTTTGACTA R: GTTTAGGATGTTGCGTGAT	5	210	
mEgCIR3567	F: AAAGAAGGAAGCGGGTGGG R: AGACCATTACCCCGATGTCAC	7	115	

Table 8: Validation of Shell traits of Pisifera, Dura and Tenera in breeding and progeny trials planted from 1999 to 2013

Palm type	Family (UV)	Origin of palm	Progeny Trial	Palm Age (years)	DNA profile after *HindIII*	
					Total Band	Expected SNP (284)
Pisifera	UV251	CBP 90 x Avros (T x P)	CPT00/1	20	1	T/T
Pisifera	UV254	CBP 90 x Avros (T x P)	CPT00/1	20	1	T/T
Pisifera	UV264	CBP 90 x Avros (T x P)	CPT00/1	20	3	A/T
Pisifera	UV263	CBP 90 x Avros (T x P)	CPT00/1	20	1	T/T
Pisifera	UV199	T X T / P Y7641	BBT99/1	21	1	T/T
Dura	UV222	D X D Y4209	CDD99/1	21	2	A/A
Dura	UV221	D X D Y4295	CDD99/1	21	2	A/A
Dura	UV267	Topi dura x Topi dura (DxD)	TDD01/1	19	2	A/A
Dura	UV219	D X D Y5080	CDD99/1	21	2	A/A
Dura	UV221	D X D Y4295	CDD99/1	21	2	A/A
Tenera	UV977	CDD99 x DRT99 (D X P)	UPT11/2	9	3	A/T
Tenera	UV979	CDD99 x DRT99 (D X P)	UPT11/2	9	3	A/T
Tenera	UV991	CDD99 x DRT99 (D X P)	UPT11/2	9	3	A/T
Tenera	UV994	CDD99 x DRT99 (D X P)	UPT11/2	9	3	A/T
Tenera	UV1002	CDD99 x DRT99 (D X P)	UPT11/2	9	2	A/A
Tenera	Commercial Tenera	D x Yangambi P	CLD13/1	7	3	A/T

D, Dura. P, Pisifera. CLD, Clonal Dura. UV, Univanich. CDD, Congo Deli Dura. CBP, Combined Breeding Programme. DRT, Drought Resistant Trial. BBT, Binga Breeding Trial. CPT, Combined Pisifera Trial. UPT, Univanich Progeny Trial. A/T, Adenine/Thymine. A/A, Adenine/Adenine. T/T, Thymine/Thymine.

Fig. 6: DNA banding patterns of shell traits by CAPS marker for confirmation of shell thickness from 7 to 21 years-old oil palms. Lane MW, molecular weight marker (100 bp ladder). Lanes UV251, UV254, UV264 and UV199, Pisifera family. Lanes UV222, UV221, UV267, UV219 and UV221, Dura family. Lanes UV977, UV979, UV991, UV994 and UV1002, Tenera family. Lane Com. T (commercial Tenera). The bar = 5 mm.



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