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OIL PALM MALE MEIOSIS PROFILING: FROM FIELD OBSERVATION TO CYTOGENETICS ANALYSIS

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ABSTRACT

Male meiosis is a principal process in microsporogenesis, important for male fertility and gamete viability in higher plants. This division ensures genome stability of sexually reproducing organisms and creates genome variation enabling diversity in a species. We present a structured profiling of male meiosis in the interspecific oil palm hybrid, Elaeis oleifera × Elaeis guineensis *(OxG), for an in-depth understanding of the process. In the pollen mother cells (PMC), interaction between the parental genomes was observed in early prophase I, via genomic* in situ *hybridisation (GISH). At this meiosis I stage, the chromosome pairing revealed 16 complete bivalents, confirming the homologous pairing of each* E. guineensis *and* E. oleifera *chromosomes. Interestingly, we found that the* E. guineensis *and* E. oleifera *pollens had distinct morphologies, which represents another feature that differentiates the two species. This could also be used as a basis diagnostic tool to evaluate sterility in oil palm interspecific hybrids.*

Keywords: chromosome pairing, meiosis, oil palm pollen.

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INTRODUCTION

Oil palm is from the genus *Elaeis* (Arecaceae), a perennial monocot with a single apical meristem. The genus has two interfertile species - the African *E. guineensis* Jacq. and Central/South American *E. oleifera*. Both species are diploid (2n=32), apparently evolved from an ancient tetraploid species (Madon *et al.*, 2005; Singh *et al.*, 2013). Their genome structure and organisation appear quite similar (Singh *et al.*, 2013). *E. guineensis* is planted commercially in most oil palm growing countries, due to its higher yield, while *E. oleifera* only in places where disease(s) devastates *E. guineensis*. Nevertheless, *E. oleifera* has interesting traits that benefit the *E. guineensis* - slower growth, resistance to certain diseases and less saturated oil with higher carotene (Hardon, 1969; Meunier, 1987; Choo *et al.*, 1997; Choo and Yusof, 1996; Amiruddin *et al.*, 2004) - and it has been the ultimate aim to introgress them into *E. guineensis* (Hardon, 1969; Rajanaidu *et al.*, 1995; Escobar and Alvorado, 2003; Amiruddin *et al.*, 2004).

Both monoecious species produce separate male and female inflorescences in alternating phases, influenced by both genetic and environmental factors. The main abiotic factor that influences the sex determination is stress. Anything that detracts from photosynthesis producing more carbohydrates for greater vigour, such as too much/little water and high-density planting, will favour the production of male inflorescences (Corley and Gray, 1976; Barcelos *et al.* 2015). The sex ratio of a palm (number of female inflorescences: total inflorescences)

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(Corley and Tinker, 2003) is an important criterion that determines its yield. A higher sex ratio is generally better for producing more fruit bunches, but excessively high ratios would mean a dearth of male inflorescence/pollen, and denying the female inflorescences of their wherewithal for pollination and fruiting. There has to be a delicate balance in the sex ratio, at any one time, for the desired yield to be realised. A detailed examination of the basic biological process that produce both male and female sex cells in oil palm will be helpful to strategise for higher level of productivity.

The reproductive cells in plants are produced via meiosis, a fundamental inheritance process that occurs in the anthers of male and ovaries of female flowers. In meiotic prophase I, two distinct events occur - homologous chromosome pairing and synapsis, in which the bivalent chromosomes become condensed and distinguishable (Hurel *et al.*, 2018).

Genomic *in situ* hybridisation (GISH) allows visualisation of the homoeologous and homologous chromosome pairing at meiosis, especially in prophase I. The technique further allows detection of chromosome rearrangement, or translocation, and commonly used to evaluate chromosome pairing during meiosis (Schubert *et al.*, 2001; Maluszynska and Hasterok, 2005; Gao *et al.*, 2016). GISH involves labeling of chromosomes or an entire genome in plant hybrids, where total genomic deoxyribonucleic acid (DNA) is used as a probe on chromosome spreads (Anamthawat-Jonsson and Heslop-Harrison, 1995). The technique is generally useful in distinguishing parental genomes in both intergeneric (Leitch *et al.*, 1991; Belandres *et al.*, 2015) and interspecific hybrid plants (Schwarzacher *et al.*, 1992; Abd El-Twab and Kondo, 2007).

Principally, pollen is the end product of meiosis in the male gametic cell, after recombination and reassertion of the chromosomes. Microspores (immature pollen) and pollen are derived from the pollen mother cells (PMC) in pollen sacs. Oil palm belongs to the tribe Cocoaeae, subfamily Arecoideae. Generally, pollen morphology is known to be distinctive for each tribe. The primary aperture type found in this subfamily is monosulcate while trichotomosulcate pollen is only found in *E. guineensis* (Rasheed *et al.*, 2016). The pollen of *E. oleifera* has yet to be described, especially its differences, if any, with that of *E. guineensis* pollen. As plant hybridisation has been known to affect the pollen features, it is anticipated that the pollen morphology of oil palm hybrid might be different than the parental types.

Despite the many studies on meiosis and pollen in other plants, similar researches on oil palm are not many. Hardon and Tan (1969) had reported on the meiotic cytology of interspecific hybrids (*E. guineensis* x *E. oleifera*) and showed that the pachytene chromosomes were normally paired except for the occasional segments. Subsequently, Madon *et al.* (2005) described the basic cytology of *E. guineensis* PMC in an effort to identify two important stages: pachytene and uninucleate stages that will facilitate research. Similarly, apart from histo-anatomical analysis on the *E. oleifera* reproductive system, De Farias *et al.* (2018) also observed the different meiosis and pollen stages, but did not describe the pollen morphology.

This study aims to provide graphical information on male meiosis in oil palm interspecific hybrids. The pairing of the homologous chromosomes of both parental species was observed. In addition, the distinct pollen shapes in the genus *Elaeis* and its interspecific hybrid are also described for the first time. Our findings will provide basic but important information on meiotic recombination in oil palm, apart from the pollen morphology, which can assist in making informed decisions in interspecific breeding of oil palm.

MATERIALS AND METHOD

Plant Materials

Young and mature male inflorescences (*Figure 1*) from *E. guineensis* and *E. oleifera* were sampled from MPOB Kluang, Johor, Malaysia. The inflorescences harvested were still enveloped in their spathes and were identified based on morphological criteria as described by Latif *et al.* (2014). The particular inflorescence to be harvested (whether containing PMC or microspores) was determined based on the description by Madon *et al.* (2005). Generally, the younger inflorescences with whitish buds of soft texture bear PMC, while those mature, with slightly yellowish hard buds should contain microspores.

The PMC are the precursors of microspores which then develop into pollen. The younger inflorescences, subtended by frond numbers 12-17 will have PMC, and those from frond numbers 18-25 bear microspores to pollen (Madon *et al.*, 2005; Latif *et al.*, 2014). The desired inflorescences were selected based on their subtending frond numbers and morphology. However, De Farias *et al.* (2018) had suggested that most meiotic stages in *E. oleifera* could be found in the inflorescence on frond 14, of which, this criterion was not used in the study.

For meiotic chromosome pairing analysis, male inflorescences from an oil palm interspecific hybrid (OxG) created from a cross between a Colombian *oleifera* and Nigerian *guineensis*, were harvested from United Plantations, Teluk Intan, Perak, Malaysia. Spear leaves of the parental palms were provided by the Genomics Laboratory, MPOB.

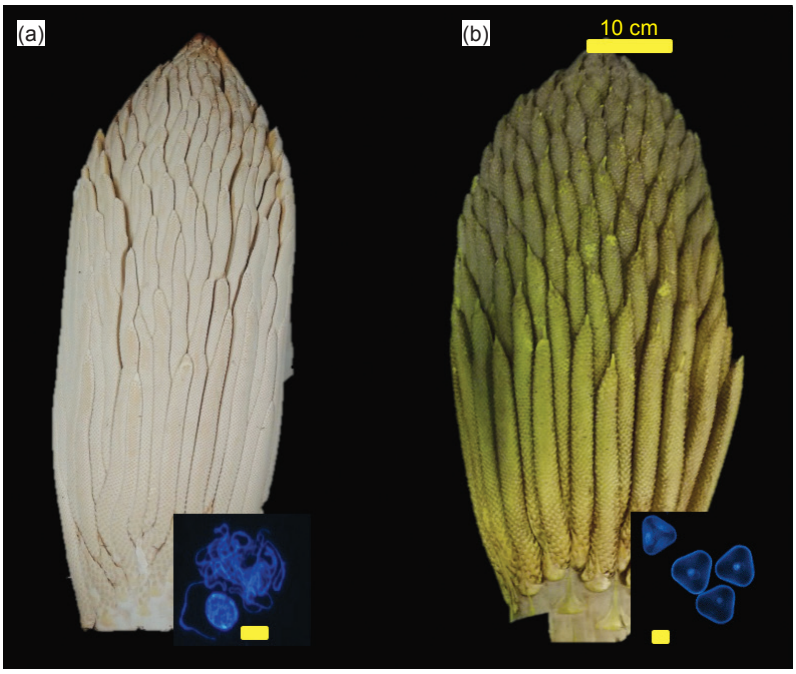

Figure 1. Oil palm male inflorescences (a) young OxG hybrid inflorescence (left) bearing pollen mother cells (PMC) (left inset), and (b) mature E. guineensis *inflorescence (right) bearing microspores (right inset) (inset bar = 20 µm).*

Microspores and Chromosome Staining with 4',6-diamidino-2-phenylindole (DAPI)

The process from harvesting of the male inflorescence to the preparation of the chromosomes spreads is shown in *Figure 2*. The young spikelet with whitish immature buds appeared crisp while the older ones with mature buds are somewhat stringy. A bud from the spikelet was put on a pre-cleaned microscope slide with a 12 µl drop of 1.25 µg ml^{-1} DAPI (Sigma, USA), then squashed with a tweezer. The husk debris was then removed and a clean coverslip was applied to cover the 'suspension'.

Chromosome Preparation

The desired meiosis stage that was stained with DAPI was observed under a fluorescent microscope. Male flower buds at the approximate desired meiotic stage were fixed in ethanol-glacial acetic acid (3:1 v/v) and stored at 4ºC for up to several months. Meiotic preparation was carried out according to Nordiana *et al.* (2016) with minor modification. Anthers from the fixed buds were thoroughly washed with distilled water and 1× citrate buffer (10× citrate buffer consists of 100 mM citric acid and 100 mM tri-sodium citrate, pH 4.6) for 10 min and then digested in 50 µl of enzyme solution containing 1% (w/v) cellulase from *Aspergiller niger* (Sigma Aldrich, USA, C-1184): 1% (w/v) pectolyase from *Aspergiller japonicus* (Sigma Aldrich, USA, P-3026): 1% (w/v) cytohelicase from *Helix pomatia* (Sigma Aldrich, USA, C-8274) for 4 hr at 37ºC. Following the digestion, the anthers were thoroughly rinsed with 1× citrate buffer for 15 min. Two anthers were macerated on a pre-cleaned glass slide in a drop of 60% acetic acid. The PMC were released and dispersed using the squashing technique after applying the coverslip on the suspension. The prepared slides were frozen, the coverslips were removed and the slides were air-dried before use for GISH analysis.

Preparation of Total Genomic Probe and Block

Total genomic DNA of the parental palms, *E. oleifera* (UP1026) and *E. guineensis* (T128), was extracted from their spear leaves using the modified cetyl trimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990), and the quality of the DNA was evaluated as described by Rahimah *et al.* (2006). The DNA of *E. oleifera* (1 µg) was directly labelled with biotin molecules using the BioPrime™ DNA Labelling System Kit (Invitrogen, USA) for use as probe. The DNA of *E. guineensis* was fragmented by autoclaving for 5 min at 15 psi for use as block, as the quantity needed was 140X (140 µg) more than the probe. The autoclaved DNA was stored at -20ºC before use.

Genomic *in situ* Hybridisation (GISH)

GISH was performed according to Madon *et al.* (1999) and Schwarzacher and Heslop-Harrison (2000) with slight modifications. The prepared slides were pre-treated with RNase A [100 µg ml^{-1} RNase A in 2× saline sodium citrate (SSC) at 37ºC for 1 hr,

Figure 2. Isolation of pollen mother cells (PMC) and microspores – from field to microscope. (a) Selection (mentioned in materials above) and harvesting of male inflorescence (left to right) (b) harvested male inflorescence brought to laboratory and processed (left to right) (c) preliminary screening by 4',6-diamidino-2-phenylindole (DAPI) staining viewed under microscope. Spikelet and buds with desired stages fixed for use (left to right).

and then soaked in 0.01M hydrochloric acid (HCl) for 2 min. The slides were then treated with pepsin solution for 10 min at 37ºC and washed in 2× SSC for 5 min. The slides were then post-fixed with 4% paraformaldehyde, washed with 2× SSC, dehydrated in different concentrations of ethanol (70%, 85% and 100%) and left to air dry. A total of 40 µl of probe and block hybridisation mixture with final concentration of 5 µg ml^{-1} was prepared per slide, containing 50% (v/v) formamide, 10% (w/v) dextran sulphate, 2× SSC, 0.125% (w/v) sodium dodecyl sulphate, 0.125 mM ethylenediamine-tetraacetic acid. The mixture was denatured for 10 min at 70ºC, then immediately cooled on ice for another 10 min. A total of 40 µl of hybridisation mixture was dropped on one slide and a plastic coverslip was applied. The hybridisation mixture and chromosomal DNA (the slides) were denatured together on Hybaid Omnislide thermal cycler (Fisher Scientific, USA) at 80ºC for 13 min, 58ºC for 35 min and allowed to cool to the hybridisation temperature of 37ºC overnight (up to 20 hr).

The following day, the slides were washed in 2× SSC for 3 min at 42ºC, rinsed twice for 5 min at 42ºC in 20% (v/v) formamide in 0.1× SSC, twice for 5 min at 42ºC in 0.1x SSC, once for 5 min in 2× SSC at room temperature (RT) and twice for 5 min in detection buffer (4x SSC/0.2% Tween) at RT. Blocking solution [5% (w/v) bovine serum albumin (BSA) in detection buffer] was applied to the slides for 5 min at RT. The biotin-labeled probe was detected with 2 µl streptavidin-CF594 conjugate antibody, 200 µg ml^{-1} (Biotium, USA) in blocking solution. Slides with

the antibody were incubated in a moist chamber at 37ºC for 1 hr. The slides were subsequently washed in detection buffer three times for 5 min each, to remove the surplus antibody at RT. A drop of 10 µl ready-to-use EverBrite™ mounting medium with DAPI (Biotium, CA, USA) was used to counterstain the chromosomes after which they were covered with a glass coverslip.

All the chromosomes slides were viewed and captured under a Leica DMRA fluorescent microscope (Leica Microsystems, Germany) with an Olympus DP73 camera and the images analysed using Olympus CellSens Dimension Imaging Software Version 1.14 (Olympus Corp., Japan).

RESULTS AND DISCUSSION

Male Meiosis Profile of Oil Palm Interspecific Hybrid

We successfully identified all the essential stages of meiosis in oil palm hybrid male inflorescences, occurring as in angiosperms (*Figure 3*). *Figures 3a-e* show prophase 1 with its five known substages - leptotene, zygotene, pachytene, diplotene and diakinesis. Synapsis (pairing) of the chromosome homologues was clearly observed at zygotene, followed by the chromosomes continuing to shorten and thicken at pachytene. The homologous recombination between the parental chromosomes occurs at this stage. The pachytene stage of early meiotic prophase has been extensively studied by cytologists as it provides a unique view of the organisation of haploid genomes (Murphy and Bass, 2012). Using GISH assay, De Farias *et al.* (2018) observed high homology between the chromosomes of the two species when the *E. guineensis* genome was hybridised on *E. oleifera* pachytene chromosomes. The elongated, synapsed fibres at this stage have been applied to assess the organisation of the rice genome (Kim *et al.*, 2005) and to build fluorescence *in situ* hybridisation (FISH) cytogenetic maps, which are ideal for comparative mapping studies and evaluating the quality of the genome sequence assembly in tomato, maize, cucumber and barley (Zhong *et al.*, 1996; Figueroa and Bass, 2012; Lou *et al.*, 2013; Phillips *et al.*, 2013). The transition from pachytene to early diplotene occurs when the elongated chromosomes transform to a compact spindly-looking bivalent fibre configuration. The bivalent fibres continue to separate along their length and eventually become detached. Further chromosome compaction occurs in the transition to diakinesis. The bivalent chromosomes at this stage have been used to study meiotic pairing, as in the intergeneric hybrids of *xBrassicoraphanus*, where the pairing of their parental genomes was observed (Belandres *et al.*, 2015).

The bivalent chromosome pairs align during metaphase I at the equator of the cell (*Figure 3f*). The PMC are in uninucleate, or one-nucleus state. Gao *et al.* (2016) evaluated the chromosome pairing in two population of *Roegneria sinica* var. *media* where it occurred mainly between homologous chromosomes. The homologous chromosomes then separate, and the two sets move to opposite poles of the cell in anaphase I (*Figure 3g*). At telophase I, the chromosome sets arrive at their respective poles and nuclear membrane reappear resulting in two daughter nuclei. A cleavage line appears and gradually divide into two daughter cells (dyad) known as successive cytokinesis (*Figure 3h*).

Following the formation of two daughter cells, the meiotic II division starts which essentially is a mitotic division. In metaphase II, the chromosomes once again orientate on the equatorial plane and divide (*Figure 3i*). The chromatids are then pulled to their respective opposite poles. At the end of telophase II, tetrad eventually gives rise to immature pollen with haploid chromosome number (n=16), also known as microspores (Madon *et al.*, 2005).

Immature pollens from the PMC are known as uninucleate microspores. Their transition to binucleate occurs through mitosis (*Figures 3l-3n*) (Latif *et al.*, 2014). At the end, these two nuclei have different functions, one as a vegetative and the other as a generative nucleus. Microspore culture has been carried out to develop haploid plants, such as in barley, hazelnut and pepper (Li and Devaux, 2001; Karasawa *et al.*, 2016; Ata *et al.*, 2019). This was also attempted for oil palm from uninucleate and binucleate microspores, but oil palm turned out to be recalcitrant to microspore culture, especially in the induction of embryogenesis (Latif *et al.*, 2013).

According to De Farias *et al.* (2018), *E. oleifera* meiosis occurs acropetally, from base to apex. A similar acropetal pattern of meiosis development was also observed for *E. guineensis* and OxG hybrids. Thus, both *Elaeis* sp. and their hybrids go through a similar meiosis pattern (Madon *et al.*, 2005; De Farias *et al.*, 2018). This also occurs in *Arabidopsis* sp. and *Triticum aestivum* (Armstrong *et al.*, 2009; Higgins *et al.*, 2014; Able *et al.*, 2009).

Chromosome Pairing in Early Prophase I in OxG Hybrid

Previously, GISH had differentiated 16 *E. oleifera* and 16 *E. guineensis* chromosomes on the mitotic metaphase spread of an OxG hybrid (Madon *et al.*, 2005). However, the composition of the parental chromosomes varied in the backcross one (BC_1) progenies (Madon *et al.*, 2018). In early meiotic prophase I, the maternal and paternal homologues of each chromosome undergo exact pairing into bivalents. In this study, bivalents are

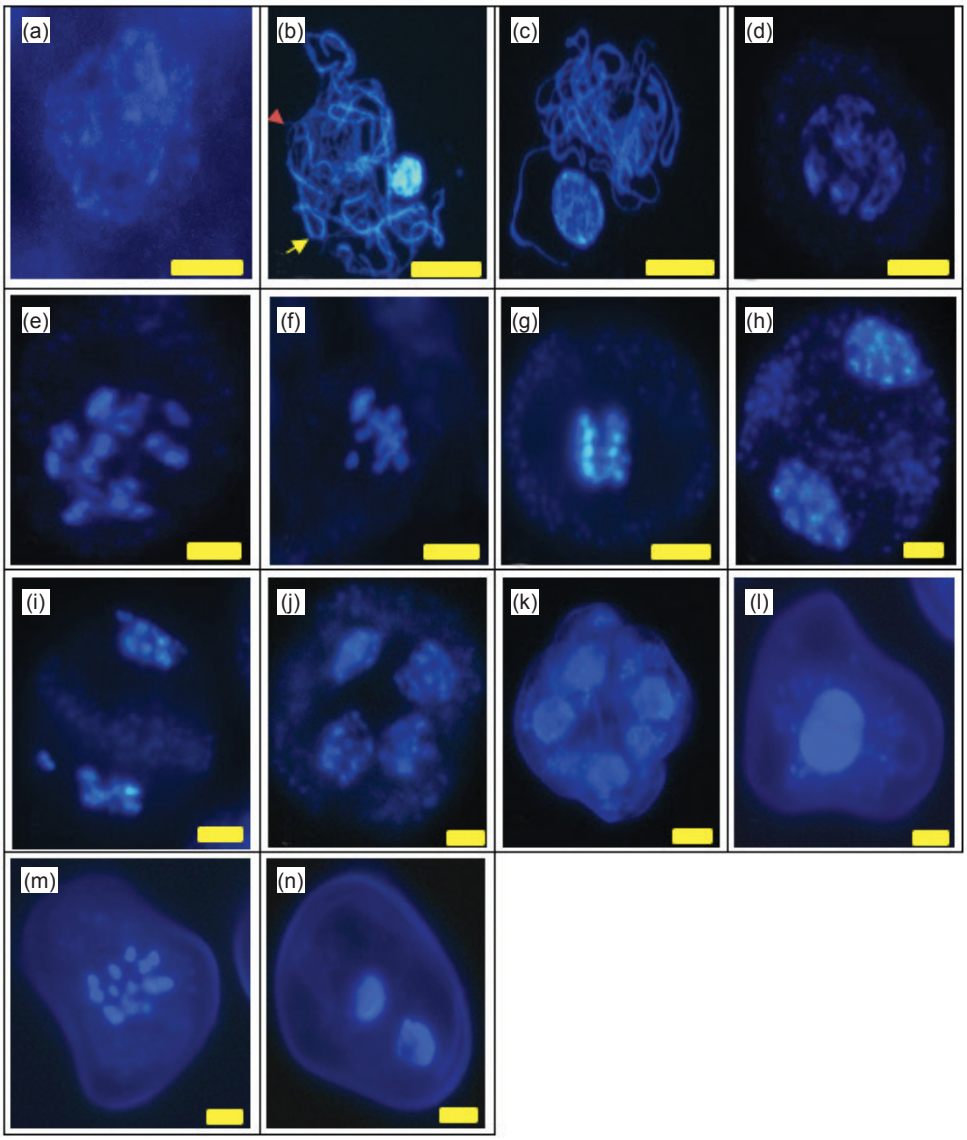

Figure 3. Cytology of male meiosis in oil palm interspecific hybrid. (a) Premeiotic interphase, (b) zygotene with paired (yellow arrow) and unpaired (red arrowhead) chromosomal regions, (c) pachytene with complete paired chromosomes, (d) diplotene, (e) diakenesis showing unaligned bivalents, (f) metaphase i, (g) anaphase i, (h) prophase ii (two daughter cells formation), (i) metaphase ii, (j) tetrad, (k) unreleased microspores, (l) uninucleate (one nucleus state) microspore (m) microspores with 16 chromosomes (n) mid-binucleate microspores with two nuclei. DNA counterstained with 4',6-diamidino-2-phenylindole (DAPI) (bar = 20 µm).

regularly observed on the chromosome spreads. GISH clearly differentiated the 16 bivalent pairs, each pair containing the red fluorescence signals of the *E. oleifera* genomic DNA used as probe, while the *E. guineensis* contributed the blocking DNA. *Figure 4* shows the homologous pairing between two chromosomes at diakinesis, one coming from each parent, forming 16 bivalents in all. These reflect the close homology between the *E. oleifera* and *E. guineensis* chromosomes. Hardon and Tan (1969) had reported the frequent formation of bivalents in meiosis I in *E. guineensis* × *E. oleifera* F1 hybrids, but there has been no information on the pairing of the parental genomes in early meiosis until now.

Cytogenetic studies on meiotic chromosome pairing have been done on other plants, for example, high frequency bivalent formation and regular meiosis in *Lolium perenne* × *Festuca pratensis* hybrids by Thomas and Thomas (1973), while Belandres *et al.* (2015) discriminated 10 pairs of *Brassica* bivalent chromosomes in a new synthetic intergeneric hybrid ×*Brassicoraphanus* line. In a related study, Fukuhara *et al.* (2016) observed frequent formation of bivalents in an interspecific hybrid of Jatropha, suggesting the presence of homoeologous chromosomes in the *Jatropha curcas* × *J. integerrima* hybrid.

Pollen Morphology of *Elaeis* sp. and OxG Hybrid

The pollen of both oil palm species have distinct shapes (*Figure 5*). *E. guineensis* pollen is triangular and *E. oleifera* pollen ellipsoid, in symmetric and asymmetric forms. The OxG pollen is a hodgepodge of the two, being triangular-ellipsoidal. Ravi Kumar

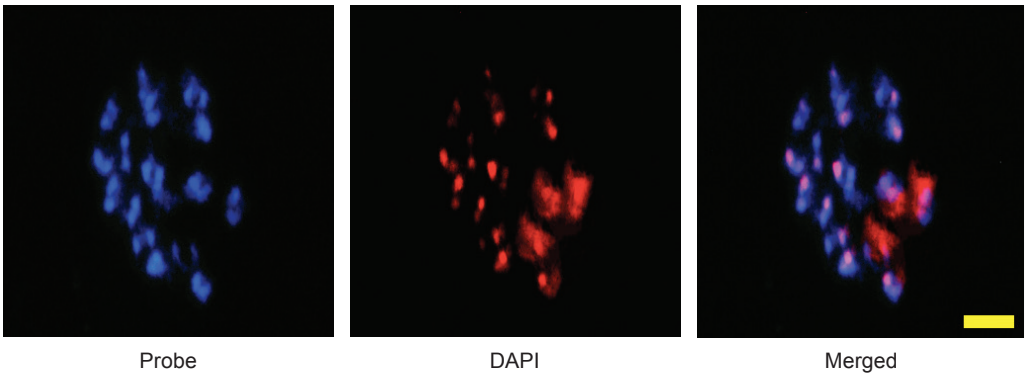

Figure 4. The genomic in situ *hybridisation (GISH) on meiotic chromosomes, probed with BioPrime-labeled total genomic DNA (1 µg) from UP1026, and detected with fluorescent dye CF594 (red) and 140X blocking DNA of T128 counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). In the merged image, there are 16 bivalents, each the pairing of one* oleifera *(red) and one* guineensis *(blue) chromosome (bar = 20 µm).*

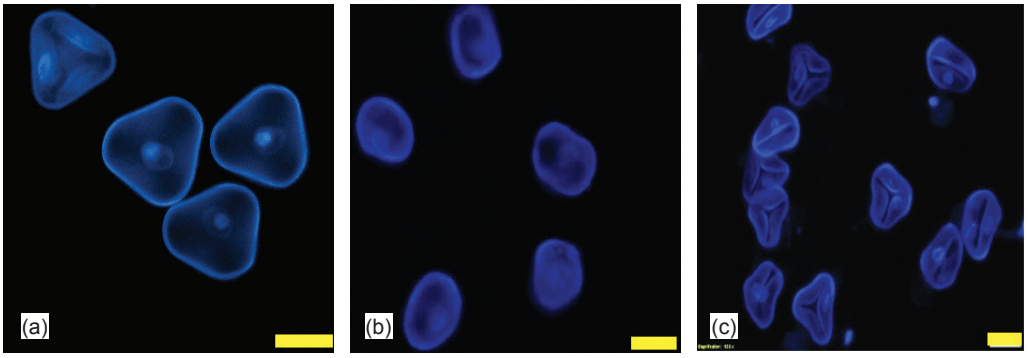

Figure 5. Pollen in (a) E. guineensis *showing triangular shape (b)* E. oleifera *showing ellipsoid shape, and (c) OxG hybrid showing a hodgepodge of both parental morphologies (bar = 50 µm).*

and Nair (1986) reported the pollen shapes in three *Gloriosa* sp. and three of their hybrids (including the reciprocal crosses). In the hybrids, 70%-100% of their pollen resembled those of their respective female parent, suggesting a strong maternal influence on the pollen shape. Similarly, Rhee *et al.* (2005) observed that the pollen morphology of interspecific hybrids of lilies after chromosome doubling highly resembled those of their mother plants. In weeds, the size of *Amaranthus* hybrid pollen was similar to that of the maternal parent but had intermediate aperture numbers between both parents (Franssen *et al.*, 2001), also suggesting a strong maternal influence.

The pollen of both species are aperturate, *i.e.*, having clear apertures on their pollen wall. Harley and Baker (2001) opined that pollen shape is often related to their aperture type, for example, monosulcate aperture (only one opening) with ellipsoidal pollen, as in *E. oleifera* and trichotomosulcate aperture (with three openings) often with trianguloid pollen, as in *E. guineensis* (*Figure 6*). In the tribe Cococeae, Rasheed *et al.* (2016) described the pollen shape are frequently elliptical, but occasionally oblate-triangular pollen are also observed. In some species, the aperture of monosulcate, and, at times, trichotomosulcate pollen can range from being symmetric to asymmetric.

E. guineensis and *Cocos nucifera* (coconut) pollen are easily distinguished by their aperture and exine pattern. *C. nucifera* has monosulcate pollen and *E. guineensis* trichotomosulcate. Cluster analysis revealed that *E. guineensis* is not in the same clade as other aeroids because of its trichotomosulcate pollen (Rasheed *et al.*, 2016). However, the triangular *E. guineensis* pollen does have some similarity with that of *Sclerosperma* sp. (Arecaceae) as described by Grimsson *et al.* (2018). Although no observation of aperture pattern was made on *E. oleifera* by Rasheed *et al.* (2016), this study found that the aperture pattern and subsequent pollen shape are similar to those in *C. nucifera*. Furthermore, the date palm (Arecaceae family) also produces elliptical-oblate and monosulcate pollen (Jazinizadeh *et al.*, 2017).

Nair (1969) suggested that the shapes of pollen could be associated to their functions, hence selection based on morpho-physiological traits may assist in breeding efforts. In *Lilium* sp., a malformed pollen shape was observed in a sterile F1 (Rhee *et al.*, 2005). Interestingly, a similar abnormality was also found in sterile interspecific hybrids of *Lagerstroemia indica* and *L. speciose* (Ju *et al.*, 2019). Hence, the distinct pollen shapes and features identified in this study could form a basis in the development of a diagnostic tool to evaluate sterility in interspecific hybrids of oil palm.

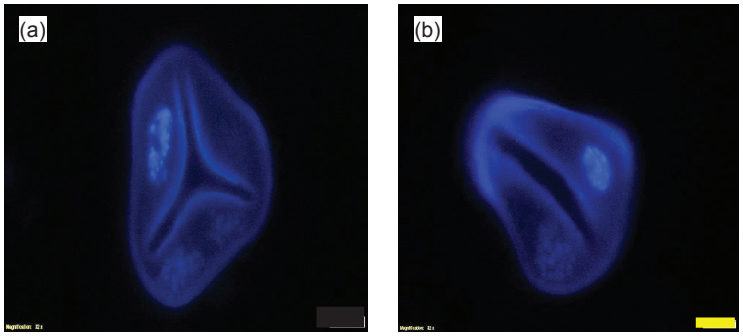

Figure 6. Trichotomosulcate pollen aperture in E. guineensis *(a), and monosulcate pollen aperture in* E. oleifera *(b) (bar = 20 µm).*

CONCLUSION

This study delves into the basic male meiosis process in an oil palm interspecific hybrid, recording its different stages. The meiosis profile of the oil palm interspecific hybrid was consistent with that observed in other plants species, including *E. guineensis* and *E. oleifera*. The information, especially the clear delineation of the different stages, will facilitate other applied experiments, such as *in situ* hybridisation of chromosomes at the particular stages in oil palm breeding. The preliminary results reported will be a spring board for future more in-depth studies on the topic. Moreover, the different pollen morphologies will greatly assist in differentiating the different oil palm types. As expected, the aperture numbers in pollen differed between *E. oleifera* (only one) and *E. guineensis* (three). Hybrid pollen was a hodgepodge of shapes between the two parental types, but more work is required to ascertain the maternal effect on the pollen shape.

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