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A HIGH-THROUGHPUT FLOW CYTOMETRY METHOD FOR PLOIDY DETERMINATION IN OIL PALM

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ABSTRACT

Non-euploid seedlings occur naturally in many plant species including oil palm but at frequencies usually considered too low for practical purposes. A flow cytometry method is described that overcomes the difficulty of exploiting such low frequency events and provides a practical plant breeding methodology to identify non-euploids/haploids within large sample sizes. In addition, the efficiency of detecting non-euploid seedlings can also be increased greatly by a pre-screen for abnormal phenotypes. Oil palm is relatively difficult to analyse via flow cytometry as tissue disruption initiates secondary metabolite production which interferes with the analyte and data capture. The addition of dithiothreitol and polyvinylpyrrolidone during sample preparation, followed by cold incubation, prior to analysis overcomes these problems. The high-throughput method developed allows the analysis of 1000 samples per day per flow cytometer. The number of haploids produced by this method rivals that of other haploid production systems and is currently the only known method of generating haploids in oil palm. Additionally, the method for oil palm is not season dependent and may be performed all year round. The method can be applied to other species and provides a practical means of harvesting naturally occurring non-euploid. The seedlings selected using this methodology can be grown, thus making the method applicable to a range of species and disciplines including evolutionary studies of speciation of polyploids, reproductive biology, embryology and the production of haploids and doubled haploids for genetic studies and plant breeding of oil palm.

Keywords: high-throughput, flow cytometry, ploidy, oil palm.

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INTRODUCTION

Higher plants vary in ploidy, but are generally stable for a particular ploidy level; this may be diploid, triploid, tetraploid or a higher ploidy level. Haploids (*n*, have one set of chromosomes) of higher plants are rare, normally non-viable and sterile. Haploids, however, are valuable in plant breeding and genetics. Haploids can be artificially induced in some species via various manipulations of gametic cells, aberrant pollination, or the action of haploid inducer genes (Palmer and Keller, 2005; Dunwell, 2010). The first haploid, arising spontaneously, in a flowering plant species was discovered in *Datura stramonium* (Blakeslee *et al*., 1922). Since then, spontaneous haploids have been detected in a wide range of plant species (Maluszynski *et al*., 2003). For plant breeding purposes, haploids of normally diploid species are converted to fertile doubled haploids which are

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extremely valuable as they are 100% homozygous (Maluszynski *et al*., 2003). Double haploids can be achieved by spontaneous chromosome doubling of haploids or treating with chemicals such as colchicine that interfere with spindle function and thereby chromosome movement during mitosis. Double haploids may be used as parental lines to develop F_1 hybrids as in maize (*Zea mays* L.), pepper (*Capsicum*) and rye (*Secale cereale* L.) (Hoecker *et al*., 2008; Ballester and Carmen, 1998).

Polyploidy has played a major role in plant evolution both in the wild and in crop development. It has been estimated that about 40% of wild species are polyploid, but that figure is higher among cultivated plants (Arumuganathan and Earle, 1991). Polyploidy can involve the multiplication of a base genome (autopolyploidy) or the addition of different genomes (allopolyploidy). Non-balanced polyploids such as triploids have an odd number of chromosome sets, are normally unable to complete meiosis, and are consequently sterile. This is an advantage in some commercial species where non-fruiting or seedless fruit are required.

The frequencies of spontaneous haploids and polyploids in nature have been considered to be too low for botanical study or practicable plant breeding purposes. However, high-throughput flow cytometry overcomes the difficulty of detecting haploids/non-euploids, at low frequency, in large sample sizes. Flow cytometry determines ploidy level by measuring the DNA content of cell nuclei released into an analyte containing a DNA specific dye (Arumuganathan and Earle, 1991).

Oil palm (*Elaeis guineensis* Jacq.) is the most important oil crop in the world. Despite its current mean yield of about 4 t ha^{-1} yr^{-1}, oil palm falls short of its estimated physiological yield potential of 18 t ha^{-1} yr^{-1} (Corley, 1983; 1998). The F_1 hybrids are a possible means of realising this potential (Pooni *et al*., 1989). Homozygous parental lines for F_1 hybrid production may be produced by repeated rounds of imposed inbreeding, but the lengthy generation time of oil palm (five years) makes this a long-term strategy, as up to eight generations of selfing are normally required. A recent breakthrough in oil palm has been the detection of naturally occurring haploids and doubled haploids which can be exploited immediately in F_1 hybrid breeding (Dunwell *et al*., 2010).

This article reports high-throughput flow cytometry analysis for examining the ploidy level of oil palm and discusses the possible applications and potential value of these results.

MATERIALS AND METHODS

Preparation of Nuclear Samples

Suspensions of oil palm nuclei were prepared by using a standard method (Arumuganathan and Earle, 1991) with the following modifications. Individual and bulks of five leaf samples were taken from fronds 1, 9, 17, 25 and 33 of palms from Bah Lias Estate research trials, North Sumatra, Indonesia. Leaf samples were chopped with a sharp clean razor-blade in plastic Petri dishes containing nuclear isolation buffer which consisted of 5 mM Hepes, 10 mM magnesium sulphate heptahydrate, 50 mM potassium chloride, 0.2% (v/v) triton-X and 2 mg $litre^{-1}$ DAPI adjusted to pH 8 or commercial CyStain® UV Ploidy solution (Partec, Germany), with or without the addition of 0.1% (w/v) dithiothreitol (DTT) and 1% (w/v) of polyvinylpyrrolidone (PVP) (Sigma-Aldrich, USA). Macerations were done in low light conditions to protect the DAPI stain in the isolation buffer. The resultant solutions were then incubated at 5°C for 30 min in darkness. Each suspension was filtered through a 40 μm Cell Trics® (Partec, Germany) sieve and collected into Röhren tubes (Sarstedt, Germany) and a further 0.5 ml of cold isolation buffer added. The final suspensions were then mixed thoroughly by pumping in and out of a pipette tip. As a check on sample quality, a drop of the analyte was placed on a glass slide and observed under a fluorescence microscope, 400X magnification. The ploidy of a standard (diploid) seedling control was confirmed by chromosome counting.

Flow Cytometry Analysis

The stained nuclei samples (analyte) were then analysed using a CyFlow® Space (Product No. 05-4020, Partec, Germany) flow cytometer by optical detection of DAPI fluorescence; a FL1 filter was used to detect the fluorescence signal intensity that was evident by pulse height, area and width. The flow cytometer was equipped with an 8 mW bulb which emits light at 375 nm. Other flow cytometers can be used. We also used a CyFlow® Ploidy Analyser (Order No. 11-01-1002, Partec, Germany) which uses UV LED excitation (with a 365 nm emission wavelength). Control diploid samples of known ploidy level were used to standardise the fluorescence channels (FL1). The graphed diploid and haploid signals were set to give a read out at a convenient position on the x-axis display. Samples were run through the flow cytometer at a low speed

(approximately 0.5 µl s^{-1}) for 30 s. Up to 10 000 single nuclei signals were acquired for each sample, which were processed by the embedded Windows™ XP/ Vista FloMax® (Partec, Germany) flow cytometry analysis software. The coefficient of variation (CV) was calculated from the histograms generated. Regions (RN) were drawn to define a certain cell or particle population. For high-throughput mass screening tissue, five young leaf samples were bulked since it was found that one non-euploid sample (*e.g.* haploid) could be detected among four diploid (euploid) ones. Bulked samples that showed the presence of non-diploid cells were de-composed and each sample re-analysed separately to determine which sample carried non-euploid cells.

Confirmation by Chromosome Counting Method

As a check on ploidy, chromosome counting was performed on randomly selected samples after flow cytometry analysis. Actively growing root tips from oil palm were sampled and pre-treated for 4 hr in 5% (v/v) saturated solution of 1-bromonapthalene (Fluka, USA), then fixed in ethanol: glacial acetic acid (3:1) for 30 min. Fixation could be stop by placement in a refrigerator (5°C) or allowed to continue immediately to the hydrolysis step: 1 N HCl for 6 min at 60°C and then stained with Schiff's reagent (Sigma-Aldrich, USA) overnight. A drop of 1% (w/v) aceto-orcein was added and incubated for a few minutes before observation. Chromosomes were observed under a light microscope 600X or 1000X magnification, Nikon, Eclipse E200, Japan.

RESULTS AND DISCUSSION

Phenolics have been shown to reduce the quality of flow cytometry analysis in many species. Phenolics, such as flavonoids and flavanols, are present *in vivo* within plant cells and become active after tissue damage (Feucht *et al*., 2004) which is particularly problematic in oil palm. The addition of DTT and PVP to the nuclear isolation buffer followed by cold incubation were found to be crucial in all flow cytometric analyses; PVP acts to strip phenolics from proteins and DNA (Bharathan *et al*., 1994), reduces secondary plant metabolite production in disrupted tissues and gives clearer flow cytometry histograms (Yokoya *et al*., 2000). The effect of phenolic compounds can be reduced further by the incubation of analyte at low temperatures, in this case 5°C. The success of this protocol could be observed under a fluorescence microscope before loading the analyte into a flow cytometer (*Figures 1a* and *1b*). Older tissues tend to produce more phenolics, starch, polysaccharides, calcium oxalate crystals and other metabolites (Lee and Lin, 2005) than younger material and therefore screening young tissues is preferred (*Figures 2a* to *2e*). Samples can be bulked to improve the efficiency of flow cytometry analysis (Cousin *et al*., 2009). An effective number of samples in a bulk was determined by the ability to differentiate one non-euploid (non-diploid) sample among several euploid (diploid) samples. All samples within a bulk were considered diploid if only one diploid peak occurred in the output histogram (*Figure 3a*). Samples within a bulk with an additional non-diploid peak in the output histogram were decomposed and re-analysed individually to identify haploids, diploids or polyploids (*Figures 3b* and *3c*). Individual diploid samples and a bulk size of five samples were found to be optimal for oil palm studied with CV values that ranged from 1.5% to 3%. The determination of ploidy was achieved by the generation of flow cytometer output histograms. The formation of single or double peaks was obvious 30 s after running the analyte into the machine. This rapid analysis allowed 200 bulked samples (1000 individual samples) to be screened in 3 hr per flow cytometer by employing two laboratory technicians for sample preparation and loading the samples. This number of analyses is unprecedented and is significantly higher than the number of samples previously (50 samples per day) recorded for high-throughput flow cytometry (Cousin *et al*., 2009). Other aspects of high-throughput flow cytometry provide potential cost savings in terms of reduced labour and laboratory consumable components. We opted to use a Partec buffer for simple nuclei extraction because it is simple, inexpensive and does not contain toxic reagents. Reducing the cost of components of flow cytometry analysis could be achieved also by washing and re-using some laboratory consumables such as filters and Petri dishes.

We have detected 1104 haploids, 184 triploids, 29 tetraploids, 20 mixoploids and 81 aneupoids from sampling 386 787 off-type seedlings in four years, from 2006 to 2010. A pre-screen can greatly reduce the number of samples taken for ploidy determination. In oil palm, for example, potential haploids may be screened out as seeds having abnormal germination *e.g*. twin seedlings, poor radicle or shoot emergence and general lack of vigour (Nelson *et al*., 2008). The frequency of 'off-type' germinated seed in oil palm is about 1 in 1000 whereas the frequency of haploids among the 'off-types' is about 1 in 100 (calculated from data in Nelson *et al*., 2009). As a consequence the number of seedlings to be screened by flow cytometry can be reduced by two orders of magnitude. Thus, the low frequency of naturally occurring haploids is no longer an obstacle to their exploitation, as the method can be used to screen large sample sizes rapidly, provided sufficient seed can be sourced. The current method is especially applicable in plants species that produced abundant seed. An oil palm inflorescence can produce over a 1000 fruits

Figure 1. (a) Application of DTT and PVP in the nuclear isolation buffer gives specific fluorescence of the nuclei (yellow arrows) in comparison with (b) where material outwith the nucleus generates additional fluorescence. Photographs were taken using fluorescence microscope, Eclipse 90i, Nikon, Japan, before loading the samples into flow cytometer. Scale bars= 20 µm.

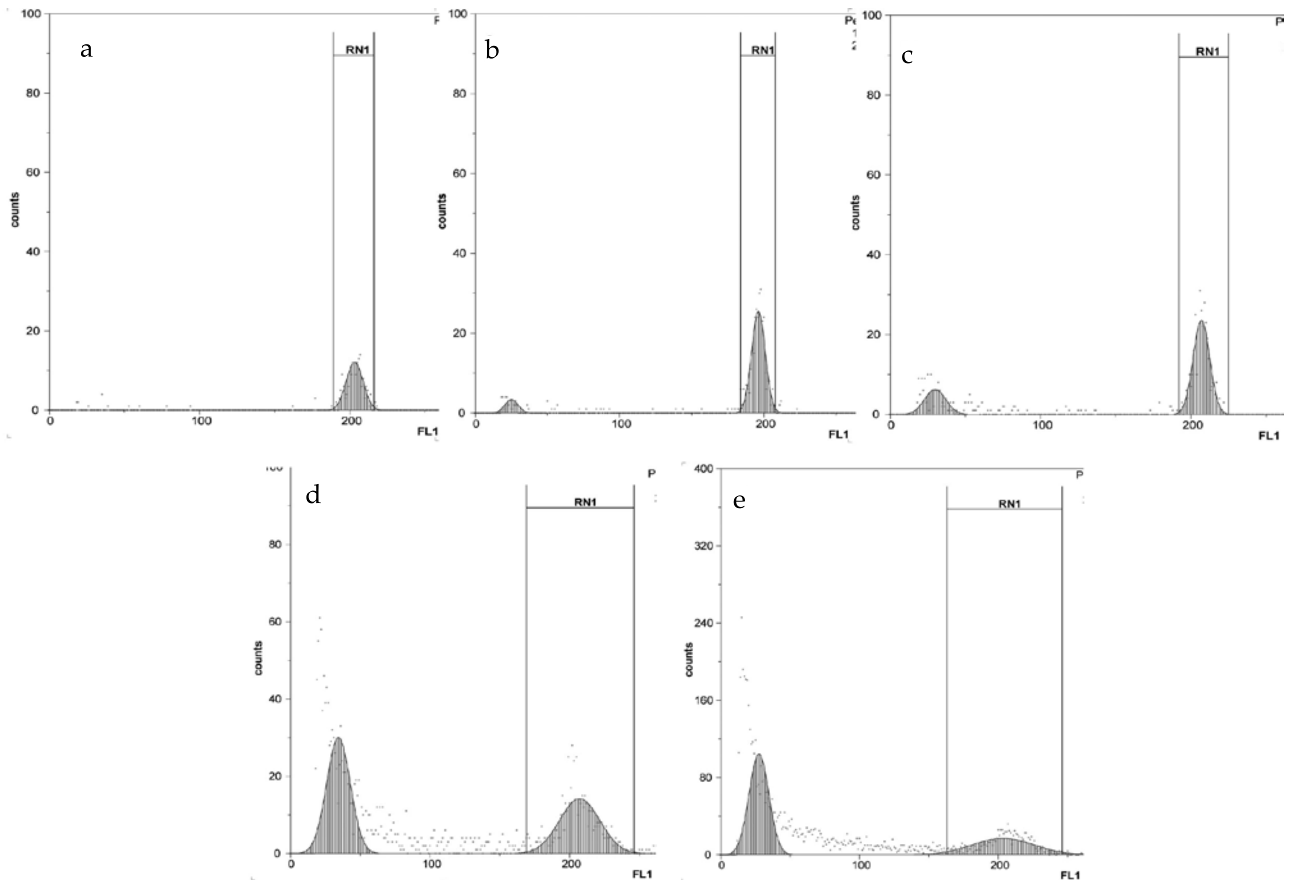

Figure 2. Use of young leaf materials for reducing 'noise' histograms. Leaf materials from different frond numbers would effect the amount of 'noise' in flow cytometry histograms. Frond 1 indicates the absence of 'noise' histogram (a), compared with frond 9 (b), frond 17 (c), frond 25 (d) and frond 33 (e) as older fronds contain more secondary metabolites that interfer with the analysis. Output histograms were produced from a Partec Cyflow® space flow cytometer and data analysed using Partec FloMax® software.

with one seed per fruit and the method is of practical value as this is currently the only known method of obtaining haploid plants in oil palm. Over 1000 haploids have been produced by this method (Nelson *et al.*, 2009; Dunwell *et al.*, 2010). In addition these haploids have been produced from a wide range of genotypes (Dunwell, 2010), indicating that haploid production is a generally occurring natural phenomenon in this species. The mechanisms involved in haploid production are currently being investigated. Successful application has been demonstrated for a relatively difficult species, oil palm, for flow cytometry analysis and may be applied to other species especially those that are recalcitrant to *in vitro* methods of haploid / doubled haploid production. In addition to haploids, the method allows the detection of other rare non-euploid plants such as aneuploids, mixoploids and polyploids (*Figures 4a* to *4e*). By combining samples with standard controls of known ploidy, the abnormal types are revealed by the presence of more than one peak in in histograms. Manual characterisation, by counting chromosomes, can be carried out to validate the flow cytometry results (Doležel and Bartoš, 2005) (*Figures 5a* to *5f*).

CONCLUSION

High-throughput flow cytometry adds a significant new tool for researchers in botany and plant breeders wishing to obtain and exploit haploids and polyploids in seed producing plant species. Current methods in haploid production focus on *in vitro* haploid production via androgenesis (*e.g.* anther culture and microspore culture) and gynogenesis (*e.g.* ovule culture), or aberrant pollination (*e.g.* wide crossing). However appealing, these three methods are, they are limited to a small number of species and there tends to be a genotypic dependency within these species (Wędzony *et al*,. 2009), and they have not been reported to have been successfully developed for oil palm. High-throughput flow cytometry offers a viable method for haploid and polyploid detection and production in oil palm, as well as opening the possibility of allowing, the efficient detection of naturally occurring ploidy off-types in a wide range of plant species. The technique as developed may be used 'aggressively' in screening for ploidy off-types or 'defensively' to assess and control seed quality.

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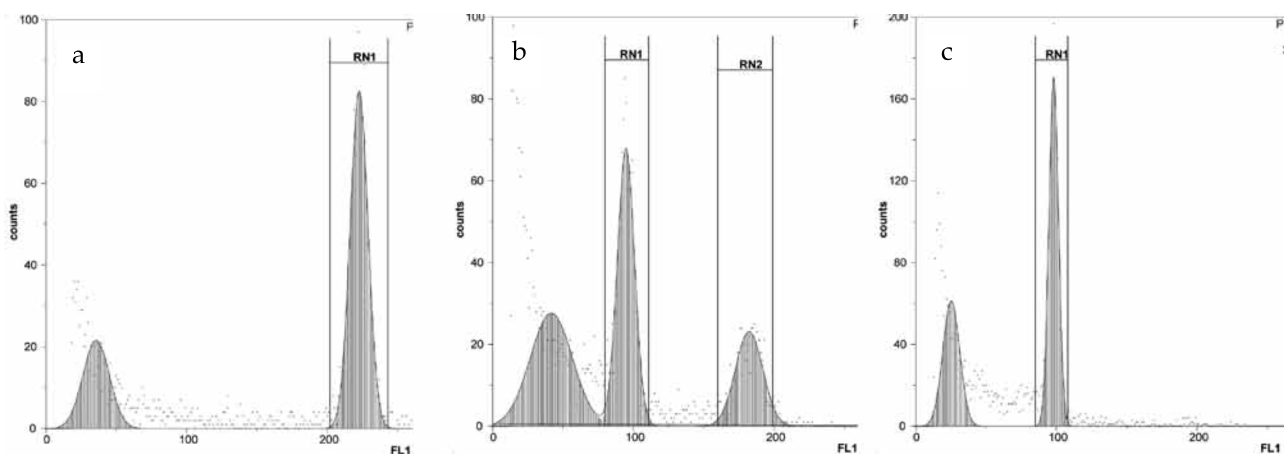

Figure 3. Bulks of five samples are effective and efficient for flow cytometry analysis. Bulking samples of all diploids (a) and mixed diploid and non-diploid tissue samples (b) show clear peaks in the determination of ploidy in oil palm. The haploid signal at 100 FL1 can be read easily from bulked samples composed of five individuals (c) after re-analysed the individual samples.

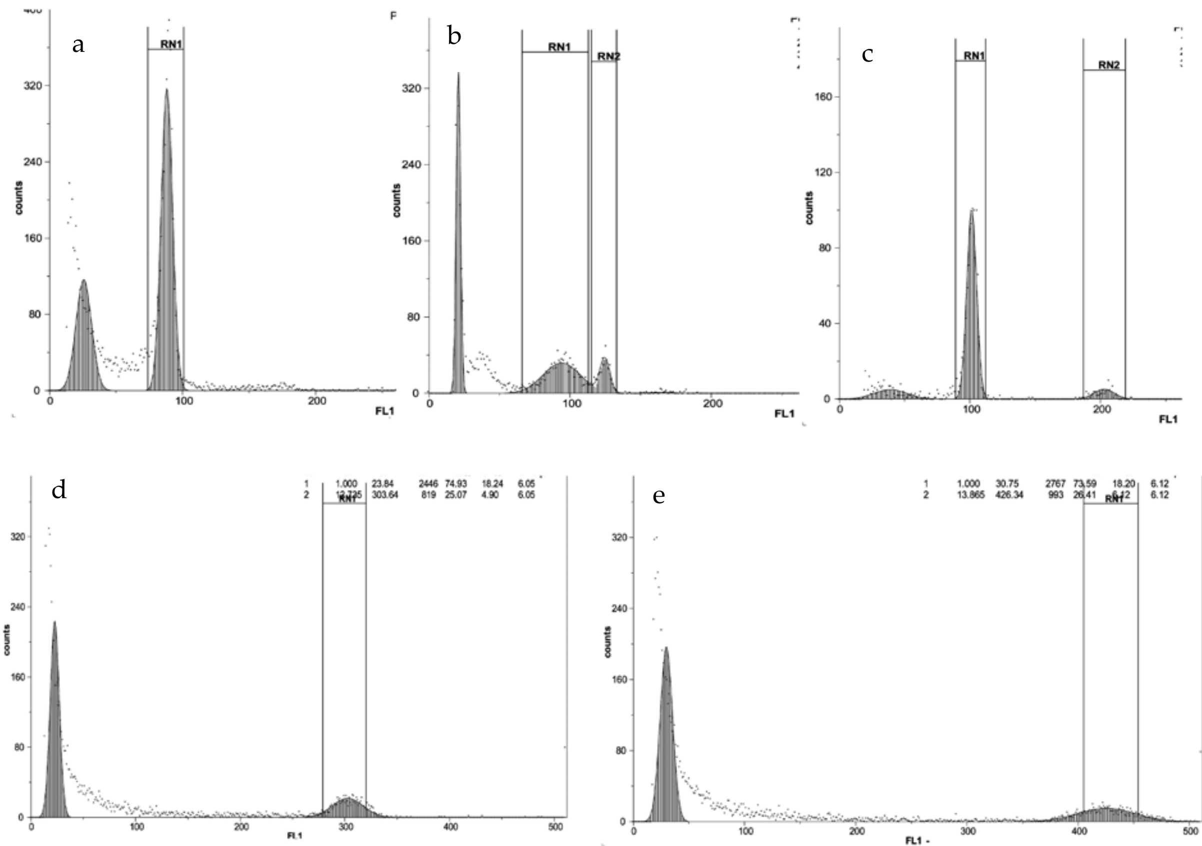

Figure 4. High-throughput flow cytometry for detecting non-euploid of oil palm samples. Clear peaks of haploid (a), aneuploid (b), mixoploid (c), triploid (d) or tetraploid (e) indicate that the high-throughput is efficient in screening of ploidy abnormalities.

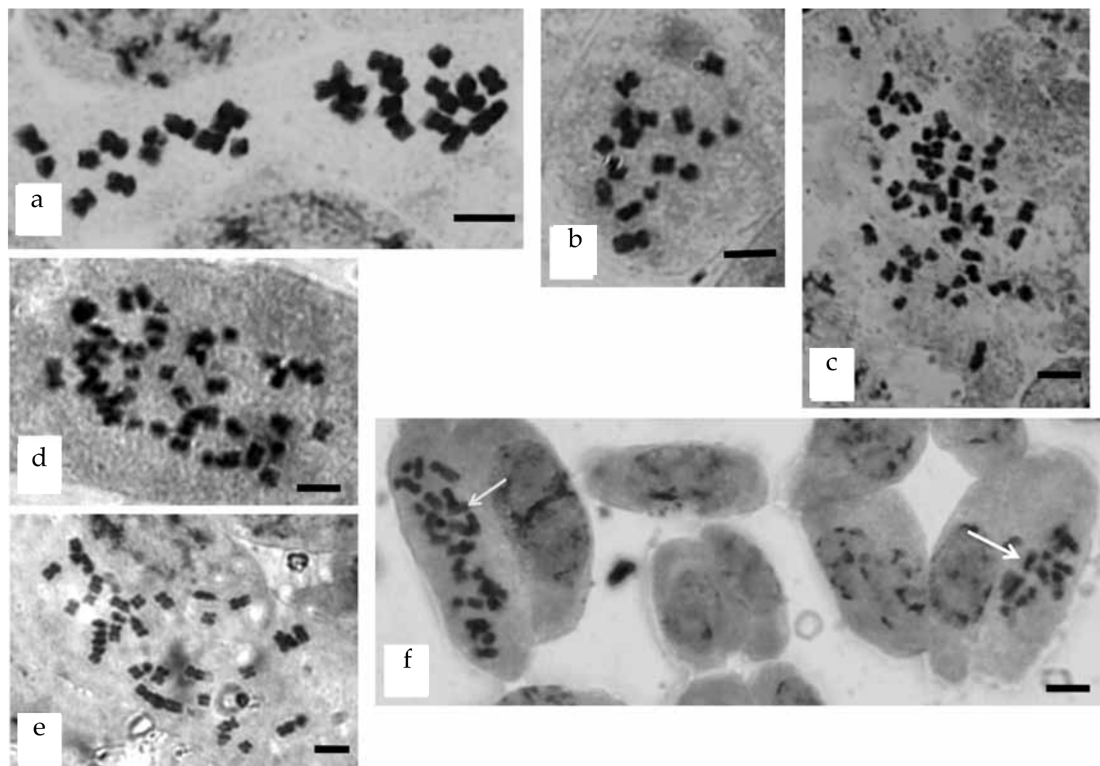

Figure 5. Chromosome counting to validate ploidy and euploidy. Chromosome counts of diploid growing root tips (32 chromosomes) were used as a standard control (a) which can be conducted to check for off-types in flow cytometry analysis, and used to confirm the chromosome complement of haploids (b) or polyploids such as triploids (c) and tetraploids (d) and aneuploids (e) which shows four extra chromosomes or mixoploidy of haploid (white arrows) and diploid (white arrows) that occurred in one individual (f). Scale bars= 5 μm.

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ERRATUM

Please note that *Figure 1* on p. 6 of the *Journal of Oil Palm Research Vol. 25(1) April 2013*, has been replaced with the following figure:

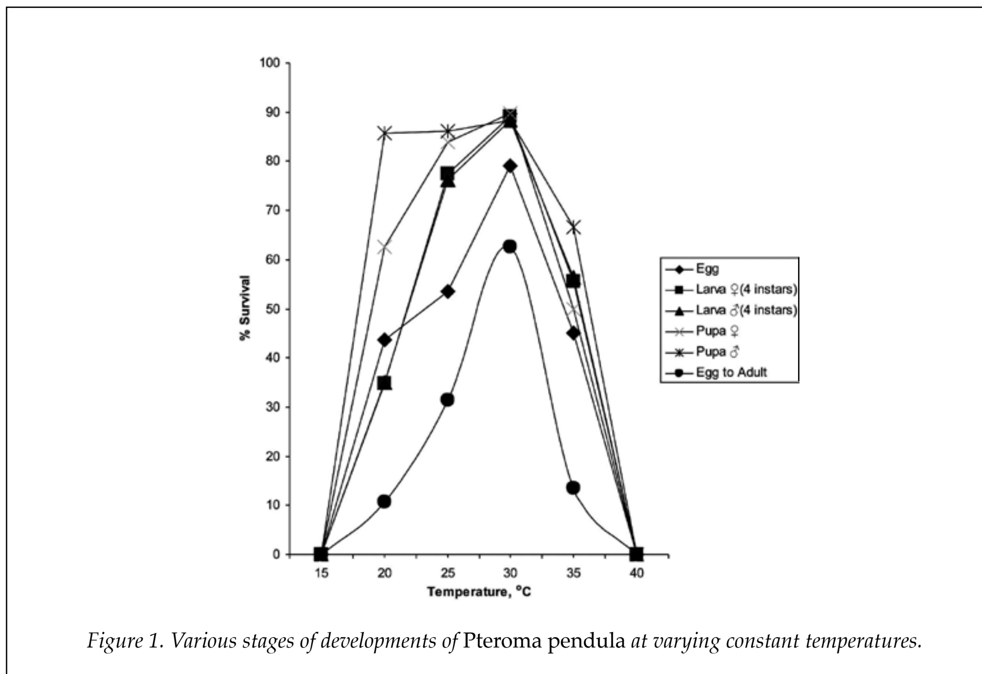

Figure 1. Various stages of developments of Pteroma pendula *at varying constant temperatures.*

The error is regretted.