ABSTRACT
Oil palm chloroplast is believed to be maternally inherited, making investigation of the chloroplast diversity an interesting endeavour. This article describes a method for extracting enriched oil palm chloroplast DNA (cpDNA) done on six palms of different origins from Angola, Nigeria, Ghana, Madagascar and Surinam. Restriction enzyme digestion was used to evaluate the successful extraction of the oil palm cpDNA. The use of a mitochondrial DNA-specific universal primer revealed that most of the cpDNA were free from mitochondrial DNA contamination. Three chloroplast-specific universal primers were also used to evaluate the cpDNA. Their amplicons were cloned and sequenced to confirm that the cpDNA was indeed amplified. A search against the public databases further confirmed that the primers amplified sequences of the Elaeis guineensis Jacq. chloroplast genome. Two of them gave consistent amplification when tested on cpDNA from the Angolan, Nigerian, Ghanian, Madagascan and Surinam palms.

Keywords: chloroplast DNA (cpDNA), Elaeis spp., oil palm chloroplast DNA diversity, chloroplast-specific universal primers.

INTRODUCTION
Oil palm, in the family Palmae, is one of the most important perennial oil-bearing crops in South-east Asia, especially Malaysia and Indonesia. It produces two oils, palm oil from the fruit mesocarp and palm kernel oil from the seed (Hartley, 1988). The crop is being improved through breeding (Hartley, 1988; Moretzsohn et al., 2002; Zaki et al., 2010; Kushairi et al., 2011). However, the lack of genetic diversity in oil palm from South-east Asia is of concern (Rajanaidu et al., 2000; Corley and Tinker, 2003), as the present large populations are descendants of just a few original palms. It was for this reason that Malaysia prospected for more germplasms from both Africa (for the African oil palm) and South America (for the American oil palm). As a result, the Malaysian Palm Oil Board (MPOB) now has the most extensive oil palm germplasm collection in the world (Kushairi et al., 2011).

In order to further improve oil palm, it is vital to have adequate knowledge on the diversity of the oil palm germplasm collections at the molecular level. Molecular markers have been developed, which can give insights into how the crop functions and also assist in achieving higher yields (Singh et al., 2007; 2008). Previously, molecular studies have mostly focused on oil palm nuclear DNA, while the chloroplast DNA or cpDNA has not been extensively studied. However, knowledge of the
cpDNA is important to get a better understanding of the evolutionary and ecological processes of the palm. The fact that chloroplast contains its own genome that is highly conserved, makes it extremely desirable for taxonomic studies (Cheng et al., 2005). Moreover, the chloroplast genome is reported to be structurally stable and ideal for population genetic studies at both the interspecific and intraspecific levels (Cato and Richardson, 1996; Mohanty et al., 2003). Recently, efforts have focused on sequencing the oil palm chloroplast genome (Uthaipaisanwong et al., 2012). The complete sequence was obtained by sequencing fragments of the genome and eventually chloroplast sequences were found as contaminants in the genomic libraries.

However, to characterise the chloroplast diversity over a range of samples would require enriched cpDNA. Although the protocols for nuclear DNA extraction from oil palm are well developed, there is none for enriched cpDNA for oil palm. We are mindful that successful cpDNA extraction is largely species dependent (Palmer, 1986), and thus, we tried several simple, robust and inexpensive protocols developed for other crops (Diekmann et al., 2008; Shi et al., 2012). Unfortunately none was successful, so we modified a method using sucrose and cesium chloride (CsCl₂) gradient separation which managed to isolate oil palm enriched cpDNA with minimal nuclear and mitochondrial DNA contamination. The extracted oil palm cpDNA were analysed for the diversity using chloroplast-specific universal primers reported by Taberlet et al. (1991).

**MATERIALS AND METHODS**

**Plant Materials**

The leaves of six *E. guineensis* palms of Angolan (2), Nigerian (1), Ghanian (1), Madagascan (1) origins and one *E. oleifera* palm of Surinam (1) origin were sampled. The sample collection included palm leaves of Fronds 7, 9 and 19. The leaf samples were stored in a cold dark room for at least four days to destarch before extraction of their cpDNA. The palm leaves were disinfected by soaking in 5% (v/v) Clorox for 10 min and then thoroughly rinsed with water. Finally, they were rinsed again with distilled water before drying at room temperature. The leaves were then cut into small pieces (approximately 2 cm × 2 cm) and kept at -80°C. The list of samples is shown in Table 1.

**Isolation of Enriched Oil Palm cpDNA**

Two established protocols (developed for other crops) reported by Mourad (1998) and Mariac et al. (2000) were tried for the isolation of oil palm enriched cpDNA.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Palm</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.311/414</td>
<td>Angola</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.311/415</td>
<td>Angola</td>
</tr>
<tr>
<td>3</td>
<td><em>E. guineensis</em></td>
<td>0.150/500</td>
<td>Nigeria</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.397/92</td>
<td>Ghana</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.240/19</td>
<td>Madagascar</td>
</tr>
<tr>
<td>6</td>
<td><em>E. oleifera</em></td>
<td>0.177/12</td>
<td>Surinam</td>
</tr>
</tbody>
</table>

*Mourad (1998) protocol.* In this protocol, sucrose gradient separation and cesium chloride gradient separation were used to isolate the purest cpDNA. Both steps require ultracentrifugation using a Beckman Coulter SW 41 Ti swinging bucket rotor to separate the cpDNA from the total genomic DNA. The protocol comprises three basic steps: separation of chloroplast organelles from the leaf tissue, lysis of plastids to release the cpDNA and isolation of pure cpDNA. All the following steps were carried out at 4°C if not otherwise stated. One thousand ml of extraction buffer (0.35 M sorbitol, 50 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 0.1% bovine serum albumin (BSA), w/v; 15 mM β-mercaptoethanol, v/v; 5 mM DIECA, 4 mM ascorbic acid) and 300 ml of suspension buffer (0.35 M sorbitol, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0) were prepared and kept at 4°C for 1 hr. Approximately 100 g of processed leaf samples were thawed and blended with 1000 ml extraction buffer using a Waring blender (2-speed, Model: E8420), at high speed for 2 s and low speed for 3 s for three cycles. The homogenate was then filtered twice through a double layered miracloth (Calbiochem) and centrifuged at 500 × g for 10 min. The chloroplast organelles were gently resuspended in 2 ml suspension buffer using a paintbrush. The chloroplast homogenate was then subjected to a three-layer sucrose gradient separation (55%; 40% and 20%) at 78 400 × g for 90 min using an ultracentrifuge (Beckman Coulter). This three-layer sucrose gradient was found to be the most suitable method for isolating oil palm cpDNA, after testing several gradients, including a two-layer sucrose gradient. The dark green band that appeared at the interphase between 40% and 55% was extracted. After isolating the organelle as a pellet, they were washed three times by gently resuspending the pellet in 20 ml of suspension buffer and centrifuging at 2000 × g for 10 min. Then, the chloroplast lysate was immediately subjected to cesium chloride gradient separation via ultracentrifugation. The lysate was mixed with 3.75 g cesium chloride (Invitrogen) and ethidium bromide was added to the solution to a final concentration of 200 µg ml⁻¹. The solution was ultracentrifuged at 19°C at 41 500 × g for 24 hr. Following that, the ultracentrifuge
tubes (Beckman Coulter) were viewed under a long wavelength UV lamp. The red band that appeared at the middle of the tubes was carefully extracted using a syringe and needle. The extracted cpDNA was washed using isopropanol saturated with sodium chloride (NaCl) and water to remove the ethidium bromide. A dialysis step was carried out for two days with one change of 2 litres fresh low TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0). The cpDNA was then precipitated in 70% volume of 3 M sodium acetate (pH 5.0) and two volumes of absolute ethanol. Finally, the cpDNA was washed using 70% ethanol and suspended in low TE buffer. The cpDNA was stored at -20°C for subsequent analysis.

Mariac et al. (2000) protocol. In this protocol, there are no sucrose and cesium chloride separation steps and it requires less substrate, only 15 g of leaf sample was ground in liquid nitrogen using a pestle and mortar to a fine powder. Then, 25 ml ice-cold sorbitol buffer (0.35 M sorbitol, 0.1 M Tris-HCl, 5 mM EDTA, pH 8.0) with 0.5% sodium bisulphite (w/v) were added. The homogenate was filtered twice through a double layered miracloth (Calbiochem). Sorbitol buffer was added to the residue of the homogenate and it requires less substrate, only 15 g of leaf sample were added. The homogenate was ground in liquid nitrogen using a pestle and mortar to a fine powder. Then, 25 ml ice-cold sorbitol buffer (0.35 M sorbitol, 0.1 M Tris-HCl, 5 mM EDTA, pH 8.0) with 0.5% sodium bisulphite (w/v) were added. The homogenate was filtered twice through a double layered miracloth (Calbiochem). Sorbitol buffer was added to the residue of the homogenate followed by filtration. This step was repeated several times to obtain 100 ml of chloroplast filtrate.

The chloroplast filtrate was centrifuged at 4000 × g for 25 min at 4°C. The pellet was gently suspended in 50 ml sorbitol buffer followed by another centrifugation at 1000 × g for 20 min at 4°C. Then, the pellet was resuspended in 4 ml NET buffer (100 mM NaCl, 80 mM Tris-HCl, 30 mM EDTA, pH 8.0). Then, 1 ml 20% Triton X-100, w/v (Sigma) and 200 μl 2-β-mercaptoethanol (v/v) were added to the chloroplast solution, mixing at slow speed for 90 min at 0°C on a three-dimension gyratory rocker. Then, the lysate solution was centrifuged at 4800 × g for 10 min at 0°C. The supernatant was transferred to 30 ml Corex tubes containing 150 μl Pronase (10 mg ml⁻¹; Sigma), 7 μl proteinase K (20 mg ml⁻¹; Novagen) and 160 μl 20% SDS (w/v). The lysate solution was incubated at 37°C for 3 hr, then centrifuged at 10 000 × g for 10 min at 25°C. The supernatant was then added with 2.2 ml CTAB buffer (20 mM EDTA, 2.8 M NaCl, v/v; 4% cetyltrimethylammonium bromide, 100 mM Tris-HCl, pH 8.0), 100 μl 2-β-mercaptoethanol and 0.05 g PVP, w/v (Sigma). This was followed by gentle shaking for 15 hr at 55°C. After that, the solution was adjusted to a final volume of 15 ml with chloroform-isooamyl alcohol and gently inverted several times to mix the solution. The mixed solution was then centrifuged at 4000 × g for 5 min at 15°C and the aqueous phase was carefully pipetted out. About 70% volume of 3 M sodium acetate (pH 5.2) was added to the aqueous solution of cpDNA, followed by centrifugation at 4000 × g for 10 min at 15°C. Once again, the aqueous phase was extracted and adjusted to a final volume of 15 ml with chloroform-isooamyl alcohol. After gently inverting the solution several times to mix, it was then subjected to a third centrifugation at 4000 × g for 10 min at 15°C.

Finally, the aqueous phase was pipetted out and added with an equal volume of isopropanol. The solution was then gently inverted several times to mix and kept at -20°C for 3 hr followed by centrifugation at 9000 × g for 10 min at 25°C. The pellet consisting of cpDNA was dissolved in 500 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The dissolved cpDNA was re-precipitated in 70% volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol overnight. This was followed by centrifugation at 9000 × g for 10 min at 25°C for DNA precipitation. The cpDNA pellet was then dried in speed vacuum for 5 min and dissolved in 200 μl TE buffer to conduct subsequent analyses.

Further Purification of Oil Palm cpDNA

The cpDNA obtained was further purified using the GeneAll Exgene Plant SV mini DNA purification kit (GeneAll Biotechnology). The purification process was carried out following the manufacturer’s instructions.

Quantification of the cpDNA

The cpDNA samples were quantified using the Multiskan GO Microplate spectrophotometer (Thermo Scientific) to measure the absorbance wavelength of the nucleic acids. The purity of the cpDNA samples were measured based on the A260/A280 value and subjected to subsequent validations.

Mitochondrial DNA Contamination Analysis

To evaluate the efficiency of the oil palm enriched cpDNA extraction protocol, an analysis was carried out using a mitochondrial DNA-specific universal primer, which had been used to amplify a 710 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene, COI (Folmer et al., 1994). This primer had also been used to amplify a conserved protein-coding region of the mitochondria in a range of invertebrates (Folmer et al., 1994). The sequences of the primer pair are:

LCO1490: 5′-GGTCAACAATATCATAAAGATATTGG-3′
HCO2198: 5′-TAAACTTCAGGGGTAGTATATGC-3′

The PCR was carried out in a total volume of 30 μl containing 30 ng cpDNA template, 1x buffer with 1.5 mM MgCl₂ (NEB), 0.2 mM dNTPs (NEB), 4.5 mM MgCl₂ (NEB), 2.5 U NEB Taq Polymerase (5 U), 10 μM forward and reverse primers. The amplification was performed in a GeneAmp PCR 9700 System
thermocycler (Applied Biosystems) programmed as follows: 95°C for 7 min, followed by 35 cycles of 95°C for 1 min, 56°C for 90 s, 72°C for 2 min and a final extension step at 72°C for 5 min. The amplicons were added with 5 μl loading dye [30% (v/v) glycerol, 1% (w/v) SDS, 0.25% (w/v) bromophenol blue] and visualised on 1.2% (w/v) agarose gel.

**Restriction Enzyme Analysis**

The cpDNA were initially analysed via restriction enzyme digestion as previously described (Mourad, 1998; Mariac *et al*., 2000; Diekmann *et al*., 2008; Shi *et al*., 2012). The analyses were carried out using 3 μg cpDNA digested with 15 units HindIII endonuclease (Invitrogen) and 50 units each EcoRI and EcoRV enzymes (Promega). The digestions were done overnight in an incubator at 37°C followed by heat-treatment at 65°C to inactivate the enzymes. Finally, the products were visualised on 1.2% agarose gel. Oil palm nuclear DNA was provided by the MPOB genomics laboratory. The analyses were carried out by digesting 3 μg DNA with 50 units each EcoRI and EcoRV enzymes (Promega). The digestion and visualisation on 1.2% agarose gel, was carried out as described above.

**Screening of Germplasm with Chloroplast Specific-universal Primers**

Three pairs of chloroplast-specific universal primers reported by Taberlet *et al*. (1991) shown in Table 2 were used to screen and evaluate the oil palm cpDNA. The primers are located at the large single-copy region, between trnT-UGU and trnF-GAA.

The PCR was carried out in a total volume of 30 μl containing 30 ng cpDNA template, 1x buffer with 1.5 mM MgCl₂ (NEB), 0.2 mM dNTPs (NEB), 4.5 mM MgCl₂ (NEB), 2.5 U NEB taq polymerase (5 U), 10 μM forward and reverse primers. The amplification was performed in a GeneAmp PCR 9700 System thermocycler (Applied Biosystems) programmed as follows: 95°C for 7 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 5 min. The amplicons were added with 5 μl loading dye and visualized on 1.2% agarose gel.

**Cloning of Oil Palm Chloroplast Amplicons**

In order to validate that the amplicons were indeed fragments of the oil palm chloroplast genome, PCR products of the Angolan samples were cloned using the TOPO-TA cloning kit (Life Technologies) following the manufacturer’s instruction. About 4-5 individual colonies were cultured on LB agar (with 50 µg ml⁻¹ Ampicillin) to obtain a pure culture. Each colony was then grown on LB broth and plasmid extraction was carried out by following the manufacturer’s instructions (Qiagen). The plasmids were checked using PCR to confirm that the expected bands were obtained. Finally, the plasmids were sequenced and searched against the public databases using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm that the PCR fragments were indeed chloroplast sequences.

**RESULTS AND DISCUSSION**

**Comparison of the Two Protocols for Extracting Oil Palm Enriched cpDNA**

Although cpDNA extraction protocols have been reported for many other crops (Mariac *et al*., 2000; Diekmann *et al*., 2008; Shi *et al*., 2012), none has been specific for oil palm. Most of the established protocols that are cost and time efficient (Diekmann *et al*., 2008; Shi *et al*., 2012) do not produce sufficiently good quality cpDNA for oil palm (data not shown) for downstream analysis, such as sequencing. In fact, in the recent publication on oil palm cpDNA by Uthaipaisanwong *et al*. (2012), the chloroplast genome was obtained incidentally while sequencing the nuclear DNA.

Two chloroplast DNA extraction protocols were tested to develop a suitable protocol to extract enriched cpDNA from oil palm. The protocols were tested on one palm from Angola (0.311/415). The advantages and disadvantages of both the protocols

<table>
<thead>
<tr>
<th>Paired primers</th>
<th>Code</th>
<th>Sequences 5’-3’</th>
<th>Loci in chloroplast genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uni1</td>
<td>B48557</td>
<td>CATTACAATAATGGATGCTCT</td>
<td>trn(T(UGU))</td>
</tr>
<tr>
<td></td>
<td>A49291</td>
<td>TCTACGATTTCGCCATATC</td>
<td>trnL(UAA) 5’ exon</td>
</tr>
<tr>
<td>Uni2</td>
<td>B49317</td>
<td>CGAAATCGGTAGACGCTACG</td>
<td>trnL(UAA) 5’ exon</td>
</tr>
<tr>
<td></td>
<td>A49855</td>
<td>GGGGATAGAGGGACTTGAAC</td>
<td>trnL(UAA) 3’ exon</td>
</tr>
<tr>
<td>Uni3</td>
<td>B49873</td>
<td>GGTTCAGGTCCCTCTATCCC</td>
<td>trnL(UAA) 3’ exon</td>
</tr>
<tr>
<td></td>
<td>A50272</td>
<td>ATTTGAACTGGTGACACGAG</td>
<td>trnF(GAA)</td>
</tr>
</tbody>
</table>

**TABLE 2. SEQUENCES OF THE CHLOROPLAST-SPECIFIC UNIVERSAL PRIMERS**
are summarised in Table 3. Technically, the Mariac protocol is less laborious, has greater versatility in the choice of leaf samples and requires less material. However, the sucrose and cesium chloride gradient separation employed in the Mourad protocol give a better quality cpDNA (Diekmann et al., 2008; Shi et al., 2012). In essence, the choice is between lower cost in time/money versus higher quality.

From spectrophotometric measurements, Mariac protocol obtained a higher yield and better quality cpDNA vis-à-vis the Mourad protocol (Table 3). The A260/A280 reading is an indication of DNA purity. In this case, both methods gave cpDNA of acceptable purity and yield. Preliminary validation via restriction analysis was carried out to ascertain the most suitable and efficient protocol for isolating oil palm cpDNA. Furthermore, the oil palm cpDNA was then evaluated via chloroplast-specific universal primers and the amplicons were also sequenced in order to verify that the extracted cpDNA samples were indeed from oil palm chloroplast genome.

In order to extract enriched oil palm cpDNA, the Mourad (1998) protocol was slightly modified by including 5 mM DIECA and 4 mM ascorbic acid to remove polyphenols and polysaccharides. An additional purification step using the GeneAll Exgene Plant SV mini DNA purification kit was also implemented for isolation of oil palm enriched cpDNA from a wide range of samples.

Evaluating the Oil Palm Enriched cpDNA

As a preliminary step, we evaluated the cpDNA using restriction enzymes as described by Mourad (1998), Mariac et al. (2000), Diekmann et al. (2008) and Shi et al. (2012). Distinct bands were observed as expected (Figure 1A, lane 2; Figure 1B, lane 4). The digested oil palm cpDNA showed similar banding patterns as described by Mourad (1998) who worked on Arabidopsis thaliana, Mariac et al. (2000) and Diekmann et al. (2008) both researched on Poaceae, in that large numbers of fine fragments were produced. The relatively well defined bands of the restriction enzyme profiles indicated that the protocols yielded good quality enriched cpDNA (Figure 1). Nuclear DNA of the oil palm, 1.8 Gb in size, would have produced a continuous smear as shown in Figures 2D and 2H. The much smaller chloroplast genome, approximately 156 kb (Uthaipaisanwong et al., 2012), produced distinct bands rather than a smear when digested with restriction enzymes (Figure 1B, lane 4; Figures 2A and 2E). Both protocols yielded enriched oil palm cpDNA, but a point of concern was that the undigested cpDNA produced using the Mariac protocol showed some smearing (Figure 1B, lane 3), possibly due to some degradation of the cpDNA or nuclear DNA contamination. The cpDNA obtained by the Mourad protocol was largely intact (Figure 1A, lane 1). The high yield and purity of cpDNA from the Mariac protocol may be due to significant nuclear DNA contamination. The cesium chloride separation in the Mourad protocol, although cumbersome, was largely successful in extracting highly enriched oil palm cpDNA. Furthermore, it seems to be more promising to extract enriched oil palm cpDNA with minimal nuclear DNA contamination.

Extracting cpDNA from Germplasm Samples

Having established the Mourad protocol as the most suitable method for extracting oil palm enriched cpDNA, it was then used to extract oil palm cpDNA from the remaining samples in Table 1. Although the cpDNA purity for some samples was acceptable (A260/A280 > 1.6), it was not so for other samples (Table 4). This was not surprising, as the leaf samples were collected from palms of different ages and

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*Figure 1. Results of HindIII digestion cpDNA extraction from the Angolan palm (0.311/415) leaf sample using the Mourad and Mariac protocols. 1: Mourad uncut cpDNA; 2: Mourad cpDNA digested with 15 units HindIII; 3: Mariac uncut cpDNA and 4: Mariac cpDNA digested with 15 units HindIII. M: 1 Kb Plus DNA Ladder (Life Technologies).*

*Figure 2. A total of 3 µg oil palm cpDNA digested with 50 units each EcoRI and EcoRV restriction enzymes (Promega). A and E: digested oil palm cpDNA using EcoRI and EcoRV enzymes respectively; B and F: uncut oil palm cpDNA; C and G: negative control; D and H: positive control (oil palm genomic DNA digested with EcoRI and EcoRV enzymes respectively).*

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TABLE 3. COMPARISONS BETWEEN THE MOURAD AND THE MARIAC PROTOCOLS FOR cpDNA EXTRACTION

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of starting material</td>
<td>~ 100 g of fresh leaves tissues</td>
<td>~ 15 g of leaf samples</td>
</tr>
<tr>
<td>Type of starting material</td>
<td>Fresh leaves or leaf stoned at -80°C are utilised and blended using an appropriate blender</td>
<td>Fresh, frozen or lyophilised leaves samples utilised and ground in liquid nitrogen using mortar and pestle</td>
</tr>
<tr>
<td>Isolation and purification of cpDNA</td>
<td>Sucrose gradient is used to isolate chloroplast from other cell components</td>
<td>Chloroplast DNA is extracted using specific lysis buffer</td>
</tr>
<tr>
<td></td>
<td>Cesium chloride (CsCl) gradient separation is used to further purify cpDNA from impurities</td>
<td>Further purification of cpDNA from polyphenols and other impurities is carried out using CTAB, polyvinylpyrrolidone (PVP) and chloroform-isoamyl alcohol</td>
</tr>
<tr>
<td>Days of extraction</td>
<td>Requires five days to obtain cpDNA</td>
<td>Four days to isolate cpDNA</td>
</tr>
<tr>
<td>Cost and simplicity of extraction protocol</td>
<td>Relatively inexpensive but tedious</td>
<td>More costly and less laborious</td>
</tr>
<tr>
<td>*Yield of cpDNA</td>
<td>4.5 μg g⁻¹ leaf sample</td>
<td>11.9 μg g⁻¹ leaf sample</td>
</tr>
<tr>
<td>*Purity of cpDNA</td>
<td>1.65</td>
<td>1.85</td>
</tr>
</tbody>
</table>

Note: *Yield and spectrophotometric measurements for Angolan oil palm (0.311/415) cpDNA.

TABLE 4. IMPROVEMENT OF THE SPECTROPHOTOMETRY MEASUREMENT (purity) OF SELECTED cpDNA USING THE GENEALL EXGENE PLANT SV MINI DNA PURIFICATION KIT

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>A260/A280 (purity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.150/500</td>
<td>1.78 1.94</td>
</tr>
<tr>
<td>0.397/92</td>
<td>1.33 1.97</td>
</tr>
<tr>
<td>0.240/19</td>
<td>1.45 1.94</td>
</tr>
</tbody>
</table>

origins, which could have influenced the quality and yield of their cpDNA. Thus, all the cpDNA samples obtained were further purified using the GeneAll Exgene Plant SV mini DNA purification kit, which significantly improved the quality of the samples (Table 4). This additional cleaning step is recommended to be included in a standard protocol for extracting enriched oil palm cpDNA.

Analysis of Mitochondrial DNA Contamination

In date palm, both the mitochondrial (715 001 bp) and chloroplast (158 462 bp) genomes are much smaller than the nuclear genome (Yang et al., 2010; Fang et al., 2012; Al-Mssallem et al., 2013). This is likely also the case for oil palm since date palm is a close relative (Uthaipaisanwong et al., 2012; Singh et al., 2013). Therefore, the mitochondrial DNA contamination analysis was conducted to investigate the reliability of the oil palm enriched cpDNA extraction protocol. The analysis was carried out using a mitochondrial DNA-specific universal primer developed by Folmer et al. (1994) that can amplify a 710 bp fragment of a conserved region from the mitochondrial genome.

Figure 3 indicates that the Mourad extraction protocol was suitable for most of the palms as no amplification products were observed in the Angolan (B-1 and B-2), Ghanian (B-4) and Madagascan (B-5) samples. However, the Nigerian (B-3) sample showed mitochondrial DNA contamination. This contamination suggests that mitochondrial DNA contamination cannot be eliminated when working with large numbers of samples. It is for this reason that any primer pairs used to amplify cpDNA, must first be validated that it can indeed amplify cpDNA but not mitochondrial DNA.

Analysis of Chloroplast-specific Universal Primers

The three chloroplast-specific universal primers were used to initially amplify cpDNA of the Angolan
to screen the cpDNA of all the six samples in Table 1. The PCR amplification was repeated three times for each primer pair. Only two (Uni2 and Uni3) produced consistent amplicons of the desired size in all the samples tested, including the purified cpDNA samples (Figure 5). However, no variation was observed within the \textit{E. guineensis} samples or between \textit{E. guineensis} and \textit{E. oleifera} samples. The lack of intraspecific and interspecific variation is not surprising as chloroplast genomes are highly conserved. A large pool of cpSSR markers are probably required to reveal any polymorphism.

CONCLUSION

This is the first report on isolating enriched cpDNA from oil palm by a combined sucrose and cesium chloride gradient separation. We present a slightly modified extraction protocol of Mourad’s (1998) method to specifically isolate oil palm enriched cpDNA from a number of palms from the African germplasm. The protocol produced sufficient enriched cpDNA (4.5 \( \mu \text{g g}^{-1} \) leaf tissue). Extraction of cpDNA was initially verified via restriction enzyme digestion followed by characterisation of the isolated oil palm cpDNA using three chloroplast universal primers sourced from the literature. The reliability of the primer pairs and cpDNA extraction protocol were evaluated by cloning and sequencing a subset of the amplicons. BLAST analysis confirmed that the \textit{Elaeis} sp. cpDNA was indeed amplified. However, only two primers produced consistent amplification for the six cpDNA samples analysed and no polymorphism was detected. Establishment of the oil palm cpDNA extraction protocol paves the way for further molecular investigations such as the

### Table 5. Summary Blast Results of Chloroplast Universal Amplicons

<table>
<thead>
<tr>
<th>Chloroplast universal primers</th>
<th>Loci in chloroplast genome</th>
<th>e-value</th>
<th>Region</th>
<th>Blast description</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uni1 (900 bp)</td>
<td>Between trnT-UGU and trnF-GAA</td>
<td>0.0</td>
<td>Large single-copy region</td>
<td>\textit{Elaeis guineensis} chloroplast genome</td>
<td>JF274081.1</td>
</tr>
<tr>
<td>Uni2 (700 bp)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uni3 (500 bp)</td>
<td></td>
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</tbody>
</table>
development of single nucleotide polymorphism (SNP) markers for the chloroplast genome. However, more samples from the oil palm accession as well as more cpSSR primers are necessary for meaningful analysis of the oil palm cpDNA diversity. It is for this reason that the chloroplast genome recently published is being mined for additional cpSSR.

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REFERENCES

AL-MSSALLEM, I S; HU, S; ZHANG, X; LIN, Q; LIU, W; TAN, J; YU, X; LIU, J; PAN, L; ZHANG, T; YIN, Y; XIN, C; WU, H; ZHANG, G; BA ABDULLAH; M; HUANG, D; FANG, Y; ALNAKHILI, Y O; JIA, S; YIN, A; ALHUZIMI, E M; ALSAHIATI, B A; AL-OWAYYED, S A; ZHAO, D; ZHANG, S; AL-OTAIBI, N A; SUN, G; MAJRASHI, M A; LI, F; TALA; WANG, J; YUN, Q; ALNASSAR, N A; WANG, L; YANG, M; AL-JELAIFY, R F; LIU, K; GAO, S; CHEN, K; ALKHALDI, S R; LIU, G; ZHANG, M; GUO, H and YU, J (2013). Genome sequence of the date palm Phoenix dactylifera L. Nature Communications, 4: 1-9.


DIEKMANN, K; HODKINSON, T R; FRICKE, E and BARTH, S (2008). An optimized chloroplast DNA extraction protocol for grasses (Poaceae) proves suitable for whole plastid genome sequencing and SNP detection. PLoS One, 3(7): e2813

FANG, Y; WU, H; ZHANG, T; YANG, M; YIN, Y; PAN, L; YU, X; ZHANG, X; HU, S; AL-MSSALLEM, S and YU, J (2012). A complete sequence and transcriptomic analyses of date palm (Phoenix dactylifera L.) mitochondrial genome. PLoS One, 7(5): e37164.


MORETZSOHN, M C; FERREIRA, M A; AMARAL, Z P S; COELHO, P J A; GRATAPAGLIA, D and FERREIRA, M E (2002). Genetic diversity of Brazilian oil palm (Elaeis oleifera H.B.K.) germplasm collected in the Amazon forest. Euphytica, 124: 25-45.


SINGH, R; NAGAPPAN, J; TAN, S G; PANANDAM, J M and CHEAH, S C (2007). Development of simple sequence repeat (SSR) markers for oil palm and their
application in genetic mapping and fingerprinting of tissue culture clones. Asia Pacific J. Molecular Biology and Biotechnology, 15: 121-131.


SINGH, R; MEILINA, O A; LOW, L E T; MANAF, N A A; ROSLI, R; NOOKIAH, R; OOI, L C L; OOI, S E; CHAN, K L; HALIM, M A; AZIZI, N; NAGAPPAN, J; BACHER, B; LAKEY, N; SMITH, S W; HE, D; HOGAN, M; BUDIMAN, M A; LEE, E K; DESALLE, R; KUDRNA, D; GOICOECHEA, J L; WING, R A; WILSON, R K and FULTON, R S (2013). Oil palm genome sequence reveals divergence of infertile species in Old and New worlds. Nature, 500: 335-339.


YANG, M; ZHANG, X; LIU, G; YIN, Y; CHEN, K; YUN, Q; ZHAO, D; AL-MSSALLEM, S and YU, J (2010). The complete chloroplast genome sequence of date palm (Pheonix dactylifera L.). PLoS One, 5(9): e12762.


ERRATUM

Please note that on p. 212 of the Journal of Oil Palm Research Vol. 27(3) September 2015, the names of the authors and corresponding address should read as below and not as printed.

KINGSLY TABI MBI*; GEORGE FRANK NGANDO EBONGUE**; LIBERT BRICE TONFACK*; GODSWILL NTSOMBOH NTSEFONG** and EMMANUEL YOUMBI*

* Plant Physiology and Improvement Unit, Lab. Biotechnology and Environment, Dep. Plant Biology, Fac. Science, University of Yaounde 1, Yaounde, Cameroon.

** IRAD – Specialised Centre for Oil Palm Research of La Dibamba, Douala-Cameroon.

The error is regretted.