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A CHLOROPLAST DNA (cpDNA) EXTRACTION PROTOCOL FOR DIVERSITY ANALYSIS OF OIL PALM (*Elaeis* spp.)

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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.

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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

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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 ($CsCl_2$) 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 1. OIL PALM LEAF SAMPLES FOR cpDNA EXTRACTION

No.	Species	Palm	Origin
1		0.311/414	Angola
2		0.311/415	Angola
3	*E. guineensis* Jacq.	0.150/500	Nigeria
4		0.397/92	Ghana
5		0.240/19	Madagascar
6	*E. oleifera*	0.177/12	Surinam

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 $\mu g\ ml^{-1}$. 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 1/10 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 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-isoamyl 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 1/10 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-isoamyl 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 1/10 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′-GGTCAACAAATCATAAAGATATTGG-3′
HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′

The PCR was carried out in a total volume of 30 µl containing 30 ng cpDNA template, 1x buffer with 1.5 mM $MgCl_2$ (NEB), 0.2 mM dNTPs (NEB), 4.5 mM $MgCl_2$ (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 *Hin*dIII endonuclease (Invitrogen) and 50 units each *Eco*RI and *Eco*RV 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 *Eco*RI and *Eco*RV enzymes (Promega). The digestion and visualisation on 1.2% (w/v) agorose 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 *trn*T-UGU and *trn*F-GAA.

The PCR was carried out in a total volume of 30 µl containing 30 ng cpDNA template, 1x buffer with 1.5 mM $MgCl_2$ (NEB), 0.2 mM dNTPs (NEB), 4.5 mM $MgCl_2$ (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 $\mu g\ ml^{-1}$ 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 2. SEQUENCES OF THE CHLOROPLAST-SPECIFIC UNIVERSAL PRIMERS

Paired primers	Code	Sequences 5′-3′	Loci in chloroplast genome
Uni1	B48557	CATTACAAATGCGATGCTCT	*trn*T(UGU)
	A49291	TCTACCGATTTCGCCATATC	*trn*L(UAA) 5′ exon
Uni2	B49317	CGAAATCGGTAGACGCTACG	*trn*L(UAA) 5′ exon
	A49855	GGGGATAGAGGGACTTGAAC	*trn*L(UAA) 3′ exon
Uni3	B49873	GGTTCAAGTCCCTCTATCCC	*trn*L(UAA) 3′ exon
	A50272	ATTTGAACTGGTGACACGAG	*trn*F(GAA)

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

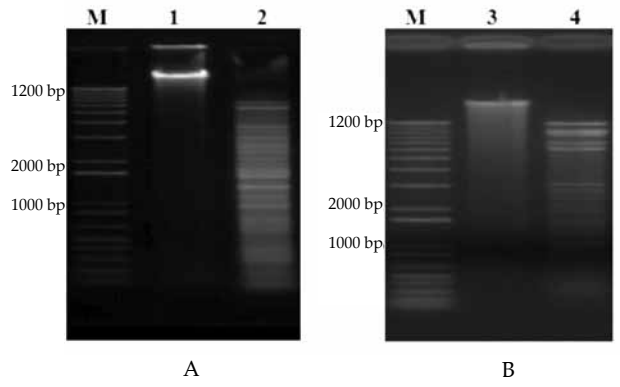

Figure 1. Results of HindIII digestion cpDNA extraction from the Angolan palm (0.311/415) leaf sample using the Mourad and Mariac protocols. 1: Mourad uncut cpDNA; 2: Mourad cpDNA digested with 15 units HindIII; 3: Mariac uncut cpDNA and 4: Mariac cpDNA digested with 15 units HindIII. M: 1 Kb Plus DNA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

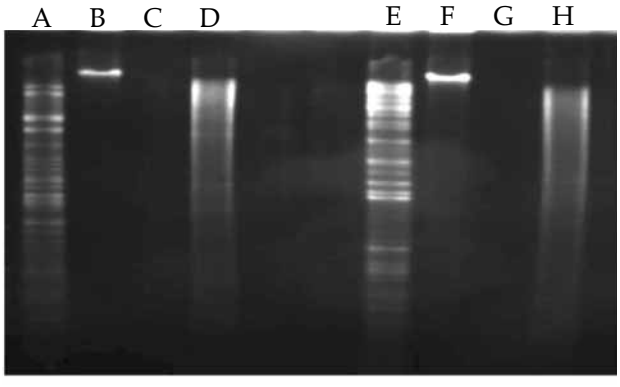

Figure 2. A total of 3 µg oil palm cpDNA digested with 50 units each EcoRI and EcoRV restriction enzymes (Promega). A and E: digested oil palm cpDNA using EcoRI and EcoRV enzymes respectively; B and F: uncut oil palm cpDNA; C and G: negative control; D and H: positive control (oil palm genomic DNA digested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

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

Comparison of features	Mourad (1998)	Mariac *et al*. (2000)
Amount of starting material	~ 100 g of fresh leaves tissues	~ 15 g of leaf samples
Type of starting material	Fresh leaves or leaf stored at -80°C are utilised and blended using an appropriate blender	Fresh, frozen or lyophilised leaves samples utilised and ground in liquid nitrogen using mortar and pestle
Isolation and purification of cpDNA	Sucrose gradient is used to isolate chloroplast from other cell components Cesium chloride (CsCl) gradient separation is used to further purify cpDNA from impurities	Chloroplast DNA is extracted using specific lysis buffer Further purification of cpDNA from polyphenols and other impurities is carried out using CTAB, polyvinylpyrrolidone (PVP) and chloroform-isoamyl alcohol
Days of extraction	Requires five days to obtain cpDNA	Four days to isolate cpDNA
Cost and simplicity of extraction protocol	Relatively inexpensive but tedious	More costly and less laborious
*Yield of cpDNA	4.5 μg g^{-1} leaf sample	11.9 μg g^{-1} leaf sample
*Purity of cpDNA	1.65	1.85

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

Sample No.	A260/A280 (purity)	
	Before purification	After purification
0.150/500	1.78	1.94
0.397/92	1.33	1.97
0.240/19	1.45	1.94

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

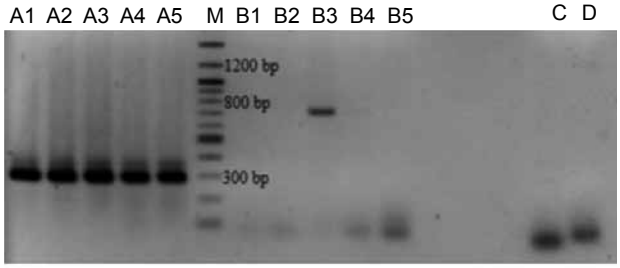

Figure 3. Five cpDNA samples from four palms of different provenances [Angola (2), Nigeria, Ghana and Madagascar] were used to test for mitochondrial DNA contamination. (A) Refers to cpDNA samples amplified using the chloroplast specific rps3 marker (unpublished), while (B) are the same cpDNA samples tested using mitochondrial DNA-specific universal primer. For the negative controls (C) and (D), cpDNA samples were replaced with MilliQ water for both primers. The 710 bp PCR product indicates mitochondrial contamination. 1 and 2: Angolan palms, 3: Nigerian palm, 4: Ghanian palm and 5: Madagascan palm. M: 100 bp DNA ladder (New England Biolabs).

palm (0.311/415). The amplicons are shown in *Figure 4*. All the primers produced amplicons of the desired sizes, although some were smaller. The amplicons of the expected size were cloned and sequenced. The sequencing results were searched against the public databases and all the primers were identified as fragments of the *Elaeis guineensis* chloroplast genome (*Table 5*).

Screening of Chloroplast-specific Universal Primers on Oil Palm cpDNA

On confirmation that the three universal primers were amplifying cpDNA, they were used 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 *E. guineensis* samples or between *E. guineensis* and *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.

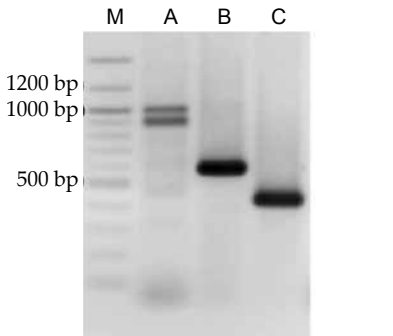

Figure 4. Amplicons of desired size from the three chloroplast-specific universal primers were sequenced. A: Uni1 (900 bp); B: Uni2 (700 bp); C: Uni3 (500 bp) and M: 100 bp DNA Ladder (New England Biolabs).

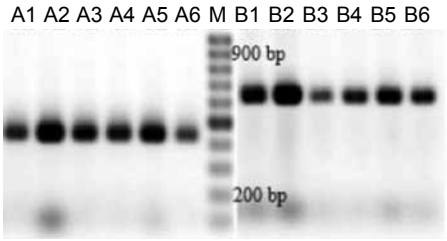

Figure 5. PCR amplification of six cpDNA samples (1 and 2: Angolans, 3: Nigerian, 4: Ghanian, 5: Madagascan and 6: Surinam) using primer Uni2 (A) and Uni3 (B). M is the 100 bp DNA Ladder (Promega).

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 μg 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 *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

Chloroplast universal primers	Loci in chloroplast genome	e-value	Region	Blast description	Accession No.
Uni1 (900 bp) Uni2 (700 bp) Uni3 (500 bp)	Between *trn*T-UGU and *trn*F-GAA	0.0	Large single-copy region	*Elaeis guineensis* chloroplast genome	JF274081.1

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 M; HUANG, D; FANG, Y; ALNAKHLI, Y O; JIA, S; YIN, A; ALHUZIMI, E M; ALSAIHATI, 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 *Pheonix dactylifera* L. *Nature Communications*, *4*: 1-9.

CATO, S A and RICHARDSON, T E (1996). Inter- and intraspecific polymorphism at chloroplast SSR loci and the inheritance of plastids in *Pinus radiata* D. Don. *Theoretical and Applied Genetics*, *93*: 587-592.

CHENG, Y P; HWANG, S Y and LIN, T P (2005). Potential refugia in Taiwan revealed by the phylogeographical study of *Castanopsis carlesii* Hayata (Fagaceae). *Molecular Ecology*,*14*: 2075-2085.

CORLEY, R and TINKER, P (2003). *The Oil Palm.* 4th ed. Oxford: Blackwell Publishing.

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 (*Pheonix dactylifera* L.) mitochondrial genome. *PLoS One*, *7(5)*: e37164.

FOLMER, O; BLACK, M; HOEH, W; LUTZ, R and VRIJENHOEK, R (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*: 294-299.

HARTLEY, C W S (1988). The botany of oil palm. *The Oil Palm.* 3rd ed. Longman: London. p. 47-94.

KUSHAIRI, A; MOHD DIN, A and RAJANAIDU, N (2011). Oil palm breeding and seed production. *Further Advances in Oil Palm Research (2000-2010)*. MPOB, Bangi. p. 47-101.

MARIAC, C; TROUSLOT, P; POTEAUX, C; BEZANCON, G and RENNO, F J (2000). Chloroplast DNA extraction from herbaceous and woody plants for direct restriction fragment length polymorphism analysis. *BioTechniques*, *28*: 110-113.

MOHANTY, A; MARTIN, J P; GOZALEZ, L M and AGUINAGALDE, I (2003). Association between chloroplast DNA and mitochondrial DNA haplotypes in *Prunus spinosa* L. (Rosaceae) populations across Europe. *Annals of Botany*, *92*: 749-755.

MORETZSOHN, M C; FERREIRA, M A; AMARAL, Z P S; COELHO, P J A; GRATTAPAGLIA, 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.

MOURAD, S G (1998). Chloroplast DNA isolation. *Methods in Molecular Biology* (Martinez-Zapater, J and Salinas, J eds.). Vol. 82: Arabidopsis Protocols. NJ: Human Press Inc. p. 71-77.

PALMER, J D (1986). Comparative organization of chloroplast genomes. *Annual Review of Genetics*, *19*: 325-345.

RAJANAIDU, N; KUSHAIRI, A; RAFII, M; MOHD DIN, A; MAIZURA, I and JALANI, B S (2000). Oil palm breeding and genetic resources. *Advances in Oil Palm Research*. MPOB, Bangi. p. 171-237.

SHI, C; HU, N; HUANG, H; GAO, J; ZHAO, Y J and GAO, L Z (2012). An improved chloroplast DNA extraction procedure for whole plastid genome sequencing. *PLoS One*, *7(2)*: e31468.

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 Biotechology*, *15*: 121-131.

SINGH, R; TAN, S G; PANANDAM, J; RAHMAN, R A and CHEAH, S C (2008). Identification of cDNA-RFLP markers and their use for molecular mapping in oil palm (*Elaeis guineensis*). *Asia Pacific J. Molecular Biology and Biotechnology*, *16*: 53-63.

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.

TABERLET, P; GIELLY, L; PAUTOU, G and BOUVET, J (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology*, *17*: 1105-1109.

UTHAIPAISANWONG, P; CHAMPRASERT, J R; SHERMAN, J R; SANGSRAKU, D; YOOCHA, T; JOMCHAI, N; JANTASURIYARAT, C; TRAGOONRUNG, S and TANGPHATSORNRUANG, S (2012). Characterization of the chloroplast genome sequence of oil palm (*Elaeis guneensis* Jacq.). *Gene, 500*: 172-180.

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.

ZAKI, N M; ISMAIL, I; ROSLI, R; CHIN, T N and SINGH, R (2010). Development and characterization of *Elaeis oleifera* microsatellite markers. *Sains Malaysiana*, *39*: 909-912.

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.

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The error is regretted.