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DNA EXTRACTION FROM MATURE OIL PALM LEAVES

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ABSTRACT

Molecular markers have a useful role in oil palm improvement as conventional breeding is limited by the large size of the palm and its long generation interval. The starting point for such applications is DNA extraction using young, immature leaves as required by common protocols. Since such leaves are sometimes not available or are difficult to obtain, a modified method of DNA extraction from older, more mature oil palm leaves kept for a period of time, has been developed. Key changes were initial extraction at ice-cool temperatures and modification of the standard buffers. The DNA obtained by this method was fully digestible for AFLP assay.

Keywords: oil palm, mature leaves, CTAB (hexadecyltrimethylammoniumbromide), DNA extraction, AFLP.

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INTRODUCTION

The oil palm sector in Malaysia is witnessing an upsurge in interest in biotechnological applications in the crop consonant with similar developments worldwide across a vast range of plant, animal and human applications. Among the various biotechnological techniques, DNA marker technology is a powerful tool that can be used for plant and animal breeding, principally through marker-assisted selection (MAS) and fingerprinting. In the oil palm, for example, economically important monofactorially inherited traits, such as shell-thickness, and fruit colour can be tagged with DNA markers which can then be identified at the seed and seedling stage, *i.e.* before the adult stage as in conventional breeding, thus saving on costs, land for planting trials and, most importantly, the time required to produce better planting materials.

Work involving markers in the oil palm started from the late 1980s and traits like shell-thickness (Mayes *et al.*, 1997; Moretzsohn *et al.*, 2000); virescens (*Vir*) and crown disease (Breure and Soebagjo, 1991) were studied with a view to increasing the yield and the quality of the oil. Mayes *et al.* (1997) reported the construction of a restriction fragment length polymorphism (RFLP) map for oil palm and subsequent identification of a marker linked to shell-thickness. Shah (1994) assessed the utility of random amplified polymporhic DNA (RAPD) markers for determining genetic variation in oil palm, and Moretzsohn (2000) produced a RAPD linkage map which included a marker for shell- thickness. In 1995, Jack *et al.* (1995) and Mayes *et al.* (1996) reported the potential of RFLP markers in identifying and characterizing oil palm populations.

The starting step in all the above studies involved obtaining highly pure samples of oil palm genomic DNA in sufficient quantities. The extraction methods for the different tissues from various plants are generally available in standard laboratory manuals but most of these relate to temperate crops. The methods used for oil palm have closely followed these protocols and mostly require young and fresh soft tissues, which are usually leaf for obvious reasons.

Young leaf tissue, however, while more readily available than say flower tissue, may itself not be conveniently obtainable. A good example is that from very tall palms. The young leaves of fronds occur in a central group of newly emerging fronds that make up the funnel part of the canopy. Below them are increasingly older fronds which are increasingly more mature and horizontal, from pressure of the expanding inflorescence and, later, developing fruit bunch in their axils as well as their own weight. The lowest layer of the canopy comprise the oldest fronds which on unattended

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palms, droop or hang downwards forming a skirt around the trunk as the oil palm, unlike the coconut, is not self-pruning. On cultivated palms, lowermost fronds are cut away when harvesting the fruit bunches so there is no skirt of brown fronds. Because of the above-mentioned structure of the palm canopy, obtaining leaf material from newly-emerging fronds, high up in the palm canopy, is difficult and collecting older leaves may be the only practical option.

Another instance when young leaves may not be available is with seedlings. The nature of the study may require non-destructive harvesting and so only the older leaves may be available. Taking young leaves would not only give less material to work with, it may also kill the seedlings.

Older leaf material also travels better and hence, where samples come from a distant breeding station and take a few days to reach the molecular marker laboratory, it may be advisable to dispatch the leaflets from older fronds. Refrigerated transport may not be available or prohibitively costly.

In a recent study of amplified fragment length polymorphism (AFLP) markers for the *Vir* gene, mature leaves had to be used since the palms used in the study were very tall and from a breeding station more than 300 km away. Difficulties with commonly used DNA extraction protocols led to the development of a modified procedure suitable for mature oil palm leaves that had been kept for a few days.

MATERIALS AND METHODS

General DNA Extraction Methods

Nine different methods for isolating genomic DNA reported in several scientific papers, as well as the present modified method and two commercial DNA extraction kits were tested. For each method, two randomly picked oil palm leaf samples (19/228 NT and 19/217 VT) of 1 g each were used. The banding pattern of the genomic DNA was compared with that of a known DNA size marker (*Hind III*-lambda DNA) when run on 0.8% agarose gel to ascertain the suitability of the particular method. Of these, method K, a modified CTAB extraction method with optimized extraction protocol and optimum chemical components in the buffers, was selected for DNA extraction for the AFLP study using mature oil palm leaves. The DNA yield from each gramme of leaf tissue was consistently above 200 mg. The DNA extracted from all the samples from both the CBP and NPC1 populations were satisfactory.

Leaf sampling and storage. An easily accessible mature frond was cut from each palm in the selected cross. This was usually from frond 35-40, depending on when the palm was last pruned or harvested, on the commonly-used system of oil palm frond numbering. The middle three pinnae on either side of the cut frond were harvested, cut into smaller pieces, placed in sample bags and kept under ambient conditions in the field laboratory. The following day the samples were dispatched by train and reached the molecular marker laboratory a day later or three days after harvesting. On arrival at the laboratory, the samples were re-checked and stored in a -80°C freezer over a six-month period during which time they were periodically taken for DNA extraction.

DNA extraction. Approximately 1 g of leaf material was used for the DNA extraction. As the materials were in storage, to retain their freshness, the initial extraction was done in 5 ml ice-cool modified extraction buffer A (0.1 M Tris-HCl, pH 8, 0.02 M Na_2EDTA, 1.4 M NaCl, 2% CTAB, 2% PVP-40, 0.35 M sorbitol, 0.02 M sodium bisulfite, 1% DIECA, 1% ascorbic acid). The mixture was swirled gently and kept on ice for 15 min, then centrifuged at 8000 rpm for 10 min at 4°C, after which the supernatant was discarded and the pellet saved. Ten millilitres modified Lysis Buffer B (0.1 M Tris-HCl, pH 8, 0.02 M Na_2EDTA, 1.4 M NaCl, 2% CTAB, 2% PVP-40, 0.14 M sorbitol, 1% Sarkosyl, 1% DIECA, 1% ascorbic acid, 1% β-mercaptoethanol) were added to resuspend the pellet. The extraction procedure then followed the normal CTAB protocol.

Quantitation of DNA. The concentration of DNA was estimated using a spectrophotometer (Ultrospec 3000 pro Beckman). The optical densities (OD) at UV wavelengths 260 nm and 280 nm were measured. The OD ratio at 260/280 was calculated to determine the DNA purity. The concentration of DNA was also estimated by comparing the intensity of the DNA bands on agarose gel (0.8%) with DNA marker of known concentration. One µg of the DNA isolated by each of the extraction methods was digested with 1 unit *Eco*RI for 2 hr in an incubator at 37°C. The digested DNA, together with undigested DNA of the same quantity and a λ-*Hind III* DNA size marker (M), was run on a 1.5% agarose gel to verify its quality.

Trial digestion. Prior to any AFLP analysis, a trial digestion was carried out for each batch of DNA. Endonuclease digestion of the DNA samples (1 µg) with both *Eco*RI and *Mse*I simultaneously was done to confirm that there was no local impurity inhibiting the restriction enzyme activity.

RESULTS AND DISCUSSION

Generally, all the techniques could be used for DNA isolation from the mature, kept oil palm leaves. However, they were not optimal for genomic DNA

for AFLP analysis because of varying degrees of degradation, low quality and high impurities in the isolated DNA.

The genomic DNA extracted by methods A, B, D, F, G, and I (*Table 1*) had high levels of impurities while C and H yielded rather low quantities of DNA and J suffered both. The quality of DNA extracted by E was better than that from the other methods due to an additional purification step. The best results, *i.e.* intact bands and no contamination by phenols or carbohydrates, were observed with procedure K. The commercial kits, L and N, while satisfactory with young leaves, were less suitable with mature stored leaves; either the yield was low or the quality poor. Good quality DNA would give a quantitation ratio of 1.8-2.0. None of the above methods gave such high values, the range obtained being 1.4-1.7. Nevertheless, the ratio of 1.4-1.6 by method K was usable for AFLP analysis.

TABLE 1.

No.	Method (reference)	Code
1	Doyle and Doyle (1987)	A
2	Chen and Ronald (1999)	B
	Chen and Ronald (1999) (modified)	E
3	Permingeat *et al.* (1998)	C
4	Chaudhry *et al.* (1999)	D
5	Aljanabi *et al.* (1999)	F
6	Bekesiova *et al.* (1999)	G
7	Barnwell *et al.* (1998)	H
8	Tel-Zur *et al.* (1999)	I
9	Dellaporta *et al.* (1983)	J
	(Modified by Benutzername 2002)	
10	Current work	K
11	Plant DNA Extraction Kit (GeneTACG)	L
12	DNeasy® Plant Kit (Qiagen)	N

The quality of oil palm genomic DNA isolated by the above methods was further verified by digestion with the enzymes *Eco*RI and *Mse*I to ensure that there was no impurity inhibiting the activity of the restriction enzymes. The DNA quality was considered satisfactory when the undigested DNA showed a clear and intact band at the high molecular weight range and also if it was effectively digested by *Eco*RI (Do and Adams, 1991). *Figure 1* shows the patterns of digested DNA, undigested DNA and a λ-*Hind III* DNA size marker (M) on a 1.5% agarose gel. The results showed that the genomic DNA extracted using method F was not digested by *Eco*RI, that extracted using methods A, B, D, G and I were partially digested while DNA obtained by methods C, E, H, J, K, L and N was fully digested. Method E produced high quality DNA when tested as above. However, it was found to be inconsistent due to oxidation which sometimes compromised the DNA quality.

⇑ Based on the above testing, the DNAs extracted from all the samples using method K were satisfactory: 1) the undigested DNA showed a clear and intact band at the high molecular weight range and there was no visible RNA contamination in the gel electrophoresis and 2) the DNA when digested with both *Eco*RI and *Mse*I simultaneously showed complete digestion when resolved on 1.5% agarose gel (*Figure 2*).

CONCLUSION

The commonly-referenced DNA extraction protocols are generally suitable for young oil palm leaves but not always for mature leaves. A modified CTAB extraction method, with extraction protocols and chemical components of buffers optimized, gave good quantities of high quality genomic DNA from mature oil palm leaves. The DNA yield per gramme of leaf tissue from many different samples was consistently above 200 mg. Furthermore, the undigested DNA showed a clear and intact band at the high molecular weight range and there was no visible RNA contamination in the gel electrophoresis. When digested with both *Eco*RI and *Mse*I simultaneously, the DNA was completely digested on 1.5% agarose gels.

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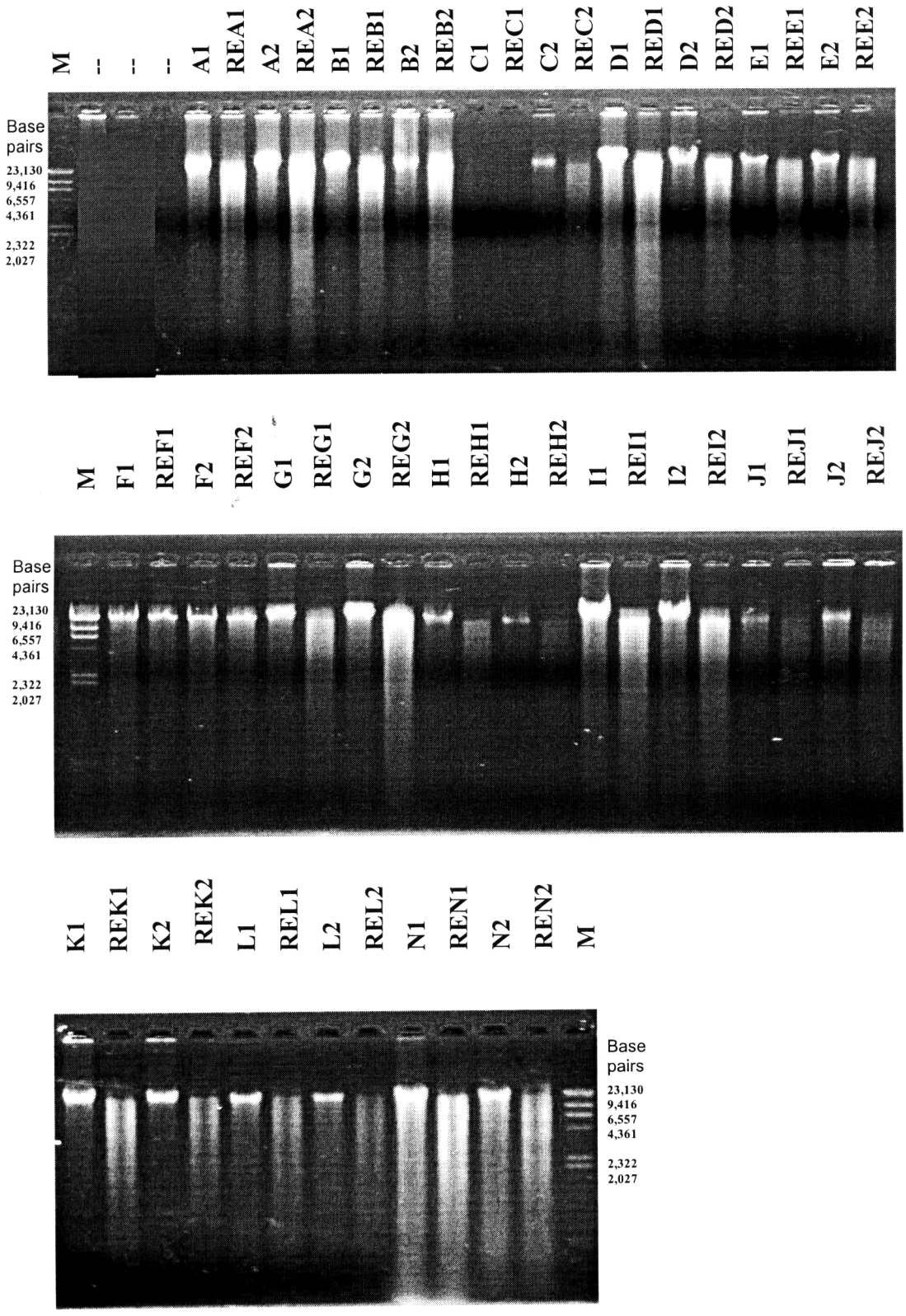

Notes: The DNA obtained by different extraction methods: A1, A2 (Doyle and Doyle, 1990); B1, B2 (Chen and Ronald, 1999); C1, C2 (Permingeat *et al.*, 1998); D1, D2 (Chaudhry *et al.*, 1999); E1, E2 (modified Chen and Ronald, 1999); F1, F2 (Aljanabi *et al.*, 1999); G1, G2 (Bekesiova *et al.*, 1999); H1, H2 (Barnwell *et al.*, 1998); I1, I2 (Tel-Zur *et al.*, 1999); J1, J2 (Dellaporta *et al.*, 1983) (modified by Benutzername, 2002); K1, K2 (current work); L1, L2 (GeneTAC™); N1, N2 (Qiagen). M, marker λ-Hind III. The letters refer to the codes in *Table 1*; 1 is sample 19/228 NT and 2 is sample 19/217 VT.

Figure 1. An ethidium bromide-stained 1.5% agarose gel with 1μg undigested DNA and DNA digested with EcoRI (RE).

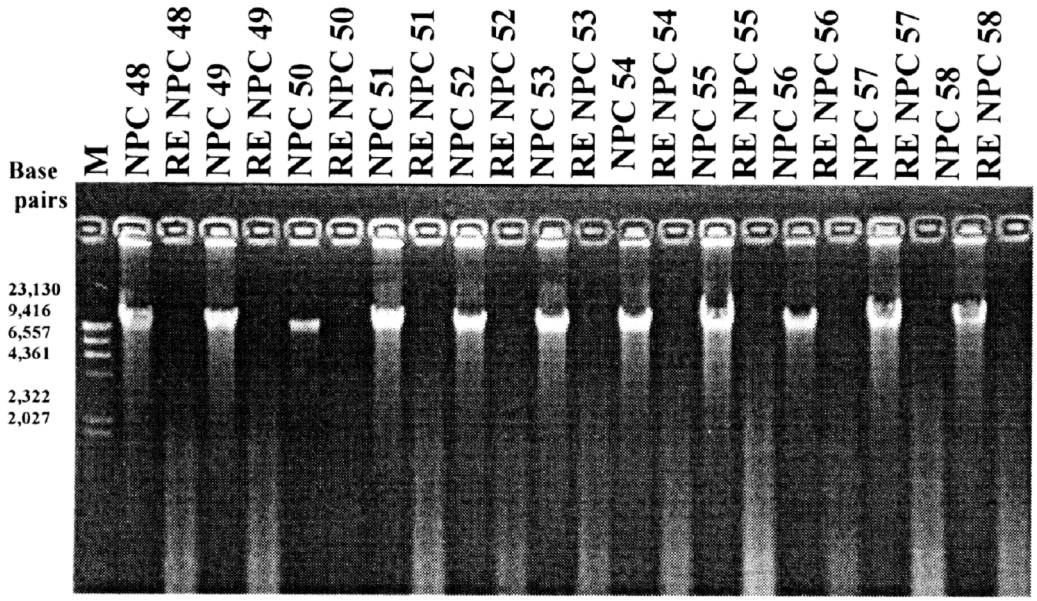

Figure 2. Gel electrophoresis of 1 µg undigested DNA and DNA double digested with two restriction enzymes Eco*RI and M*seI *(RE) from some oil palm (NPC1) individuals, extracted by method K. M, marker λ-Hind III.*

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Appendix 1

- **2X CTAB buffer**

  0.1 M Tris-HCl (1 M)
  0.02 M Na2EDTA (0.5 M)
  1.4 M NaCl
  2% CTAB
  dH_2O to 1 L
  The above mixture was sterilized by autoclaving.

- **Extraction buffer A**

  0.1 M Tric-HCl, pH8 (1M)
  0.02 M Na_2EDTA (0.5 M)
  2% PVP-40
  Stir or put in 50˚C water bath to dissolve the PVP first.

  0.35 M Sorbitol
  0.02 M Sodium bisulfite
  1% DIECA
  1% Ascorbic acid

- **Lysis buffer B**

  2 X CTAB buffer
  2% PVP-40
  0.14 M Sorbitol
  1% Sarkosyl
  1% Ascorbic acid
  1% DIECA
  1% β-mercapthoethanol (add before use)