DEVELOPMENT OF MODIFIED COCOA DNA EXTRACTION METHOD

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ABSTRACT

This is the first attempt to study Papua New Guinea cocoa at the genetic level using molecular markers. To study cocoa at the molecular level, the first the step is to extract clean good quality Deoxyribonucleic Acid (DNA) to be used for DNA profiling. This paper described a modified method developed to extract good quality DNA, after attempts to use the conventional Cetyltrimethylammonium bromide (CTAB) based methods failed. The modified procedure developed was successful and used to extract high quality DNA and used to evaluate the genetic diversity of selected hybrid cocoa planting materials from Papua New Guinea.

Key Words: DNA fingerprinting, molecular markers, genetic diversity

INTRODUCTION

Protein (isozyme) analysis was first used to determine the polymorphism among cocoa genotypes (Atkinson *et al.*, 1986). The isozyme method was simple, but the limiting factor was the low level of polymorphism detected. In order to obtain a more accurate description of genetic diversity of genotype, molecular markers were used to analyse the DNA sequences. Variation at the DNA level using Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) markers were used to study genetic diversity of cocoa. The use of these two markers were informative and comparable (N'Goran *et al.*, 1994), however, RAPD markers were chosen and used in the current study because the technique is fast, inexpensive, avoids the use of radioisotopes and requires minute amount of DNA for Polymerised Chain Reaction (PCR), compared to the RFLP technique. The technique is also less laborious and more suitable for use in developing countries.

MATERIAL AND METHODS

Twenty five accession of *Theobroma cacao*, which included 9 parental and 16 progeny genotypes were collected from one of the breeding trial conducted at Papua New Guinea Cocoa and Coconut Research Institute (Kuman, 2005). Healthy young fresh leaves were randomly sampled from each row of selected genotypes in the breeding trial. The sampled leaves from each genotype were packed separately inside plastic bags and labelled (which included the name of the genotype and date of harvest) and placed inside a 10 L polystyrene container half filled with ice. At the end of all collections, a full plastic bag of ice was gently laid on top of the samples and the polystyrene container was securely enclosed and transported to the University of Melbourne, Molecular Plant Genetic Germplasm laboratory for DNA isolation.

Sample treatment

The sample once brought into the laboratory were removed from the polystyrene container and stored immediately inside a -20 °C freezer. Prior to DNA extraction, one frozen leaf of each genotype was thawed, washed with distilled water and blot dried with paper towel. The midveins and petioles of the leaves were removed, and approximately 0.1 g of sample excised from each of the leaves and was used for DNA

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extraction. The remaining samples were stored in the freezer for later use.

DNA extraction

Many attempts were made to extract DNA from cocoa leaves by adopting different Cetyltrimethylammonium bromide (CTAB) methods, but none of them were successful. The first CTAB protocol adapted to extract cocoa DNA was based on the modified method of (Taylor *et al.*, 1995). The second CTAB protocol adopted was based on Crouzillat *et al.*, (1996) with the quality of reagents and chemicals specified reduced by 1/10 to extract approximately 0.1 g sample. The third CTAB method adopted was based on Laurent *et al.*, (1993) with exclusion of the last step of DNA purification that used Cesium chloride-Ethidium bromide. The forth CTAB method adopted was based on a protocol used to extracted DNA from mango (1999, S.Chungwongse, pers. comm.). The fifth CTAB method adopted was a modified of method 4. This involved doubling the strength of extraction buffers and inclusion of polyvinylpyrrolidone to purify the DNA extracted.

The last method adopted was the modified method described in this paper to successfully extract DNA after various prescribed methods from the literature used, failed to extract good quality DNA. This method involved using spin columns and propriety buffers from QIAGEN, Pty Ltd, Australia with modifications made to suit the requirement of the experiment. All the buffers, enzymes, spin column tubes and collection tubes used in this extraction were supplied as a kit including the standard DNA extraction protocol.

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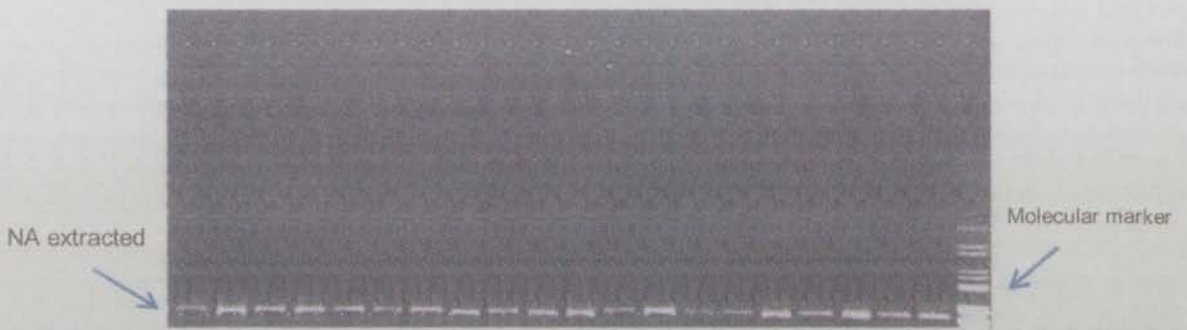
Modified DNA extraction method

The weight leaf (approximately 0.1 g) sample was ground in liquid nitrogen using a sterile molar and pestle and then transferred immediately into an Eppendorf tube. 100 µL of AP1 buffer and 4 µL Rnase A enzyme (100 mg /mL) were added to the ground sample and the mixer was vortexed vigorously. The sample was incubated for 10 min. at 65 °C and gently inverted and mixed three times during the incubation. After incubation, 130 µL of AP2 buffer was added to the lysate (mixture), mixed and incubated on ice for 5 min. The lysate was transferred into a QIA shredder spin column sitting on a 2 mL collection tube, and centrifuge at 1400 rmp for 2 min. The flow-through from the spin column was transferred into a new Eppendorf tube without disturbing the pellets of cell debris accumulated at the bottom of the tube. Buffer AP3 (250 µL) and 100 % ethanol (450 µl) were accurately measured and added to 450 µL clear lysate (flow- through) that was collected. The mixture (1150 µL) was thoroughly mixed by pipetting and gentle vortexing. The mixture was left at room temperature for 5 min. and then 650 µL of the mixture (AP3 buffer + ethanol + lysate), including any precipitate was loaded into the DNeasy mini spin placed inside a new 2 mL collection tube. The mixture was centrifuged at 800 rpm for 1 min. and the flow through discarded. This step was repeated for the remainder of the mixture. The DNeasy mini spin column was placed inside a new collection tube and 500 µL of AW buffer was added into the column and centrifuge at 800 rpm for 1 min. This step was repeated, and the mixture was centrifuge for 2 min. at 1400 rpm, to dry the column membrane. Ethanol (500 µL) was added into the column and centrifuged at 1400 rpm for 2 min. This step was repeated 5 times, to clean the DNA. The column was transferred to a 1.5 mL Eppendorf tube and 100 µL of preheated (65 °C) AE buffer was added directly into the column membrane and incubated for 5 min. at room temperature. The column was centrifuge for 1 min. at 800 rpm and the flow-through was collected. The elution step was repeated twice. The flow-through from each elution step was combined and precipitated with volume of chilled isopropanol (100 %). The DNA was precipitated and diluted with 30 µL of TE buffer (pH 8.0).

Extracted DNA was quantified using a spectrophotometer by measuring the absorbance at 260 nm; the approximate DNA purity was estimated by measuring the A260 nm/A280 nm ratio. Double stranded DNA that showed an OD of 1.00 is known to have a concentration of approximately 50 µg /mL and a pure preparation of ds DNA has an OD 260/OD 280 value between 1.80 and 2.00 (Maniatis *et al.*, 1982). Extracted DNA samples of the highest quality were used and each individual DNA stock concentration was diluted 10 ng /µL for PCR reaction. The RAPD-PCR amplification was performed after amplification of the DNA.

tion protocols were evaluated to extract cocoa DNA. None of the protocol was successful. All the (OD) reading for the stranded DNA extracted using the CTAB based methods were below pure OD range, which infers that extracted DNA could be contaminated (results not shown). When extracted DNA was separated by electrophoresis and visualized under UV, there was no DNA visible on the gel (results not shown). However the modified method using the QIAGEN Kit method produced good quality DNA.

Figure 1: Good quality DNA extracted shown on the gel after electrophoresis and visualized under UV light.



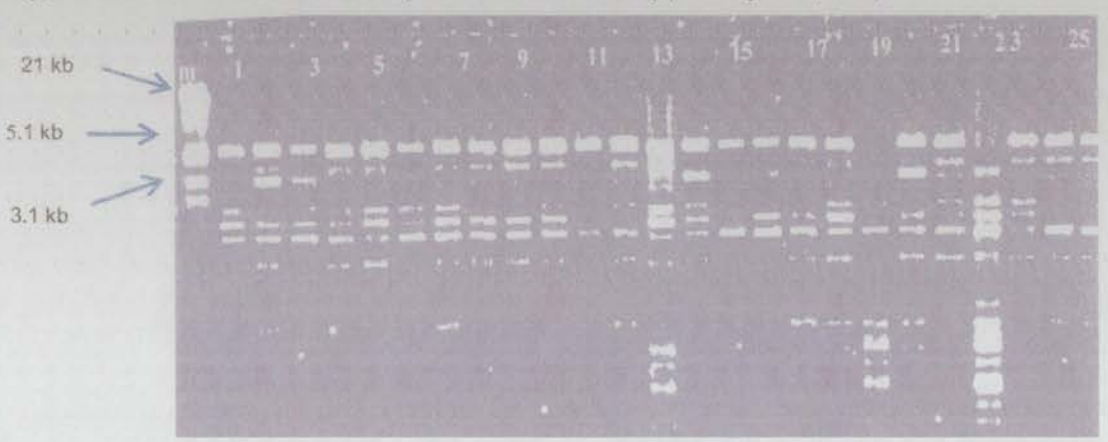
RESULT

DNA extraction

The biggest obstacle to any successful isolation of plant DNA is overcoming the problems of secondary products such as phenolic, quinones and protein contamination. In this experiment different CTAB-based DNA extrac-

The PCR amplification of genomic DNA with RAPD primers produce reproducible and distinct marker profile for genomic DNA extracted from each of the genotype. Each primer amplified between 4 to 15 markers, ranging from 0.56 to 21 kb (Figure, 2). A total of 122 useful markers were produced, of which, 79 % were polymorphic, an average of 5 loci per primer.

Figure 2: RAPD band after amplification of genomic DNA of cocoa genotype with OPW-04 from DNA of genotype KEE12. M = molecular marker (λ EcoRI/Hind III ladder) (Promega Inc, USA)



DISCUSSION AND CONCLUSION

The difficulty encountered in extracting cocoa DNA using CATB methods may be due to the inefficiency of the protocols to purify ubiquitous polysaccharides gum present in the cocoa leaves (Figueira *et al.*, 1994). Increasing the strength of detergents to releases DNA from cells, combined with Polvinylpyrrolidone (PVP) to remove phenolic compounds that formed strong H-bonded complexes, failed to purify the DNA. Mercaptobenzothiosazide and Metabisulfite were also used as antioxidants and phenol oxidase inhibitors, but this does not improved the quality of DNA.

However, good quality DNA was extracted using the modified method described using QIAGEN kit. The success of this method depended on the design of the QIAGEN kit to allow absorption of DNA onto a silica gel membrane and optimized removal of carbohydrate polyphenolic and other plant metabolites. The advantage of using the kit is that it has a unique microfilteration and homogenization unit that removes protein, polysaccharides and cell debris in a single step (QIAGEN protocol, 1977).

The modified DNA extraction protocol developed can be used to extract good quality DNA for PCR amplification and profiling especially, for materials that are difficult to extract using other CTAB based extraction protocols. The procedure can be used to extract DNA from any parts of plants for DNA profiling.

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