Chapter Three

Materials and Methods

3.0 Material and Methods

3.1 Banana samples

Wild *Musa* species samples were collected from different parts of Peninsular Malaysia by Dr. Asif Javed, 2001(R.Y. Othman pers.comm.) and were part of the collection at University of Malaya.

Samples of cultivated bananas were collected from Malaysian Agriculture Research and Development Institute (MARDI) germplasm collection.

The banana samples are listed in Table 3.1.

Table 3.1: The accession, ploidy level and genome constitution of 18 varieties of banana.

Accession	Ploidy	Genome Constitution
Textilis	2x	(TT)
40 hari	2x	AA
BC 1	2x	AA
BC 3	2x	AA
W. Johol	2x	AA
P. Johol	2x	AA
Musa 133	3x	AAA
Berangan	3x	AAA
Novaria	3x	AAA
Minyak Laut	3x	AAA
Valery	3x	AAA
Nain de China	3x	AAA
Giant Cavendish	3x	AAA
Chinese Cavendish	3x	AAA
Pisang Masak Hijau	3x	AAA
Robusta	3x	AAA
Raja	3x	AAB
Kelat Siam	4x	ABBB

3.2 DNA Extraction

The following solutions and reagents were prepared for DNA extraction.

1. Liquid Nitrogen
2. Extraction Buffer
    - 5% w/v CTAB (Cetytrimethylammonium bromide)
    - 1.4M NaCl
    - 20mM EDTA
    - 100mM Tris-HCl (pH 8.0)
    - 0.4% β-mercaptoethanol (added just before use)
3. Chloroform-isoamyl alcohol (24:1)
4. 100% isopropanol
5. Washing solution
    - 76% ethanol
    - 10mM ammonium acetate
6. TE buffer
    - 10mM Tris HCl
    - 1Mm EDTA (pH8.0)
7. 7.5M ammonium acetate

Fifteen gram of fresh young plant leaves were taken, wrapped with aluminum foil and stored on ice immediately. The plant material was grounded to a fine powder, using liquid nitrogen, mortar and pestle. Ground powder was immediately transferred to the extraction buffer to prevent degradation of DNA by cellular enzyme.

The powder was transferred to 35ml of pre-warmed (60°C) extraction buffer in polypropylene tube. The tube was incubated at 60°C for 120 minutes in water bath. One volume of chloroform-isoamyl alcohol was added. The tube was inverted gently and thoroughly by hand for 10 min to ensure emulsification of the phases. The tube was then centrifuged at 10,000 rpm for 10 min. After centrifugation, the aqueous phase was re-extracted once with fresh chloroform-isoamyl, inverted for 10 min and centrifuged again. The supernatant was transferred into another clean tube, and then 0.6 volume of ice-cold isopropanol was added and mixed gently and thoroughly by inverting the tube several times. The DNA/CTAB complex was precipitated as a fibrous network and was lifted out from solution and transferred to 20 ml of washing solution. The tube contained washing solution was mixed gently for a few time and then DNA fibrous network was transferred to one ml of ice-cold 76% ethanol. Again the tube was inverted gently. The same process was repeated twice with 76% ethanol and finally with 95% ethanol.

The clean DNA was dissolved in one ml deionised water and then kept at –20°C for further analysis.

A total of 18 DNA samples from different Selected Banana Varieties were extracted using the above procedure.

3.2.1 Determination of DNA Quality and Concentration

The amount of 990 µl of distilled water was added to 10 µl of DNA solution from stock to make 100x dilution. The quality and concentration of DNA then was determined by using spectrophotometer (Eppendorf, BioPHotometer. AG 22331Harmburg).

The calculation of concentration of DNA in the stock was made as follow:

If absorption at 260nm light value, L = 1.00

DNA concentration = 50 µg/ml

Dilution factor = 100

Thus, DNA concentration of sample = Lx 50 µg/ml x 100

The quality of DNA was determined by absorption of 260nm light / absorption of 280nm light. Good DNA quality has value in between 1.80-2.00.

3.2.2 Dilution

The samples were further diluted to 20ng/µl after the concentration and quality of DNA were detected.

The calculation was made as below:

L = absorption value of sample to 260nm light

Dilution factor, f = 100

First, the DNA concentration should be converted to ng/µl

Thus, concentration of stock DNA is ng/µl $= \frac{L \times 50 \times 100}{1000}$ µg/µl

$= 5\ L$ µg/µl

$= 5000\ L$ ng/µl

Secondly, volume needed from DNA stock solution to make 5000 µl of 20ng/µl was calculated as below:

Volume needed from DNA stock, $V = \frac{20 \text{ ng/µl} \times 500 \text{ µl}}{5000\ L \text{ ng/µl}}$

$= 2/L$ µl

Thus, the dilution was made:

2/L µl of DNA stock solution + (500 µl – 2/L µl) of distilled water.

3.3 Polymerase Chain Reaction (PCR)

3.3.1 Preparation of PCR

Listed materials were obtained from the respective companies for use in PCR.

- *Taq* DNA Polymerase -Promega Corporation, USA
- Mg free buffer - Promega Corporation, USA
- $MgCl_2$ - Promega Corporation, USA
- dNTP mix - Promega Corporation, USA
- All primers – Gibco BRL, USA

3.3.2 PCR Technique

All the thin wall PCR tubes, tips and deionised water were autoclaved at 121°C, 15psi for 20 minutes. Method and primers used were based on the protocols by *Kaemmer* et al (1997).

Three sets of primers were selected for PCR in this study. Table 3.2 listed the sequence, annealing temperature and length of each expected PCR product.

Table3.2: Sequence and annealing temperature of studied *Musa* STMS primers.

Templet	5'-primer sequence-3'	Annealing Temp.	Length of Product
AGM193	AACAACTAGGATGGTAATGTGTGGAA	55°C	128bp
AGM19	GATCTGAGGATGGTTCTGTTGGAGTG		
AGMI103	ACAGAATCGCTAACCCTAATCCTCA	55°C	181bp
AGMI10	CCCTTTGCGTGCCCCTAA		
AGMI105	TCCCAACCCCTGCAACCACT	53°C	267bp
AGMI108	ATGACCTGTCGAACATCCTTT		

Source: *Kaemmer* et al., (1997)

The reaction volume was 25 µl for each sample. The concentration of template DNA is 50 ng, final concentration in the reaction solution was 1.5 mM $MgCl_2$, 150 µM dNTP, and 0.2 µM of each primer and 0.5 µl of a thermo stable polymerase (Promega; 500 units per 100 µl).

The master mix (all components mentioned above except DNA) was prepared for all the samples to be run at the same time. It was then transferred into separate PCR tubes. Lastly, the genomic DNA for each sample was transferred in different PCR tubes.

Amplification of all samples was carried out using a thermocycler (Ependorf. AG 22331 Hamburg).

PCR was performed at 94°C for 4 min as the initial denaturing steps, followed by 35 cycles of 30 seconds 94°C denaturing, 30 seconds at annealing temperature, T_a depending on the primer pair and finally 30 seconds 72°C extension and a final extension at 72°C for 10 minutes.

After PCR, all products were analyzed by small sized polyaclrylamide gel electrophoresis. All positive products were then kept at –20°C and later run on large gels for further analysis. Negative products were repeated by PCR with same primers together with positive control under the same conditions and run again to reconfirm the amplification process.

3.3.3 Optimization

Primer sets required optimization due to unexpected negative results from all PCR reactions under standard condition as recommended by Kaemmer *et al.*, 1997.

The annealing temperature was set in a gradient between 55°C to 65°C and analyzed by using small sized polyacrylamide gel electrophoresis. The PCR products at each temperature were compared and the appropriate annealing temperature was selected for further use.

3.4 Polyacrylamide Gel Electrophoresis (PAGE)

3.4.1 Preparation of Gel

Special equipment

- Electrophoresis power supply
- Gel plates and spacer for vertical gel, washed with detergent
- Vertical gel apparatus
- Comb (15 well)
- Thermal circulator

All equipments were from Bio-Rad (Protein Xi)

Reagents

- 30% bis-acrylamide solution (35:1)
- 10X TBE buffer
- Urea
- 10% w/v ammonium persulfate
- Acrymide gel loading and denaturing solution (stop mix), containing:
    1. 95% formamide
    2. 0.05% xylene cynole
    3. 0.05% bromophenol blue
    4. 12.5% sucrose
    5. 10mM NaOH
- TEMED

In this study, 3 different concentrations of 8M urea-polyacrylamide gels, 6%, 7% and 8% were used for different primer sets depending on the allele sizes.

For each 40 ml of gel mix:

- 30% bis-acrylamide solution

6%	7%	8%
8 ml	9.3 ml	10.7 ml

- 10X TBE 4 ml
- Urea 19.25 g
- 10% Ammonium per sulfate (APS) 330 µl
- TEMED 33 µl
- Water to adjust the volume

Firstly 40 ml the gel mix containing 30% bis-acrylamide solution, 10X TBE, urea and water was prepared, this amount was sufficient to fill the gap between the gel plates, immediately before addition of the gel mix into the chamber, 330 µl of ammonium persulfate and 33µl of TEMED were added and mixed gently by swirling. The mixture was dispensed into the chamber using a 5ml micropipette. Then the comb was inserted into the top of the chamber and the gel was allowed to polymerize for 1 hour.

After polymerization, the bottom spacer and the comb were removed. The gel was then mounted in the electrophoresis apparatus connected to the thermal circulator with the temperature set at 55°C. The tank and the upper part of the gels were filled with 1X TBE buffer to provide the electric connection through the whole system.

3.4.2 Preparation of Samples and The Running of PAGE

Before gel analysis, a 1:3 volume "stop mix" was added to the sample and the mixture was denatured for 3 minutes at 94°C. Immediately after denaturation, the samples were kept on ice.

Polyacryamide gels were pre-run for 30 minutes at 55°C and at 220V. After pre-running, each sample was loaded into a different well. Molecular weight marker, which was used, was100bp DNA ladder (Promega). The gel was run for 2.5 hour at 210V and 55° C. According to *kaemmer*'s paper, the amount of 8% urea-polyacrylamide gel was used for primer set AGMI 9/93, 7% for AGMI 10/103 and 6% for primer set AGMI 105/108.

3.4.3 Silver Staining of Denaturing Polyacrylamide Gels

Silver staining of denaturing polyacrylamide gels will then be done. After electrophoresis, the gel was soaked in a fixing solution containing 5% acetic acid and 10% ethanol for 20 min, washed once for one minuet in distilled water and stained for 30 min in 0.2% $AgNO_3$. After a brief rinse in distilled water, signals were developed by soaking the gel in solution containing a volume (250ml) of 3% NaOH and 3 ml of 38% formaldehyde. Gel was then rinsed and photographed by Image Kit Digital- Alpha 2200.

3.5 Data Analysis

3.5.1 Chi-Square Test (χ^2)

Statistical analysis was conducted for all data. χ^2 test was used to determine the significance level for existing specific suitable allele according to the primers.

The χ^2 test was carried out using the formula listed below:

$$\chi^2 = \sum \frac{(\text{Obs} - \text{Exp})^2}{\text{Exp}}$$

Obs = Observed frequencies **Exp = Expected frequencies**

The significance level used was P = 5%

3.5.2 Polyacrylamide Gel Analysis

High intensity and highly reproducible bands resulting from the electrophoretic separation of amplified product for each sample were recorded.

The banding patterns of each sample were recorded base on the presence or absence of bands. Individual PCR products detected for each primer set, each corresponding to an allele. Then the results of scoring of the bands were represented in a table for each primer.

3.5.3 Combination of Data

The data from chi-square test and polyacrylamide gel analysis were combined. The relationship between the source of samples and their banding pattern for each individual and primer was then determined.