Chapter Five

Discussion

5.0 Discussion

5.1 STMS General Discussion

If Short Sequence Repeat (SSR) loci are cloned and sequenced, primers to the flanking region can be designed to produce a Sequence-Tagged Microsatellite Site (STMS). Each primer pair identifies a single locus. However, because of the high mutability of SSR loci the primers may amplify different size in number of alleles. Table 5.1 gives a comparison between different DNA markers.

It has been shown that STMS markers have special characteristics such as locus specificity, potential to amplify multiple alleles and co dominant nature. Further more their transferability makes STMS markers a powerful tool for genetic mapping, diversity analysis and genotyping. These markers were also chosen because they have been successfully used for wheat, barley and rice and also, for *Cavendish* banana and recently been successfully utilized in an undergraduate project for analysis of somaclonal variation in *Mutiara* banana, a variant of *Rastali* banana. (R.Y. Othman pers.comm.).

In this study, banana samples were analyzed using STMS primer pair: AGMI 9/93, AGMI 10/103 and AGMI 105/108. The studied primers had been developed by Kaemmer *et al.*, (1997), thus the major expenses for utilizing STMS is only limited to running cost.

Table 5.1: A comparison of the main feature of different DNA markers techniques.

Feature	RFLP	MAAP	AFLP	STMS	Sequencing
Development cost	Medium	Low	Low	High	High
USD/sample	100	None	None	500	500
Samples/day	20	50	50	50	20
Level of skill required	Low	Low	Medium	Low-Medium	High
Radioactive necessary	Yes-No	No	Yes-No	Yes-No	Yes-No
Reliability	High	Low-Medium	High	High	High
Dominant or Co-dominant	Co-dominant	Dominant	Dominant	Co-dominant	Co-dominant
Poly morphism	Medium	Medium	Medium	High	Medium-low

Source: Karp (1997).

RFLP : Restriction Fragment Length Polymorphism

MAAP : Multiple Arbitrary Amplicon Profiling

AFLP : Amplified Fragment Length Polymorphism

STMS : Sequence-Tagged Microsatellite Site

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5.2 Discussion Based On Results

5.2.1 Extraction of DNA

The DNA extracted from leaves was quantitated using spectrophotometer (Eppendorf AG 22331Harmburg) and then diluted to get final concentrations of 50 ng/μl.

The data showed that the ratio of (260nm/280nm) values were almost between 1.8-2.0. This is the desired ratio and indicates low protein and other substance contaminations in the DNA preparations.

5.2.2 Optimization of PCR and Gel Electrophoresis

Condition for primer set AGMI 9/93, AGMI 10/13 and AGMI 105/108 had to be optimized due to the unsatisfactory initial screening result using small sized polyacrylamide gel electrophoresis after PCR. The annealing temperatures, which were chosen after optimization were different from that recommended by Kaemmer *et al.*, (1997). Different lab conditions such as different type of PCR machine, room temperature, chemicals, PCR tube etc. will cause this variation. Hence, optimization may be required for every primer pair used.

Due to unsatisfactory initial screening results, the lower percentage of gels from what has been suggested in Kaemmer's paper (1997) for each length of fragment (6% instead of 7% and 7% instead of 8%) was determine if better separation or clarity could be achieved, but the result was not very satisfactory. Although the mobility of the products would be faster by decreasing the percentage of the gel, comparison of resolution showed that the higher percentage of polyacrylamide gel would give a better separation. This could be because of different mobility of each fragment in different concentration of gel.

According to the previous studies, the electrophoresis was carried out in 210 Volts, 400 Ampere for 2.5 hours (Kaemmer *et al.*, 1997). It was found in our study that, a longer running period up to 3-3.5 hours with the same amount of voltage and ampere, would give a better separation and results that are more desirable. This is excluding pre-running of the gel, which was in the same voltage and Ampere condition, but for 30 min.

Pre-running the gel was to apply the equal temperature conditions to the whole gel, otherwise the bands will appear unclear due to its effect on the samples mobility.

Another technical problem was with breaking of the glass plates during electrophoresis of the big gels due to high temperature. Overcoming the problem achieved by letting the TBE buffer to cover the whole surface of the glasses during the running period instead of covering half of the glass or less which usually applied before. In this way, an equal temperature condition would apply throughout the surface of the glasses.

Although polyacrymide gels needs many handling procedure, takes long time to be prepared, and cast, run and its neurotoxicity required it to be handled with care, a high intensity and highly reproducible bands resulting from the electrophoretic separation were recorded all the time and the diffusion rates of polyacrylamide gel was very low. Moreover, silver staining technique is much more sensitive compared to other methods like ethidium bromide. These reasons are encouraging enough to utilize the polyacrymide as an ideal choice in this study. It is believed that the sequencing gel method is more useful and sensitive although it was rejected here due to its high cost and high level of skill required.

After electrophoresis, gels were stained in three steps, as mentioned in material and method chapter.

Although most of the staining reagents could be reused with less loose of equality for a few times, in this study fresh reagents were used for each staining stage. A better and clearer result was achieved from freshly prepared solutions especially in the developing stage. All the solutions were kept and used at 4°C. It was found that low temperature of the developing solution would result in clearer bands and faster appearing comparing to result of a solution at higher temperature.

5.2.3 STMS Analysis

Based on most published researches three alleles have been detected for each primer set. All three alleles conformed to the equilibrium distribution of genotype (Hardy-Weinberg Equilibrium).

From the chi-square test results, two of three primer sets produce high levels (≥ 50%) of heterozygocity (AGMI 10/103 & AGMI 9/93) however; the level of heterozygocity for AGMI 105/108 appears to be very low. This shows that this primer may not be useful for examining population diversity.

It was noticed that for all three primer sets, *Textilis* showed a different pattern. Although the bands appear to be at the defined area but a small differences between the exact locations of the band representing *Textilis* was observed consistently (arrows in Figures 4.3 and 4.5). This was expected because *Textilis* are completely different species from the other samples, which was examined in this study; *Musa Textilis* is a species from *Australimusa* whereas all other samples come from *Eumusa, which* is another section in genus *Musa* division. The only suspicious case, which shares the same allele with *Textile*, was *Musa 133* in PCR amplification using primer AGMI 9/93 and this band could be considered as shadow band or could be created through the possibility of contamination or repetition due to thawing of the primers or samples.

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In general, STMS analysis in this study is not clearly implied judging from the resulting banding patterns. The inconsistency of banding patterns produced could be cased by some technical problems in the preparation of electrophoresis materials. For instance, the thickness of the gel used in this experiment was 1.5 millimeter, which is most suitable for protein analysis. However considering that this work dealt with DNA, it is recommended to use a gel thickness of one millimeter. Due to unavailability of specific page electrophoresis set for DNA analysis, thickness of 1.5 had to be used as substitute. Besides the 100 base pair ladder used is not suitable in this experiment as a smaller range (ex. 50 base pair) is preferred.

Some triploid genotypes presented more alleles than expected for microsatellite loci. Crouch *et al.,* (1999a) had also detected an unexpected higher number of alleles amplified from the diploid genotype (AA type), the triploid plantain (AAB type) and their progeny, and proposed that a high frequency of duplicated alleles or duplicated chromosomal region may occur in genomes A and B of *Musa*. Silvana *et al.,* (2003) also reported the same extra bands observed in their study with cultivars banana.

It was observed that most of the AAA type genome varieties showed a similar banding pattern; Valery, Giant Cavendish, Chinese Cavendish and Pisang Masah Hijau, all belong to the Cavendish subgroup derived from *Eumusa* section of genus *Musa.* However, Novaria and Robusta, which also come from the same subgroup, were very different. Although in other study in Brazil, Cavendish subgroup exhibited 100% similarity (Creste, s. *et al.,* 2003).

It was also observed that there is a band, which appeared only in the varieties containing the B group genome (ABB, AABB) but was absent in the pure A genomic varieties (AA, AAA)(Figure 4.4.).

Although BC1, BC3 and W.Johol are all wild type banana and BC 1 and W.Johol are both known as *Musa acuminata ssp. Malacensis,* in one case with STMS 9/93, no similar banding pattern were observed.

In general, it was not possible to come to any conclusion on the relatedness of the sample tested due to the small number of alleles generated. For instance Kaemmer *et al.*, (1997) used 69 accessions to test microsatellite markers. Also in other study cases, Silvana *et al.*, (2003) and Coto, O. *et al.*, (2002), each work at least with 35 samples for banana and sugarcane respectively.

However, the results showed that it was possible to differentiate between some varieties using the three primers studied while several alleles were common to many of samples. Much more primers need to be used and analyzed before any phylogenetics relationships can be inferred. However, the study showed the potential of such an approach for studying diversity in bananas.