GENETIC CHARACTERIZATION OF CULTIVATED BANANAS AND PLANTAINS *(Musa ssp)* IN KENYA

BY

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Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Botany, Faculty of Science at Kenyatta University

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DECLARATION

I Justus Mong'are Onguso do hereby declare that this thesis is my own original work and has not been submitted for a degree in any other university.

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We do confirm that the work reported in this thesis was carried out by the said candidate under our supervision and has been submitted for examination with our approval as university supervisors.

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DEDICATION

To my Parents Mr. Johana Onguso and Mrs. Alexina Mokeira Onguso. Secondly to my eldest brother Samwel Onguso, my wife Callen, son, daughter, other brothers and sisters for their love and support.

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ABSTRACT

Bananas and plantains (*Musa ssp*) are a major staple food for millions of people in the tropical world. World banana production has been estimated at 80.6 million tonnes (FAO, 1994) of which only 15% is exported. The dessert banana is a major export crop of the Caribbean countries where the Cavendish bananas account for 10% of the world's production of bananas and Plantains. In the East African highlands, the annual per capita consumption is about three hundred kilogrammes, the highest consumption figure in the world.

In Kenya, banana and plantain nomenclature is not standardized. This is because different communities refer to the local cultivars by different names. The conventional method of describing *Musa* germplasm is based on fifteen morphological traits. The field testing requires elaborate experimentation and one or two years for the plant to flower and fruit. However, the subjective nature of the scoring process makes it prone to error (Bhat *et al.*, 1995). Environmental effects on gene expression make direct comparison of clones growing in different locations impossible.

Cytoplasmic genetic effects on morphology may be an additional factor contributing to ambiguities encountered in the results of key -derived genomic classification. The extent to which cytoplasmic genetic effects confound efforts to differentiate clones based upon their morphological characteristics, especially within the hybrid group AB, ABB and AAB, has not been systematically examined.

In this study, a polymerase chain reaction (PCR)-based approach, namely, random amplified polymorphic DNA (RAPD) was used to estimate the genetic relationships of twenty selected banana cultivars from different regions of Kenya. Nineteen random primers were used. The polymorphism in PCR amplification products were scored as discrete variables. The data was used to calculate Jaccard's similarity coefficients. The similarity measures were subjected to cluster analysis based on the unweighted pair group method analysis (UPGMA) for classification of the cultivars.

Cluster analysis separated the twenty cultivars into three clusters. The first cluster consisted of Ng'ombe, Nusu Ng'ombe, Ntobe, Sialamule, Kimuga, Kibuzi, Sibusi, Bukamba, Mfupi and Gitigi. They were of the genome AAA. The second cluster had Manyatta, Mokoya, Murure, and Horn plantain whose genome was AAB. The third cluster consisted of Pekera, Muraru, Spambia, Matumbo, Nyar Sausett and Mtama whose genome was AA. The cultivars that clustered together were thought to be similar and distinctly different from those that clustered far away from them. The study revealed that RAPD analysis is useful for clonal identification. This is very important for researchers who do *Musa* germplasm classification and management.

It is recommended that further work be done using more cultivars and more specific molecular markers such as restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs) or simple sequence repeats (SSRs).

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Abbreviations

AFLP	Amplified fragment length polymorphism
cDNA	Random complementary DNA
CTAB	Cetyltrimethylammonium bromide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleaic acid
DSCP	Double strand conformational polymorphisms
EDTA	Ethylenediaminetetraacetic acid
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KARI	Kenya Agricultural Research Institute
PCO	Principal coordinate analysis
PCR	Polymerase chain reaction
RAHM	Random amplified hybridization microsatellites
RAMPs	Random amplified microsatellite polymorphisms
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RPM	Revolutions per minute
SSCP	Single strand conformational polymorphisms
SSRPs	Simple sequence repeat polymorphisms
TAE	Tris acetate EDTA
TE	Tris-EDTA
TNE	Tris sodium EDTA
UPGMA	Unweighted pair group method analysis

VNTR	Variable number tandem repeats

CHAPTER 1

1:0 INTRODUCTION

1:1 Background

Bananas and plantains are major staple foods for millions of people in the tropical world. World banana production has been estimated at 80.6 million tonnes (FAO, 1994) of which only 15% is exported. In the East African highlands, the annual per capita consumption is about three hundred kilogrammes, the highest consumption figure in the world. The dessert banana is a major export crop of the Caribbean countries where the Cavendish bananas account for 10% of the world's production of bananas and Plantains (Daniells, 1990).

Bananas constitute the fourth most important starchy staple after cassava, sweet potatoes and yams (Table, 1). It is estimated that over 70 million people subsist on this crop (INIBAP, 1994). For many countries the banana is an important import substitution crop without which the producer countries would have to import food.

Table 1: Production levels of some major starchy staples (million metric tonnes) FAO, 1994

Region	Cassava	Yam	Sweet potato	Cooking bananas
Africa	72.8	29.1	6.9	20.7
Asia	48.4	0.2	1.1	0.8
South America	30.1	0.3	1.2	5.6
Central America	1.0	0.4	114.3	1.6
World total	152.3	30.1	124.5	29.7

Bananas include diverse types such as dessert, cooking, roasting and beer bananas. Dessert bananas are those bananas consumed raw at ripeness and are usually distinguished by the sweet flavour of the fresh fruit when ripe. Cooking bananas are consumed when cooked and in much of the world they have always been referred to as plantains (Swennen and Vulysteke, 1987). However, there are two types of cooking bananas. The first type is cooked when the fruits are green and provides a starchy staple nutritionally similar to potato (Simmonds, 1966). Bananas of this type can be allowed to ripen and then eaten as dessert bananas. For this reason they can not be called true plantains. The second type of cooking banana is unpalatable raw even when the fruits are ripe and therefore requires cooking before being consumed. These are the true plantains (Swennen and Vulysteke, 1987). The beer bananas are those whose pulp is bitter and astringent. They can be eaten neither raw nor cooked. However, juice and alcohol can be made from this type of bananas, hence the name.

Cultivated bananas and plantains (cultivars) belong to the Eumusa section of the family *Musaceae*. They are natural polyploids (diploids, tripolids or tetraploids) of two wild seedy species of *Musa*: *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). While spontaneous polyploidization within *M. acuminata* formed the triploid (AAA) cultivars, the natural interspecific hybridization of both species formed plantains (AAB) and cooking bananas (ABB) characterized by starchy fruits and the diploid (AB). The latter hybridization occurred when the diploid (AA) and triploid (AAA) *Musa acuminata* cultivars were taken by man to areas where *M. balbisiana* is native (Simmonds, 1962). The resulting genome groups were classified as AA, AB, AAA, AAB, ABB, AABB, AAAB with the letters A and B representing the contributions of *M.*

acuminata (2n=22) and *M. balbisiana* (2n=22) respectively. Domestication of edible forms was associated with the emergence of sterile parthenocarpic types, which have been selected and propagated vegetatively. Somatic mutation that occurred during long-term asexual propagation represents an additional source of genetic variation for the development of new clones (Karamura, 1998).

1:2 Uses and composition

The cultivation of bananas in East Africa is a key component in both the food security and the agricultural sustainability of the region. It has an extended harvest period which ensures food and income throughout the year. In addition, farmers in the tropics can intercrop bananas with legumes and can feed animals on by-products (peels and pseudostems) of the crops.

Bananas can be eaten as a dessert fruit, when cooked or roasted. In Uganda, large quantities of beer are brewed from *Musa.* Other products derived from *Musa* are figs (dried slices of ripe bananas) powder grinded from unripe fruits, flakes, juices and purees. In parts of Asia, male parts are eaten as vegetables. Green leaves are often used as umbrellas, plates or wrapping fibre (made from *Musa textilis*). Dry leaves can be used for making bags, ropes, baskets, carpets, shoes and a host of indoor decorations (Price, 1994).

The highland bananas reduce soil erosion on steep slopes and are principal sources of mulch for maintaining and improving soil fertility (INIBAP, 1986). The leaves as well as stems rot as they are being broken down by micro-organisms which gives good aeration to the soil and this also adds organic matter to the soil.

The pulp of the ripe banana contains about 70% water and for the rest mainly carbohydrates. One hundred grams of banana produce 100 calories. There is a little fat and protein but it is a fairly good source of vitamin A, B_1 and C.

1:3 Origin and distribution

Banana and plantains belong to the genus *Musa* whose centre of origin is South East Asia, stretching from India to Papua New Guinea and including Malaysia and Indonesia (INIBAP, 1998). The first and probably the most crucial step in the evolution of edible bananas was the development, and, subsequently, the selection of parthenocarpy and seed sterility in *Musa acuminata,* giving rise to the edible diploid (AA) cultivars (Karamura, 1998). Long ago some of these species crossed resulting in an increase in female sterility, parthenocarpy and triploidy. Hybridization probably took place repeatedly as the cultivation of edible triploids continued.

Inhabitants of that region then discovered that some of the plants had edible fruits and could be propagated by suckers. Selection by man has, over millenia, profoundly altered the properties of *M. acuminata* in the humid tropics. Meanwhile, plants had also been taken by man to drier monsoon areas where *M. balbisiana* (BB) is native. The two species crossed resulting in the genome groups AB, AAB and ABB. There are a few edible forms of *M. balbisiana.* The *M. balbisiana* confers hardness and drought resistance to its hybrids. The hybrids extended their geographical ranges from the wetter tropics to the seasonally drier zones. At present there are many clones of cultivated bananas in existence, belonging to different genome groups, of which AA, AAA, AAB and ABB are the most numerous (Karamura, 1998).

Malayan sailors probably took bananas to Madagascar about the fifth century AD. The bananas then spread to the East Coast and mainland of Africa from there. The two main centres of banana production in Africa are the wet tropical zones of West and Central Africa and the East African Highlands. The East African Highlands produce distinct types of bananas which are thought to have been introduced between the 5th and 10th centuries and a wide range of unique varieties now exists in this region (INIBAP, 1998)

In Uganda, the crop is a staple food in all parts that do not experience a pronounced dry season, i.e. the area within about 80km of the shore of Lake Victoria. It is also important in the South Western highlands, the slopes of Mt. Elgon in the East and well watered areas of the Western part of the country. In Tanzania, bananas are a staple food in the high rainfall areas, located in the higher altitudes and low lying places around Lake Victoria in addition to valley bottoms, where they are a major constituent of the local diet (Acland, 1971; Ngeze, 1994). In Kenya, bananas are widely grown in the Western part, in areas surrounding Lake Victoria, on the slopes of Mt. Kenya and along the coast. Maize, however, is the staple food in these areas (Baker and Simmonds, 1952; Sebasigari; 1987).

1:4 Cultivars

The East African bananas, whose names are too numerous to catalogue, have evolved to suit the local environment. They can be grouped into three categories: dessert bananas, plantains and the East African Highland (cooking and beer) bananas. East

Africa to the West and North of Lake Victoria is regarded as a secondary centre of diversity for bananas (Simmonds, 1966).

Lack of clear clone identity in the crop has resulted in unnecessary duplication with regard to collection, conservation and research (Karamura, 1998). For instance, in Uganda banana germplasm offers challenges in that, since there is no test for clonal identity, many clones may have been collected several times under different names because there is a long list of different vernacular names attached to each clone (Kyobe, 1981; Karamura and Karamura, 1994). Some names may be synonyms due to the many languages spoken, others may just refer to environmental effects on the phenotype of the crop or somatic mutations. Close genetic relationships among cultivars as well as frequent spontaneous somatic mutations and the instabilities generated during *in vitro* culture (somaclonal variation) contribute further major obstacles to the correct identification of a clone. The detection and elimination of clonal variation represents an essential step in the application of micropropagation of true-to -type *Musa* clones for *in vitro* germplasm conservation and planting of high quality material (Daniells, 1997).

Traditionally, characterization and classification of bananas has been accomplished by use of morphological descriptors (Stover and Simmonds, 1989). This method is based on comparing genetic material by visible phenotypes such as fruit colour or on measured traits such as yield. Although classical phenotypic features are still extremely useful, the efficiency of selection may be reduced by age, developmental stage or environmental effects on measured traits. Simmonds and Shepherd (1955) gave a list of 15 characters used in scoring of *Musa* cultivars. For each character corresponding with the wild *M. acuminata* a score of 1 was given; characters corresponding to *M.*

balbisiana got a score of 5 and intermediate cultivars were given intermediate scores. Thus, a cultivar got a total score of at least 15 and at most 75. The AA and AAA cultivars obtained scores of 15-24, AAB cultivars 25-54 and ABB 55-64.

Simmonds and Shepherd's (1955) system of *Musa* classification permits the grouping of cultivars based upon the relative genetic contribution of *Musa acuminata* (A) and *M. balbisiana* (B) to the genome of any given cultivar. However, the subjective nature of the scoring process makes it prone to error (Bhat *et al.*, 1995b). Environmental effects on gene expression makes direct comparison of clones growing in different locations impossible (Swennen, 1990)

Due to the subjective nature of Simmonds and Sherperd's classification, scientists are now complementing this classification with molecular techniques to characterize *Musa* groups (Bhat *et al.*, 1995b). Genetic characterization is essential for scientifically sound germplasm conservation.

1:5 Statement of the problem

In Kenya, bananas and plantains are widely confused in different growing areas. It is common to find the same local banana or plantain having been assigned different names in different regions. It is difficult to differentiate the bananas from the different regions. Traditionally, characterization and classification in *Musa* germplasm has been accomplished by use of morphological descriptors. These descriptions are greatly influenced by the environment and are subject to individual bias. Genetic characterization is therefore the only sure way of differentiating between the available banana cultivars and establishing genetic relationships within the cultivated germplasm (Bhat *et al.*, 1995a). A good understanding of genetic relationships is also critical for the effective organization and management of a *Musa* germplasm collection.

1:6 Justification

This research was based on the already existing banana project on mass propagation of clean banana seedlings in the Institute for Biotechnology Research of Jomo Kenyatta University of Agriculture and Technology. Local banana cultivars are sourced from different regions for *in vitro* multiplication in the laboratory. Since different communities refer to them by different names, it is very difficult to establish how to handle these materials for mass propagation. This is because different cultivars require different protocols. It is very expensive and time consuming for a cultivar to go through a multiplication process only to turn out to have been the wrong one. This usually happens with the cooking types. In the East African Highlands, the farming community complicates the banana nomenclature by conferring local names (in local dialects) which has resulted in over 300 synonyms for banana cultivars. Neither the farmers in the next village, nor researchers, extension workers or the international community know the synonyms among these many. Yet, scientists need to use the same name for the same genotype to enable them to publish results which can be compared with results obtained by others working on the same crop (Karamura, 1998).

1:7 Research Hypothesis

1. All the cooking bananas have the same genetic make up.
2. All the ripening types have the same genetic make up.

1:8 Objectives

1. To determine determine the optimal conditions for the polymerase chain reaction.

2. To characterize the locally cultivated bananas and plantains using different random primers.
3. To determine the genetic relationships among locally cultivated bananas and plantains.

CHAPTER 2

2:0 LITERATURE REVIEW

The genus *Musa* consists of edible bananas and plantains that are among the major fruits of the tropical countries. *Musa* consists of the sections Australimusa (chromosome number, $n = 10$), Callimusa ($n=10$), Eumusa ($n=11$) and Rhodochlamys ($n=11$). The common edible bananas and plantains belong to the section Eumusa and its members are distributed from India to Japan and Samoa (Bhat *et al.,* 1995b) This section comprises of nine to ten species and includes fruit, fibre and vegetable yielding plants. Under this section, *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) are of great economic importance (Bhat *et al.,* 1995a).

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Cytoplasmic genetic effects on morphology may be an additional factor contributing to ambiguities encountered in the results of key derived genomic classification. The extent to which cytoplasmic genetic effects confound efforts to differentiate clones based upon their morphological characteristics, especially within the hybrid group AB, ABB and AAB, has not been systematically examined (Jarret *et al.*, 1993).

Spontaneous somatic mutations in banana are of great agricultural significance in giving rise to several clonal forms of important cultivars. Due to extensive proliferation of numerous mutants, however, the identification of original parental forms available in India, Hawaii and East Africa has not been easy (Stover and Simmonds, 1989). The cultivar identification on the basis of morphological traits is time consuming and is not always accurate because the system is subjective (Bhat *et al.*, 1995b).

Due to the subjective nature of Simmonds and Sherperd's (1955) classification, scientists are now complementing this classification with molecular techniques to characterize *Musa* groups. Genetic characterization is essential for scientifically sound germplasm conservation. Characterization permits the estimation of genetic relatedness and diversity. Traditionally, characterization and classification of *Musa* germplasm has been accomplished by the use of morphological descriptors (Simmonds and Shepherd, 1955, IBPGR, 1991).

Several molecular techniques have been used for *Musa* germplasm characterization and classification. These include: diversity for anthocyanin content and profile (Horry and Jay, 1988), enzyme polymorphisms (Bonner *et al.*, 1974; Jarret and Litz, 1986; Bhat *et al.*, 1992), rRNA spacer-length heterogeneity (Lannaud *et al.*, 1992), chloroplast DNA polymorphisms (Gawel and Jarret, 1991) restriction fragment length polymorphisms (RFLPs) (Jarret *et al.*, 1992; Bhat *et al.*, 1995b) and variable number tandem repeats (VNTRs) (Weising and Langridge, 1991; Kaemmer *et al.*, 1993).

2:1 The polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique for *in vitro* amplification of specific DNA sequences by simultaneous primer extension of complementary strands of DNA. The PCR method first described by Mullis and Faloona (1987) involves three steps: denaturation, primer binding and DNA synthesis. Target duplex DNA is first denatured followed by the hybridization and annealing of two oligonucleotide primers that flank the specific DNA segment. Each primer hybridizes to one of the two separate strands such that the 3'OH ends are facing each other. The annealed primers are then extended (5'-3') on the template strands by a DNA polymerase (Taylor, 1991). These three steps constitute a PCR cycle.

Repeated cycles generate an exponential accumulation of multiple copies of DNA fragments approximately 2^n where n is the number of cycles. The exponential amplification arises because under appropriate conditions, the primer extension products synthesized in cycle n, function as templates for other primers in cycles n+1 (Erlich and Arnheim, 1992). Requirements for the reaction include; deoxyribonucleotides and nucleosides for DNA synthesis, primer, DNA templates, DNA polymerase and buffer containing magnesium (Wachira, 1996).

The efficiency of PCR has been greatly improved in the advent of identification of thermostable DNA polymerases like taq polymerase from the bacterium *Thermus aquaticus* which is used extensively (Saiki *et al.,* 1988). The use of such thermostable DNA polymerases has eliminated the need to add fresh enzymes after the denaturation step which is characterized by high temperature (90-95°c) in the thermal profile. In addition, the high temperature optimum of this enzyme (70-75°c) significantly increases the yield and length of targets that can be amplified.

The PCR method has been automated and thermal cycling blocks are now widely used. Coupled with this automation, the use of high temperatures for primer annealing and extension has significantly increased the specificity (target vs non-target product) of the PCR technique (Erlich and Arnheim, 1992). It has found a wider use as a tool for genetic analysis because it takes a comparatively shorter time than southern blotting. It requires extremely small quantities (nanograms) of crude DNA for amplification, and its products can be visualized on ethidium bromide stained agarose gels (Orrego, 1990).

2:2 Restriction fragment length polymorphism (RFLP)

Another method that could be used to characterize *Musa* groups is restriction fragment length polymorphisms (RFLPs). Natural variations in DNA sequence can be detected in several ways. One way can be to directly sequence the DNA and make detailed comparisons between sequences of two test specimens but this method is laborious and time consuming. Genetic variability can however be readily assessed using restriction enzymes. These are nucleases produced by a variety of micro-organisms that cleave DNA at particular restriction (target) sites made up of specific base sequences in the DNA.

Restriction enzymes digest and reduce large pieces of DNA to a series of defined size. Since the number and position of the restriction sites could be specific to a group of individuals, the number of fragments produced and the size of each fragment will reflect the genetic variation within groups of individuals. Thus, differences in fragment size and number (RFLP) arising from restriction digestion of nuclear, organeller, or total DNA, are called restriction fragment length polymorphisms (RFLP) and can be used as a fingerprint for a given target DNA or as a direct measure of variability.

Grozdicker *et al.*, (1974) used fragment length polymorphisms generated after digesting adenovirus DNA with restriction endonucleases to characterize mutant isolates that exhibited a temperature sensitive phenotype. RFLPs have been demonstrated to be ubiquitous and exceedingly numerous in comparison with all other genetic traits (Beckmann and Soller, 1986). The DNA RFLPs can be treated as any other Mendelian phenotypic trait and alleles are usually codominant. They can be mapped by standard genetic crosses in much the same way that differences in protein electrophoretic mobility have been used to show the loci coding for the proteins (Beckmann and Soller, 1986).

However, certain requirements must be met for the successful detection of RFLPs. DNA from genetically distinct individuals are digested with a restriction enzyme and separated by gel electrophoresis. The DNA is then transferred to a membrane by Southern blotting (Southern, 1975) and hybridized to a radioactively labeled DNA probe (Powell, 1992).

2:3 Random complementary DNA (cDNA)

Another class of clones, the random complementary DNA (cDNA) have been shown to detect significantly more variations than random genomic clones selected on the basis of hypomethylation. Random complementary DNA (cDNA) clones are derived from gene transcripts that are good sources of single copy clones. This is a contrast to hypomethylation derived clones which are not always coincidental with coding regions since surrounding regions to coding genes may also be undermethylated. It has been observed that cDNA clones detect significantly larger genomic restriction fragments (Wachira, 1996).

2:4 Amplified fragment length polymorphism (AFLPs)

The amplified fragment length polymorphism (AFLP) is a technique that combines some features of RFLP and PCR technologies. The products generated are, however, inherited in a dominant manner and polymorphism is dependent upon the random arrangement of specific sequences adjacent to restriction sites (Zabeau and Voss, 1993). AFLP is a highly informative assay because high levels of polymorphism are often inherited in the random placement of restriction sites between the different genomes. The nucleotide sequence variability within a short stretch of DNA flanking these restriction sites is easier to detect. In a comparative study evaluating the potential utility of several molecular marker systems, Powell *et al.,* 1996) demonstrated that AFLPs had the highest information content or effective multiplex ratio when compared to RFLPs, RAPDs and SSRPs (simple sequence repeat polymorphisms). Depending on the reaction conditions, it is possible to simultaneously display more than 100 loci per gel lane, a high proportion of which may be polymorphic between genomes (Draaistra *et al.*, 1995).

Despite its high information content, the utility of AFLP markers has not yet been widely reported in literature though it is likely that its use will become widespread as can be inferred from the few reports made on it so far (Draaistra *et al*., 1995, Thomas *et al*., 1995).

2:5 Simple sequence repeat polymorphisms (SSRP)

These are also known as microsatellites and consist of stretches of tandemly repeated mono-, di-, tri-, tetra-, penta- or hexa nucleotide motifs. Such repeats are extremely abundant and randomly distributed in a eukaryotic genome (Rogstad *et al*., 1998).

Although a function has yet to be determined for such non-coding tandemly repeat DNA families, they are often associated with high levels of polymorphism in both animals (Weber, 1990,) and plants (Thomas *et al*., 1995). They are co-dominant markers and coupled with their high information content (Powell *et al.,* 1996) will become popular genetic markers.

2: 6 Random Amplified Hybridization Micro-satellites (RAHM)

Efforts to develop the use of micro-satellite markers can be quite laborious, requiring library construction, screening and sequencing of clones. A new system for micro-satellite DNA detection and isolation which combines RAPD amplification and oligonucleotide screening has been developed (Richardson *et al*., 1995).

The technique is termed as random amplified hybridization micro-satellites (RAHM) or random amplified micro-satellite polymorphisms (RAMPs). It is based on prior RAPDs amplification of plant genomic DNA following standard procedures, transfer of amplified DNA (on a gel) onto Hybond N+ filters using southern blot procedures (Southern, 1975) and hybridization with labelled oligonucleotides probes carrying simple sequence repeats. The method has been tested on a number of plants which include sugar beet, olive, sunflower and yams. The efficiency of detection of micro-satellite sequences is dependent on their relative abundance. In the tested material several hybridizing bands containing micro-satellite sequences were identified (Richardson *et al.*, 1995)

2: 7 Single strand conformation polymorphism (SSCP)

Detection of mutation is an important aim in molecular biology and genetic research. As sequencing to identify base changes is not always possible, simple procedures for detection of mutations have been devised. These include enzymatic cleavage or denaturing gradient gel electrophoresis (DGGE) and double and single strand conformational polymorphisms (DSCP and SSCP, respectively). Resolution of DSCP and SSCP are based upon secondary and tertiary structural conformation differences in double stranded DNA (heteroduplex analysis) and single stranded DNA (SSCP analysis), respectively (Ohmori, *et al.*, 1995).

Separation of DNA fragments in SSCP analysis is carried out at low temperature on non-denaturing polyacrylamide gels. Resolution of alleles is however often dependent upon electrophoretic conditions. SSCPs and DSCPs have been employed to study point mutations (Sheffield *et al.*, 1993) and short tandem repeats. The former have been used to study phenetic relationships in coffee (Orozco-Castillo, *et al.*, 1995) and genetic differentiation and pedigree relationships in the trees *Gliricidia sepium* (Dawson,1995). The resolution of analysis can however be limited by the size of fragment since the larger the fragment (e.g.>1kb) the more difficult it is to be effectively differentiated (Sheffield *et al.*, 1993).

2: 8 Random amplified polymorphic DNA

Williams *et al.,* (1990) described a method for the identification of genetic polymorphisms in plants based on PCR. The method was based on arbitrary amplification of specific DNA sequences scattered throughout the genome by use of single short (10 mer) synthetic oligonucleotide primers. Sequences amplified yield stable genetic markers.

While classical PCR requires nucleotide sequence information for the design of primers, Williams *et al.,* (1990) demonstrated that priming with random oligonucleotides would give genetic (RAPD) markers. This provided an innovative technology for DNA mapping, fingerprinting and related research (Waugh and Powell, 1992; Tingey del Tufu, 1993). Primers used had no palindromic sequences and had at least 60% G+C content to ensure efficient annealing and were designed so as to reduce internal pairing which can produce PCR artifacts.

The procedure described by Williams and his colleagues ensured that fingerprints upto 2.0 kilobases in length were obtained which would be visualized after electrophoresis and staining with ethidium bromide. The size and number of polymorphic fragments was a function of the sequence variability between termini of primers which resulted in changes in priming sites. Such changes in priming sites could be due to point mutations or changes that modify the size or block the amplification of the target DNA, such as insertions, deletions, and inversions (Wachira, 1996).

RAPDs have been used for clone identification in cocoa (Wilde *et al.*, 1992; Russel *et al.*, 1993), banana (Bhat *et al.*, 1995a, Kaemmer *et al.*, 1993) and population differentiation in nitrogen fixing tree Gliricidia (Chalmers *et al.*, 1992; Dawson, 1995).

They have been used for estimating genetic relationships in coffee (Orozco-Castillo *et al.*, 1994), chinese water chestnut (Li and Mildmore, 1999), genetic mapping of conifers and betula (Carlson *et al.*, 1991) and wild wheat (Fashima *et al.*, 1999).

Random amplified polymorphic DNA (RAPD) has also been used to characterize germplasm in several important crops including papaya L (Stiles *et al.*, 1993), celery (Yang and Quiros, 1993) and mallus (Koller *et al.*, 1993). RAPD analysis is a desirable technique for detection of genetic diversity since it is technologically simple, rapid and does not require use of radionucleotides in contrast to other methods such as restriction fragment length polymorphism (RFLP) or variable number tandem repeats (VNTRs) (Bhat *et al.*, 1995b). The potential applicability of RAPDs for detection of genetic diversity in wild species and cultivars of bananas was demonstrated by Kaemmer *et al.*, (1993).

Several attempts have been made classify *Musa* germplasm using such markers as RFLPs (Gawel *et al.*, 1992, Bhat *et al.*, 1995a), isozymes (Bhat *et al.*, 1992) and anthocyanins or flavinoids (Horry and Jay 1988). These valuable methods may, however, be laborious or expensive (Howel *et al.*, 1994).

Howel *et al.*, (1994) investigated the applicability of RAPD for distinguishing banana cultivars. Using the technique of RAPD, 116 amplification products were identified in *Musa* germplasm using nine primers. This enabled them to identify RAPD markers that were specific to each of the nine genotypes of *Musa* representing AA, AAA, AAB, ABB and BB genomes.

Polymorphisms have been detected by RAPDs at different levels ranging from that between genotypes of cocoa, maize and celery (Wilde *et al.*, 1992; Yang and Quiros,

1993) to varietal differences in barley and soybean (Bhat *et al.*, 1995b). Different species of peanut could also be distinguished using RAPD (Koller, *et al.*, 1993). RAPDs are cheaper than other molecular markers, fast, reliable and use arbitrarily defined oligonucleotide primers that are applicable to serve as " probes" for a wide range of material (Williams *et al.*, 1990).

RAPD analysis has been used to develop genetic maps for plant species (Reiter *et al.*, 1992), to identify molecular markers linked to important genes (Gawel *et al.*, 1992) and identify cultivars of papaya (Stiles *et al.*, 1993). They have also been used to evaluate relationships within onions (Wilkie *et al.*, 1993), lens (Abo-elwalfa *et al.*, 1995) and lentils (Sharma *et al.*, 1995).

Cytoplasmic genetic variability within and between *Musa* genomic groups has been reported (Gawel and Jarret, 1991). Thus, descrete variables such as those generated by RAPD analysis are preferable for clonal identification and classification.

Based upon the clustering relationships of previously unclassified cultivars with characterized ones, it is possible to tentatively assign the unclassified ones to specific genomic groups (Bhat *et al.*, 1995). Similarly, the pattern of variation observed by Howel *et al.*, (1994) following the application of multivariate analyses to the RAPDs banding data was very similar to the variation defined using morphological characteristics and used to assign material into the different genome classes.

RAPD makers are readily detected and analyzed in *Musa* and this technique lends itself to germplasm characterization. RAPD analysis does not require large amounts of DNA and is thus ideally suited for characterization of *in vitro Musa* collections. DNA sufficient for RAPD analysis can be extracted from one or two leaves of *in vitro* plants.

Analysis of *in vitro* plants eliminates the need to grow germplasm to maturity for morphological characterization which in the case of bananas and plantains, requires more than one year (Bhat *et al.*, 1995a).

Since somaclonal variation is common in *Musa in vitro* cultures (Vulysteke and Swennen, 1990) the development of true to type RAPD profiles for principal varieties might be useful in monitoring genetic stability. Bhat *et al.*, (1995a) suggested the use of a few cultivars and species as reference clones for classification of *Musa* germplasm using RAPD analysis. Appropriate cultivars with well characterized RAPD profiles include: *Musa malaccensis* (AA group), Red banana (AAA group), Njali Poovan (AB group), 'Mysore Bale' and 'Agni Rasthali' (AAB group), Bluggoe (ABB group) and Tani (BB group). Their data suggested that these groups were ideal reference cultivars and reflected the major RAPD polymorphisms characteristic of the respective groups identified in their analysis.

CHAPTER 3

3:0 MATERIALS AND METHODS

3:1 Plant materials

Banana suckers of the most popular cultivars were collected by the Kenya Agricultural Research Institute (KARI) from farmers' fields throughout the major banana growing regions in Kenya. The materials were planted at the KARI –Kisii demonstration farm for germplasm conservation and research. The names of the collected suckers were maintained as they were named in the local dialects by the farmers in the various collection areas. The collection areas were: Murang'a, Nyeri, Mtwapa, Kakamega, Embu and Kisii districts. Suckers were requested from the Institute and grown at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) demonstration field to serve as a source of leaf tissue for DNA isolation. The same accessions were also maintained *in vitro* at the Institute for Biotechnology Research Laboratory, JKUAT. The cultivars used in this study, location where collected, their genome, use and morphological characteristics are shown in table 2.

Table 2: *Musa* germplasm analyzed for RAPD genetic markers

No.	local name	Origin	Characteristics	Morphological Classification*
1.	Bukamba	Machakos	-Dark brown pseudostem -Short broad leaves -Small bunch -Small well packed fingers	

		-Cooking type	AAA
2. Gitigi	Murang'a	-Pale yellow pseudostem -Long narrow leaves -Small bunch -Big sparsely packed fingers - For beer making	AAA
3. Horn plantain	Kisii	-Pale yellow pseudostem -Long narrow leaves -Medium sized bunch -Short well packed fingers - Roasting type	AAB
4. Kibuzi	Kakamega	-Dark brown pseudostem -Short thick leaves -Big bunch -Long thickly packed fingers - Cooking type	AAA
5. Kimuga	Murang'a	-Dark brown pseudostem -Long narrow leaves	

		-Big bunch -Big well packed fingers - Cooking type	AAA
6. Manyatta	Rongo	-Dark brown pseudostem -Long broad leaves -Big bunch -Long fingers tapering at the ends - Dessert type	AAB
7. Matumbo	Mtwapa	-Dark brown pseudostem -Long broad leaves -Big bunch -Long fingers tapering at the ends -Cooking type	ABB
8.Mfupi	Mtwapa	-Pale green pseudostem -Short and thin leaves, sharp at the ends -Medium bunch -Short well packed fingers -Cooking type	AAA
9. Mokoya	Kakamega	-Dark brown pseudostem	

		-Long broad leaves -Big bunch -Thick well packed fingers curved at the end -Dessert type	AAA
10.Mtama	Oyugis	-Dark to black pseudostem -Short thick leaves -Medium bunch -Thick well packed fingers -Cooking type	AAA
11.Muraru	Nyeri	- Pale brown to black pseudostem -Long broad leaves, rounded at the ends -Big bunch -Thick well packed fingers -Dual purpose	AAA
12.Murure	Bungoma	- Dark green pseudostem -Long broad leaves -Medium bunch -Short, thick well packed fingers -Cooking type	AAA

13.Ng’ombe	Kisii	- Dark green to black pseudostem -Long broad leaves -Very big bunch -Thick loosely packed fingers -Cooking type	AAA
14.Ntobe	Kakamega	- Dark green pseudostem -Short narrow leaves -Medium bunch -Short well packed fingers -Cooking type	AAA
15.Nusu Ng’ombe	Kisii	- Dark green pseudostem -Short narrow leaves -Medium bunch -Short well packed fingers -Cooking type	AAA
16. Nyar Sausset	Homa Bay	-Pale brown pseudostem -Long narrow leaves -Big bunch -Medium well packed fingers - Dessert type	AAA

17. Pekera	Embu	-Pale green pseudostem -Short broad leaves -Medium bunch -Short well packed fingers -Dessert type	AAA
18. Sialamule	Busia	-Pale brown pseudostem -Long narrow leaves -Big bunch -Thick well packed fingers -Cooking type	AAA
19. Sibusi	Uganda	-Pale green pseudostem -Long narrow leaves -Medium bunch -Short well packed fingers -Cooking type	AAA
20. Spambia	Uganda	-Pale green pseudostem -Long broad leaves -Small bunch -Long sparsely packed fingers	

	-Roasting type	AAB

*Morphological classification by the Kenya Agricultural Research Institute (KARI)

Standard genetically characterized reference cultivars e.g. *Musa macaccencis* (AA), Red banana (AAA), Njali Poovan (AB), Rasthali (AAB) and Tani (BB) were obtained from the National Bureau for Plant Genetic Resources, New Delhi, India. The cultivars were in form of preserved DNA.

The laboratory work was carried out at the National Bureau for Plant Genetic Resources, New Delhi, India.

3:2 Analysis procedure

Ten samples of young unfurled leaves were randomly collected from healthy plants of each cultivar, labeled and transported as accompanied luggage in a cooler box to India. In the laboratory, the material was kept at -80°C and taken out for DNA extraction as required.

3:3 DNA extraction

The general procedure used was that described by Saghai-Maroof *et al.,* (1984). Five grams of clean young leaf tissue was ground to a fine powder with a mortar and pestle after freezing in liquid nitrogen to degrade the cell wall. To remove polysaccharides, the powder was transferred to a 50 ml centrifuge tube with 20ml cetyltrimethylammonium bromide (CTAB) buffer maintained at 60°C in a water bath. The contents of the tube were mixed vigorously for about one minute. The CTAB buffer contained 4M sodium chloride, 1 M Tris HCl, 0.5 M EDTA, 2% 2 Mercaptoethanol and 10% CTAB. The pH of the Tris HCl was adjusted to 8.0 with hydrochloric acid. All the reagents were autoclaved at 121^{0}C for 15 minutes before use. Unautoclaved 2 mercaptoethanol was added just before use.

The mixture was then incubated at 60°C for one hour with intermittent mixing. To remove the lipid bilayer, the tube containing the mixture was then filled with chloroform : isoamyl alcohol (24: 1) and gently mixed by inverting for ten minutes. The mixture was then spun at 17,000 revolutions per minute (rpm) for ten minutes at 25°C. The rotor used was SS34 in sorval RC-5C (Germany) centrifuge.

The upper aqueous phase was transferred to a fresh 50 ml test tube. To this aqueous phase was added 0.7 times volume of isopropanol and mixed gently to precipitate DNA. The DNA was spooled out with a bent pasteur pippet and placed in a 1.5 ml eppendorf tube. To wash off any remaining cell wall and salts, 0.75 ml of 70% ethanol was added, mixed gently and the tubes incubated at 37°C for 30 minutes. This process was repeated twice. The 70% ethanol was finally decanted off and the pellet dried under a vacuum for half an hour to get rid of ethanol and enable the DNA to dissolve in T.E (Tris:EDTA 10: 1). The DNA was then dissolved in 10µl of T.E (10:1) and stored in the fridge (4°C) awaiting purification.

3:4 Purification of the extracted DNA

To 500μl of DNA in Tris- EDTA was added 2μl of Rnase A and mixed well to remove RNA. This mixture was then incubated at 37°C for one hour. To remove the proteins, 3μl of proteinase k was then added, mixed well and incubated at 37°C for another one hour. To remove any organic debris, an equal volume of saturated phenol: chloroform was then added and mixed well. The mixture was then spun at 10,000 rpm for five minutes.

The upper aqueous layer containing the DNA was collected in a fresh tube and an equal volume of chloroform: isoamyl alcohol (24:1) added and mixed gently. The mixture was then spun at 10,000 rpm for five minutes. The upper aqueous layer was collected in a fresh tube. The chloroform extraction was repeated twice.

The upper aqueous layer was collected in a fresh tube, one-tenth volume of 3M sodium acetate of pH 5.2 was added and mixed well. To the mixture of the upper aqueous layer and sodium acetate, 2.5 volume of chilled ethanol was added to precipitate the DNA. The DNA was spooled out with a bent pasteur pippet. The DNA was then placed in a clean, sterile 1.5ml eppendorf tube.

Extra salts were removed by adding 70 % ethanol and allowing the samples to stand for two hours. The treatment was repeated once more.

The pellet was dried under vacuum at –60°C for half an hour. The dry pellet was dissolved in a minimum volume of Tris: EDTA 10:1 and kept at 4°C in a fridge.

3:5 Estimation of DNA concentration in the samples by fluorimetry

3:5:1 Buffer preparation

A tenfold concentration of Tris sodium EDTA (TNE) buffer was prepared by dissolving 12.11g Tris, 3.72g EDTA and 116.89g sodium chloride in 800ml of distilled water. The pH was adjusted to 7.4 with concentrated hydrochloric acid. The final volume was made up to 1000ml with distilled water. The solution was filtered with a watman filter paper No. 42 and stored in the fridge at 4°C.

Low range assay solution was prepared by mixing 10 µl of 0.1 µg/ml Hoest 33258 dye, 10ml of 10x TNE and 90 ml of distilled water. The solution was then stored in the fridge at 4°C.

3: 5: 2 Preparation of the DNA standard

The DNA standard was prepared by mixing 100 µl of 1 mg/ml calf thymus DNA standard, 100µl of 10x TNE buffer and 800 µl of distilled water. The solution was stored in the fridge at 4°C.

A minifluorometer (TKO 100, Hoefer) was turned on at least 30 minutes before using for the lamp to stabilize prior to taking measurements. The scale knob was set fully clockwise.

The working solution was prepared by taking 200ml of 1x TNE from the 10x stock. The solution was filtered and placed in a graduated dispenser. To this solution was added 20µl of 1mg/ml Hoechst 33258 dye stock and mixed well. The dye formed a complex with double stranded DNA whose fluorescence was measured by the fluorometer.

3:5:3 Calibration of the fluorometer

To calibrate the fluorometer, 2 ml of the working solution was pipetted into the fluorometer cuvette. The sides of the cuvette were dried with a low-lint tissue paper and inserted into the well. The well was closed and the fluorometer adjusted to zero.

The cuvette was removed and 2µl of the lower reference standard (calf thymus DNA 10µl/ml) added. Mixing was done with parafilm on the top of the cuvette.

The cuvette was inserted into the fluorometer well, the small knob adjusted until the LED read the same concentration as the standard.

The cuvette was washed once with working solution. Two millilitres of working solution was added and the above steps repeated with 3 standards: 50, 100 and 250 µg/ml of calf thymus DNA.

Once the last standard had been used, the cuvette was washed with the working solution, rinsed with distilled water, dried with low-lint tissue paper and reused.

Two millilitres of the working dye solution was pippeted into a fluorometer cuvette and inserted into the well. The cover was closed and the instrument adjusted to zero.

The cuvette was removed, 2µl of the sample added and Mixed by inverting with a parafilm at the top. The fluorometer showed the concentration of the samples in ng/ml. The readings were recorded.

3:6 Determination of the optimal conditions for the polymerase chain reaction

Optimal reaction conditions for the polymerase chain reaction (PCR) were determined before the actual random amplified polymorphic DNA (RAPD) analysis was carried out to ensure repeatable results. Various concentrations of Mg^{2+} ion, primers, template DNA and Taq polymerase were experimented. Each experiment was repeated five times.

3:6:1 Experiment 1: Determination of the optimal Mg^{2+} concentration

The experiment was set up with different concentrations of Mg^{2+}, i.e. 0, 1, 2, 3 and 4 mM. All the other reagents were kept constant.

3:6:2 Experiment II: Determination of the optimal template DNA concentration

The experiment was set up with different concentrations of template DNA, i.e. 0, 10, 15, 20 and 25ng. The optimum Mg^{2+} concentration of 3mM established from experiment 1 was used. All the other reagents were kept constant. The optimum template DNA concentration was 20ng.

3:6:3 Experiment III: Determination of the optimal primer concentration

The experiment was set up with different concentrations of. 0., 0.2, 0.3, 0.4 and 0.50 μM of 10-mer primer opc-15 (Operon technologies, Alameda, CA). The optimum concentrations of 3mM Mg^{2+} and 20ng template DNA established in experiment 1 and II respectively were used. All the other reagents were kept constant

3:6:4 Experiment IV: Determination of the optimal Taq DNA polymerase concentration

The experiment was set up with different concentrations of Taq DNA polymerase, i.e. 0, 0.65, 0.7, 0.75 and 0.8 U. The optimum concentrations of 3mM Mg^{2+}, 20 ng of template DNA and 0.4μM of primer OPC-15 established in experiment 1, II and III respectively, were used. All the other reagents were kept constant.

3:7 PCR amplification using random primers

The thermal cycler (Perkin Elmer Gene Amp 9600) was switched on at least 15 minutes before use to allow the temperature to rise to 94°C. From the preliminary results, the optimum concentrations of the reagents established were: 3mM of Mgcl$_2$, 0.4μM of primer, 0.75U of Taq DNA polymerase and 20ng/μl of template DNA. These were used in all subsequent PCR amplification reactions. The reagents were pipetted out accurately using appropriate pipettes into 0.5ml microtubes in the following order:

Original concentration	Required volume	Final concentration
10x PCR buffer II	2.5μl	
25 mM Mgcl$_2$	3.0μl	3.0mM
5 μM primer	2.0μl	0.4μM
2.5 mM mix of dNTPs	2.0μl	0.2mM
Taq DNA polymerase 3U/μl	0.2μl	0.75U
Template DNA (10ng/μl)	2.0μl	20ng
Double distilled water	13.3μl	
Total reaction volume	25.00μl	

The contents were mixed by repeated pippeting. They were then spun down at 10,000 rpm for five seconds.

The tubes were placed firmly in the wells of the thermal cycler and the program started. The temperature cycling conditions were set as follows:

Step 1. Denaturation at 94°C for 4 minutes

Step 2. Denaturation at 94°C for 1 minute

Step 3. Annealing at 35°C for 1 minute

Step 4. Polymerization at 72 °C for 1 minute.

Step 5. Repeat from step 2 to 4: 40 times

Step 6. A final extension period at 72 °C for 8 minutes.

At the end of the run, the tubes were taken out and 2.5 μl of 10x loading dye (bromophenol blue and xylene cyanol, 1:1) added. The tubes were spun for two to five seconds and stored at 4°C until electrophoresis was carried out.

3.8 Agarose gel electrophoresis

The gel tray was prepared by taping the open ends. A comb was placed on and the tray levelled.

One point eight percent agarose (metaphor) gel in 1x TAE (Tris Acetate EDTA) was prepared and boiled. It was cooled to 60°C (hand warm) and gently poured into the gel tray avoiding any air bubbles. It was allowed to set for 30 – 40 minutes.

The adhesive tapes were removed and the trays placed in the electrophoresis tank filled with 1x TAE buffer. The comb was then removed carefully. More buffer was poured till the gel got fully emmersed.

The samples were loaded carefully. There were fifteen samples per gel. A one kilobase DNA size marker (Fermentas) was loaded. The leads of the powerpack were connected and electrophoresis started at 60v.

The run was stopped after 3 hours when the bromophenol blue had travelled 2/3 of the length of the gel.

3:9 Visualization of amplification products

The power pack was put off and the leads disconnected.

The gel was stained in 0.5 to 1 µg/ml ethidium bromide in distilled water for 45 minutes.

It was viewed under UV light on a transilluminator and photographed.

The PCR amplification was repeated with twenty primers. These were:

OPA-03	5'-AGTCAGCCAC-3'
OPA-04	5'-AATCGGGCTG-3'
OPA-06	5'-GGTCCCTGAC-3'
OPA-09	5'-GGGTAACGCC-3'
OPA-11	5'-CAATCGCCGT-3'
OPA-14	5'-TCTGTGCTGG-3'
OPA-20	5'-GTTGCGATCC-3'
OPC-02	5'-GTGAGGCGTC-3'
OPC-06	5'-GAACGGACTC-3'
OPC-08	5'-TGGACCGGTG-3'
OPC-11	5'-AAAGCTGCGG-3'
OPC-13	5'-AAGCCTCGTC-3'

OPC-20	5'-ACTTCGCCAC-3'
OPD-03	5'-GTCGCCGTCA-3'
OPD-04	5'-TCTGGTGAGG-3'
OPD-07	5'-TTGGCACGGG-3'
OPD-08	5'-GTGTGCCCCA-3'
OPD-10	5'-GGTCTACACC-3'
OPD-11	5'-AGCGCCATTG-3'
OPD-16	5'-AGGGCGTAAG-3'

The amplification products were scored across the lanes by comparing their respective molecular weights.

3:10 Data analysis

The polymorphism in PCR amplification products were scored as discrete variables. A score of '1' showed the presence of bands while a score of '0' showed the absence of bands. Data were used to calculate Jaccard's (1908) similarity coefficients. The formula used was a/n where 'a' was the number of bands in the two lanes being compared, and 'n' was the total number of bands in the two lanes being compared. The similarity measures were subjected to unweighted pair group method analysis (UPGMA) for classification of the accessions. All data analyses were performed using NTSYS- pc ver. 1.9 (Exeter software, New York, USA).

CHAPTER 4

4:0 RESULTS

4:1 Determination of the optimal reaction conditions for the polymerase chain reaction

4:1:1 Amplification with different concentrations of Mg^{2+} ions

Figure 1 shows the results obtained with different concentrations of Mg^{2+} ions. The amplification with 2 mM Mg^{2+} gave clearer bands than that with 1mM of Mg^{2+} as can be seen from lanes 3,8 and 13. More distinct amplification was observed with 3mM Mg^{2+} ion concentration than1mM and 2mM Mg^{2+} concentration as can be seen from lanes 4, 9 and 14. There was no major difference between amplification with 4mM and 3mM Mg^{2+} concentration as shown on lane 9 and 10. The results showed that 3mM of Mg^{2+} produced bands in all the cultivars used. Four millimoles of Mg^{2+} gave no amplification with cultivar Pekera (lane 5). The amplification with 4 mM of Mg^{2+} was clearer than that with 3mM of Mg^{2+} for cultivar Ntobe as can be seen on lane 14 and 15. The optimal concentration of Mg^{2+} ion was 3mM.

4:1:2 Amplification with different concentrations of the primer

More distinct amplification was given by 0.4μM of 10-mer primer as opposed to 0.3μM. The amplification with 0.4μM gave clearer bands than 0.3μM and 0.2μM of the 10-mer primer (figure 2). There was no big difference in the amplification products between 0.4μM and 0.5 μM of the primer as shown on lane 5,10 and 15. With cultivar Mokoya, 0.3μM of the primer did not give any amplification as shown on lane 8. The optimum primer concentration was 0.4μM

4:1:3 Amplification pattern with different concentrations of taq DNA polymerase

Figure 1 shows the results obtained with different concentrations of taq DNA polymerase. Amplification obtained with 0.7U gave more consistent results than 0.65U as shown in lanes 3, 8 and 13. The bands were clearer and more distinct than those obtained with 0.65U. There was no distinct difference between 0.75U and 0.8U for cultivar Mokoya and Ntobe as can be seen from lanes 9, 10, 14 and 15. For cultivar Pekera, 0.8U never yielded any amplification as shown in lane 5. The optimum taq DNA polymerase concentration was 0.75U.

4:1:4 Amplification with different concentrations of template DNA

The amplification with 20ng of template DNA yielded more clear bands than that with 15ng and 10ng as shown on lane 10, 14 and 15 in figure 4. With cultivar Pekera, 25ng of template DNA did not show any amplification as shown on lane 5. Increasing the template DNA concentration from 20-25ng did not yield any major difference in amplification products as can be seen from lanes 10 and 15. Therefore, 20ng of template DNA was used for all subsequent amplification work.

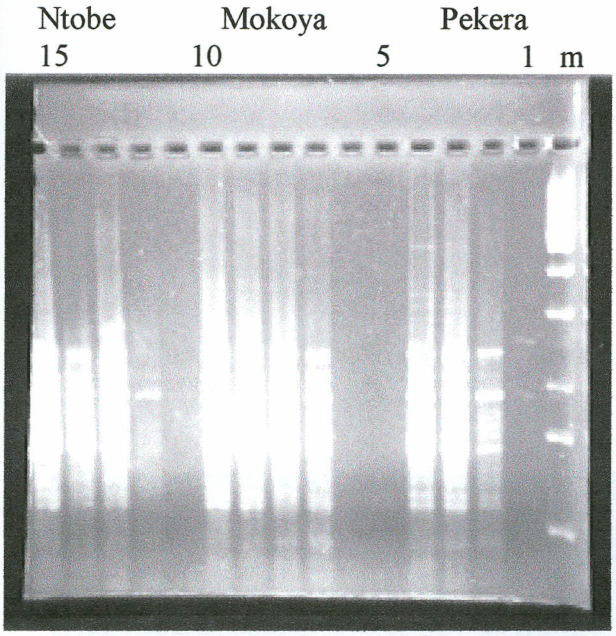

Fig. 1. Amplification with different concentrations of Mg^{2+} using primer OPC-15

Lanes1-15 (Right-Left) are: (1-5) Pekera, (6-10) Mokoya, (11-15) Ntobe

(m) 1 Kb DNA fermentas marker was used

Lanes 1, 6, 11 contained 0 mM Mg^{2+}, Lanes 2, 7, 12 contained 1 mM Mg^{2+}

Lanes 3, 8, 13 contained 2 mM Mg^{2+}, Lanes 4, 9, 14 contained 3 mM Mg^{2+},

Lanes 5, 10, 15 contained 4 mM Mg^{2+}

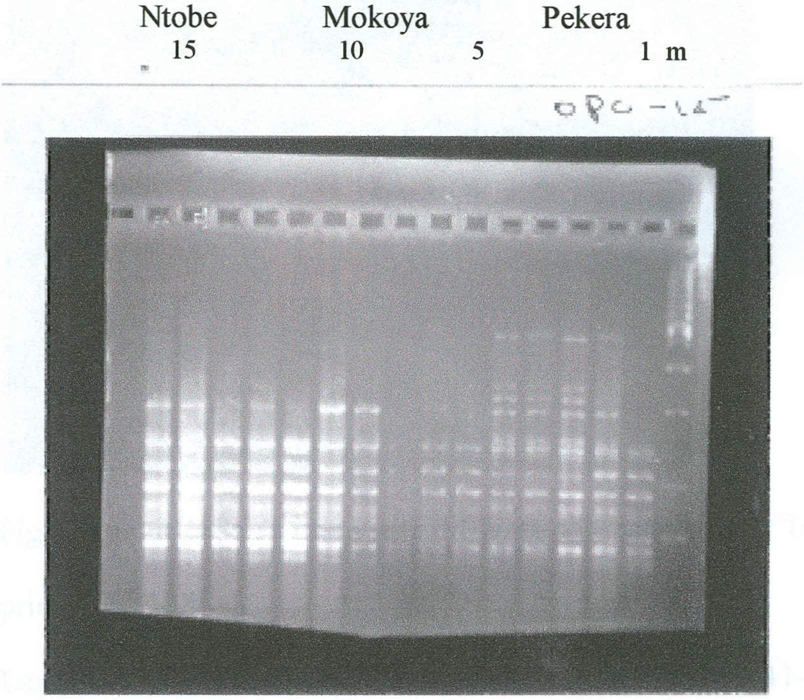

Fig. 2 Amplification with different concentrations of the primer using primer OPC-15

Lanes1-15 (Right-Left) are: (1-5) Pekera, (6-10) Mokoya, (11-15) Ntobe

(m) 1 Kb DNA fermentas marker was used.

Lanes 1, 6, 11 contained 0 µM of primer OPC-15, Lanes 2, 7, 12 contained 0.2 µM of primer OPC-15, Lanes 3, 8, 13 contained 0.3µM of primer OPC-15, Lanes 4, 9, 14 contained 0.4µM, Lanes 5,10,15, contained 0.5µM of primer OPC-15.

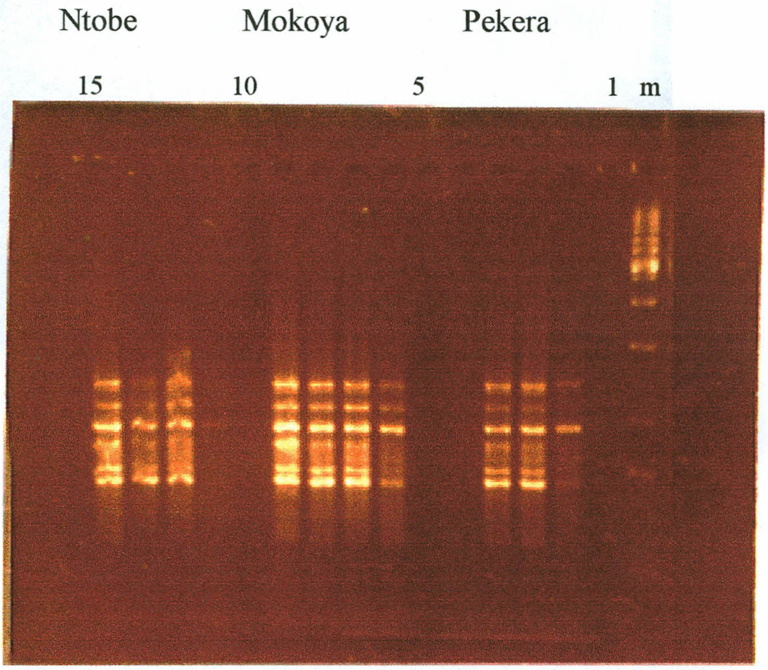

Fig.3. Amplification pattern with different concentrations of Taq DNA polymerase using primer OPC-15.

Lanes1-15 (Right-Left) are: (1-5) Pekera, (6-10) Mokoya, (11-15) Ntobe

(m) 1 Kb DNA fermentas marker was used.

Lanes 1, 6, 11 contained 0 U of Taq DNA polymerase, Lanes 2, 7, 12 contained 0.65U of taq DNA polymerase, Lanes 3, 8, 13 contained 0.70U of taq DNA polymerase, Lanes 4, 9, 14 contained 0.75 U of taq DNA polymerase, Lanes 5, 10, 15 contained 0.80U of Taq DNA polymerase.

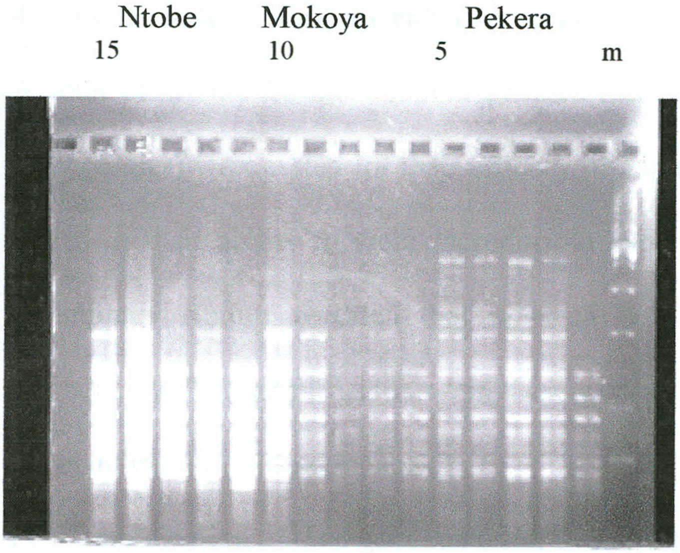

Fig. 4. Amplification with different concentrations of template DNA using primer OPC-15

Lanes1-15 (Right-Left) are: (1-5) Pekera, (6-10) Mokoya, (11-15) Ntobe

(m) 1 Kb DNA fermentas marker was used

Lanes 1, 6, 11 contained 0 ng of template DNA, Lanes 2, 7, 12 contained 10ng of template DNA, Lanes 3, 8, 13 contained 15ng of template DNA, Lanes 4, 9, 14 contained 20ng of template DNA, Lanes 5, 10, 15 contained 25ng of template DNA

4: 2 PCR analysis of all the cultivars using random primers

A total of twenty 10-mer random primers (selected from sets A, C and D) (Operon technologies) were tested for their efficacy in initiating PCR product formation. Primers varied in their ability to yield reproducible banding patterns with the template DNA. Preliminary studies revealed that nineteen out of the twenty tested primers gave reproducible PCR amplifications and these were subsequently utilized for the analysis of the plant materials listed in table 2

PCR amplification of total genomic DNA from twenty *Musa* cultivars using twenty random 10-mer primers was carried out. However, only data from the nineteen primers that gave reproducible products were included in the statistical analyses. The size range of amplification products also differed with selected primer sequence and ranged from 0.1 to 3.0 kilobases (kb) as shown in table 3:

Table 3: The Nineteen most discriminatory primer sequences and the characteristics of their amplification products

Primer No.	Sequence	No. of poly. bands	Size of products(kb)
OPA-03	5'-AGTCAGCCAC-3'	5	0.3-2.0
OPA-04	5'-AATCGGGCTG-3'	5	0.2-2.0
OPA-06	5'-GGTCCCTGAC-3'	3	0.3-2.0
OPA-09	5'-GGGTAACGCC-3'	4	0.1-1.5
OPA-11	5'-CAATCGCCGT-3'	5	0.1-0.2
OPA-14	5'-CTCGTGCTGG-3'	4	0.2-2.0
OPA-20	5'-GTTGCGATCC-3'	7	0.1-3.0
OPC-02	5'-GTGAGGCGTC-3'	7	0.2-2.5
OPC-06	5'-GAACGGACTC-3'	8	0.1-3.0
OPC-08	5'-TGGACCGGTG-3'	8	0.1-2.5
OPC-13	5'-AAGCCTCGTC-3'	10	0.1-3.0
OPC-20	5'-ACTTCGCCAC-3'	6	0.15-3.0
OPD-03	5'-GGTCTACACC-3'	7	0.1-3.0
OPD-04	5'-TCTGGTGAGG-3'	10	0.1-3.0
OPD-07	5'-TTGGCACGGG-3'	8	0.1-2.5
OPD-08	5'-GTGTGCCCCA-3'	5	0.2-2.0
OPD-10	5'-TGTCTGGGTG-3'	6	0.1-2.5
OPD-11	5'-AGCGCCATTG-3'	5	0.2-2.0
OPD-16	5'-AGGGCGTAAG-3'	7	0.1-3.0

The banding patterns obtained from the RAPD analysis were as shown in figures 5-11.

4:2:1 Amplification with primer OPA-04

All the cultivars had a uniform 3 kb band as shown in figure 5. Cultivars Ng'ombe (lane 3-4), Sialamule (lane 23-25) and Nyar sausett (lane 38-40) had a clear 3.5 kb band. Most cultivars had a 5kb band except Ng'ombe (lane 3-4), Nusu Ng'ombe (lane 14-16), Sialamule (lane 23-25) and Nyar sausett (lane 38-40). Only Sialamule (lane 23-25) and Muraru (lane 50-52) had a 2.5 kb band.

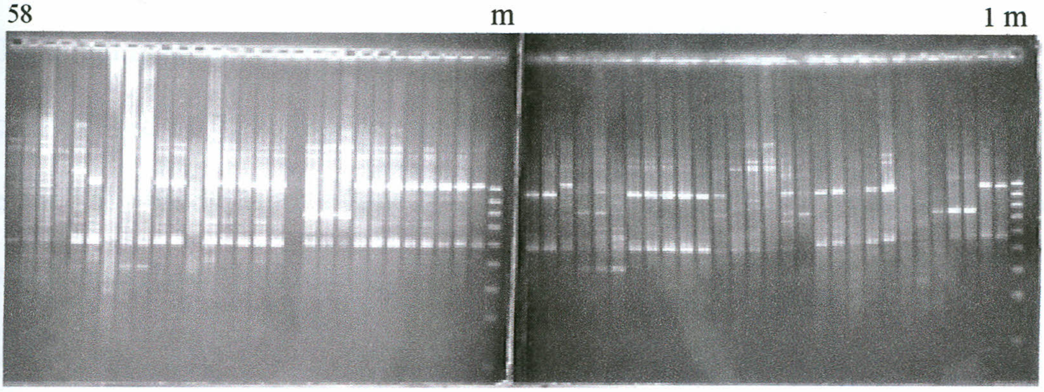

Fig. 5. Amplification with primer OPA-04

Lanes 1-58 (Right-Left) are: (1- 2) Kimuga, (3-4) Ng'ombe, (5-7) Pekera, (18-10) Mokoya, (11-13) Ntobe, (14-16) Nusu Ng'ombe, (17-19) Spambia, (20-22) Murure, (23-25) Sialamule, (26-28) Matumbo, (29-31) Manyatta, (32-34) Sibusi, (35-37) Bukamba, (38-40) Nyar Sausett, (41-43) kibuzi, (44-46) Mfupi, (47-49) Gitigi, (50-52) Muraru,(53-55) Mtama, (56-58) Horn Plantain.(M) 1 kb DNA Fermentas marker was used.

4:2:2 Amplification with primer OPA-06

The amplification pattern produced by Primer OPA-06 is shown in figure 6a. All the cultivars had a 0.75 kb band except Manyatta (lane 29-31). The following cultivars had extra 2.5kb bands: Murure (lane 20-22), Bukamba (lane 35-37), Nyar sausett (lane 38-40) and Kibuzi (lane 41-43).

4:2:3 Amplification with primer OPA –11

The banding pattern produced by primer OPA-11 showed that all the cultivars had a similar 1.5 kb band except Sialamule (lane 23-25) (figure 6b). All the cultivars had a 2 kb band except Sialamule (lane 23-25) and Mtama (lane 53-55). Most cultivars had a 2.5 kb band except Nusu Ng'ombe (lane 14-16) and Mtama (lane 53-55). The cultivars with a clear 3 kb band were: Kimuga (lane 1-2), Pekera (lane 5-7), Ntobe (lane 11-13) Matumbo (lane 26-28) and Gitigi (lane 47-49) shown in figure 6b.

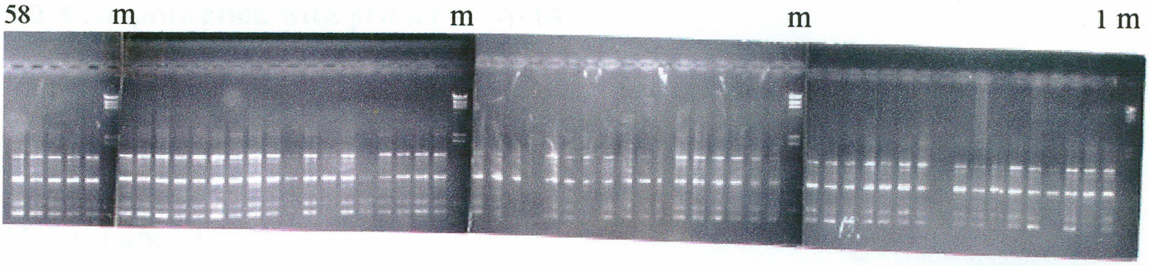

Fig. 6a). Amplification with primer OPA-06

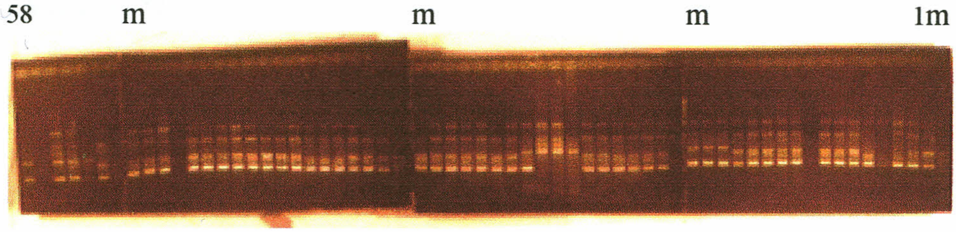

Fig. 6b). Amplification with primer OPA –11

Lanes 1-58 (Right-Left) are: (1- 2) Kimuga, (3-4) Ng'ombe, (5-7) Pekera, (18-10) Mokoya, (11-13) Ntobe,(14-16) Nusu Ng'ombe, (17-19) Spambia, (20-22) Murure, (23-25) Sialamule, (26-28) Matumbo, (29-31) Manyatta, (32-34) Sibusi, (35-37) Bukamba, (38-40) Nyar Sausett, (41-43) kibuzi, (44-46) Mfupi, (47-49) Gitigi, (50-52) Muraru, (53-55) Mtama, (56-58) Horn Plantain. (m) 1 kb DNA Fermentas marker was used.

4:2:4 Amplification with primer OPA-14

Primer OPA-14 revealed that all the cultivars had a uniform 2.5 kb band as shown in figure 7. Most of the cultivars had a common 2 kb band except Muraru (lane 50-52) and Mtama (lane 53-55).

4:2:5 Amplification with primer OPC-02

All the cultivars had a 0.5 kb band except cultivar Mtama (lane 53-5) and Horn plantain (lane 56-58). Most of the cultivars had a 0.75 kb band except Ng'ombe (lane 3-4) and Mtama (lane 53-55). Generally, all the cultivars had a 1.25 kb as well as a 0.7 kb band as shown in figure 8a. All the cultivars had a 2kb band except Pekera (lane 5-7), Matumbo (lane 26-28)

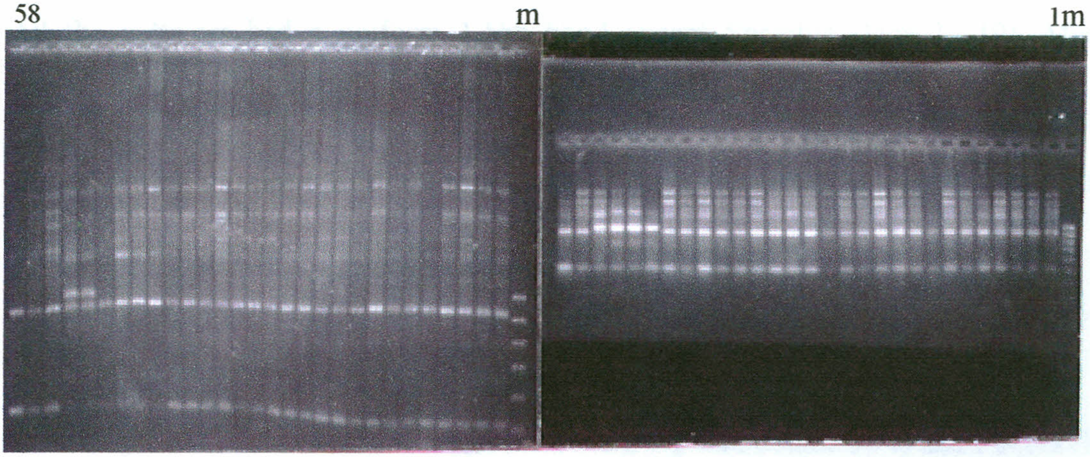

Fig.7. Amplification with primer OPA-14

Lanes 1-58 (Right-Left) are: (1- 2) Kimuga, (3-4) Ng'ombe, (5-7) Pekera, (18-10) Mokoya, (11-13) Ntobe,(14-16) Nusu Ng'ombe, (17-19)Spambia, (20-22) Murure, (23-25) Sialamule, (26-28) Matumbo, (29-31) Manyatta, (32-34) Sibusi, (35-37) Bukamba, (38-40) Nyar Sausett, (41-43) kibuzi, (44-46) Mfupi, (47-49) Gitigi, (50-52) Muraru, (53-55) Mtama, (56-58) Horn Plantain. (m) 1 kb DNA Fermentas marker was used.

4:2:6 Amplification with primer OPC-06

All the cultivars had a uniform 1.5kb band except Spambia (lane 17-19), Matumbo (lane26-28), Nyar sauset (lane 38-40) Mtama (lane 53-55) and plantain (lane 56-58). The cultivars with a unique 2.25 kb band were: Pekera (lane 5-7), Matumbo (lane 26-28), Nyar sausett (lane 38-40) and Muraru (lane 50-52) as shown in figure 8b.

4:2:7 Amplification with primer OPC-08

Primer OPC-08 revealed that Kimuga (lane 1-2), Ng'ombe (lane 3- 4) and Pekera (lane5-7) could be distinguished from the other cultivars by the absence of the a 0.25 kb band that was common to all the other cultivars as shown in figure 8c. All the cultivars had a uniform 0.75 kb band except Spambia (lane 17-19). The cultivars that had a common 0.4 kb band were: Mokoya (lane 8-10), Ntobe (lane 11-13), Nusu Ng'ombe (lane 14-16), Sibusi (lane 32-34) and Gitigi (lane 47-49).

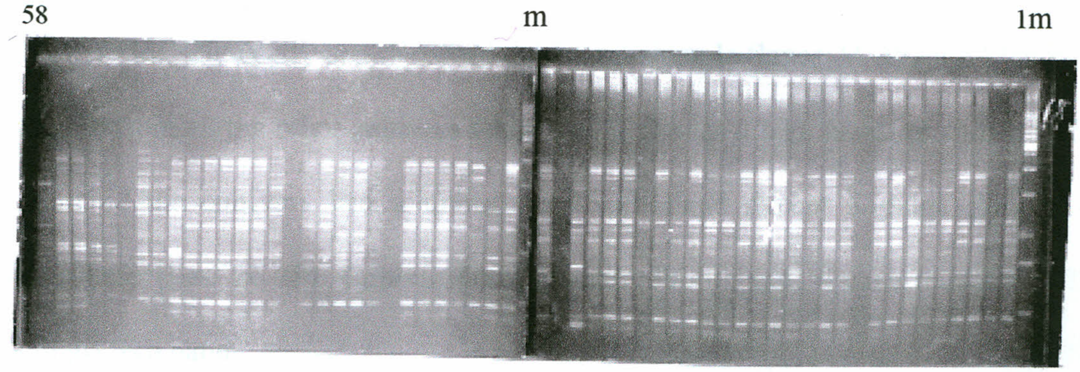

Fig. 8a). Amplification with primer OPC-02

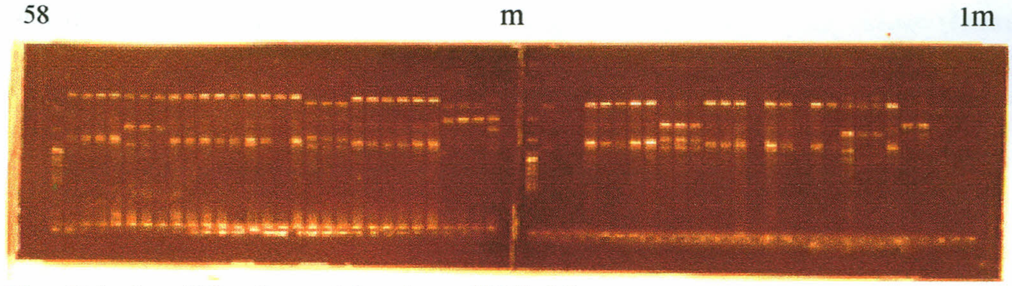

Fig. 8b). Amplification with primer OPC-06

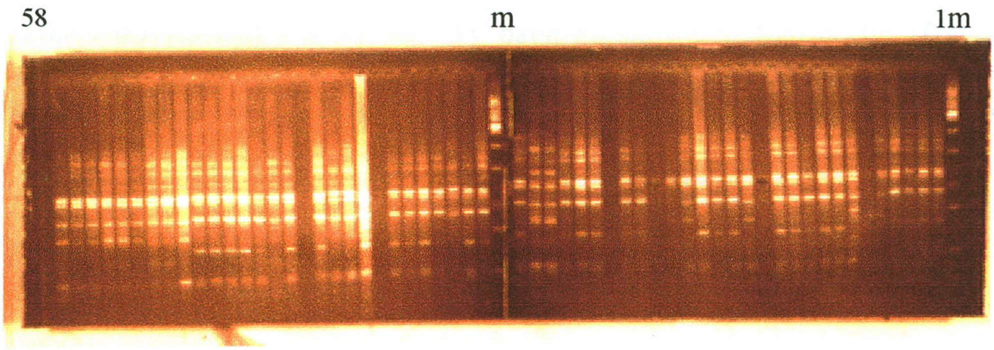

Fig. 8c). Amplification with primer OPC-08

Lanes 1-58 (Right-Left) are: (1- 2) Kimuga, (3-4) Ng'ombe, (5-7) Pekera, (18-10) Mokoya, (11-13) Ntobe,(14-16) Nusu Ng'ombe, (17-19)Spambia, (20-22) Murure, (23-25) Sialamule, (26-28) Matumbo, (29-31) Manyatta, (32-34) Sibusi, (35-37) Bukamba, (38-40) Nyar Sausett, (41-43) kibuzi, (44-46) Mfupi, (47-49) Gitigi, (50-52) Muraru, (53-

55) Mtama, (56-58) Horn Plantain. (m) 1 kb DNA Fermentas marker was used.

4:2:8 Amplification with primer OPC-20

Primer OPC-20 clearly distinguished cultivar Sialamule (lanes 23-25) and Matumbo (lane 26-28) from all the other cultivars which had a similar 0.8 kb band as shown in figure 9. Most of the cultivars had a uniform 1.25 kb band except Kimuga (lanes 1-2) and Matumbo (lane 26-28). Cultivar Nyar sausett (38-40) and Muraru (lanes 50-52) had a unique 3kb band.

4:2:9 Amplification with primer OPD-03

All the cultivars had a 0.75 kb band except Kimuga (lane 1-2), Sibusi (lanes 32-34) and Gitigi (lanes 47-49) (figure 10a). Cultivars Kimuga (lane 1-2), Ng'ombe (lane 3-4) and Nusu Ng'ombe (lane 14-16) had a clear distinguishing 2 kb band.

4:2:10 Amplification with primer OPD-04

Three cultivars had a clear 0.25 kb band as shown in figure 10b. These were: Nusu Ng'ombe (lane 14-16), Sialamule (lane 23-24) and Horn plantain (lane 56-58). All the cultivars had a 0.4 kb band except Nusu Ng'ombe (lanes 14-16), Sialamule (lane 23-25) and Muraru (lanes 50-52).

4:2:11 Amplification with primer OPD –07

All the cultivars had a 0.5kb band except Ng'ombe (lanes 3-6) which could easily be singled out as shown in figure 11a. Most of the cultivars had a 0.4 kb band except Ng'ombe (lane 3-4), Mokoya (lanes 8-10), Nusu Ng,ombe (lane 14-16), Nyar sausett (lanes 38-40), Murarau (lanes 50-52), Mtama (lanes 53-55) and Horn plantain (lanes 56-58). The cultivars with a 0.75 kb band were: Ntobe (lane 11-13), Matumbo (lanes 26-

28), Manyatta (lanes 29-31) and Mtama (lanes 53-55).

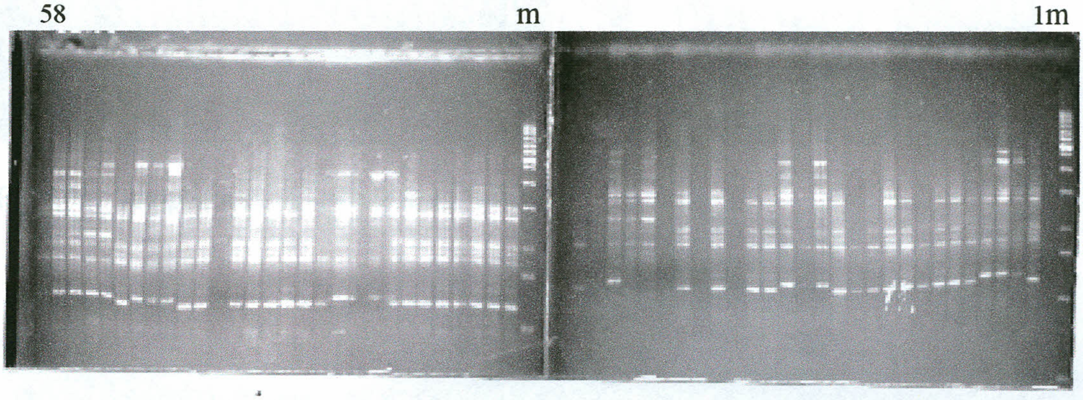

Fig. 9. Amplification with primer OPC-20

Lanes 1-58 (Right-Left) are: (1- 2) Kimuga, (3-4) Ng'ombe, (5-7) Pekera, (18-10) Mokoya, (11-13) Ntobe,(14-16) Nusu Ng'ombe, (17-19) Spambia, (20-22) Murure, (23-25) Sialamule, (26-28) Matumbo, (29-31) Manyatta, (32-34) Sibusi, (35-37) Bukamba, (38-40) Nyar Sausett, (41-43) kibuzi, (44-46) Mfupi, (47-49) Gitigi, (50-52) Muraru, (53-55) Mtama, (56-58) Horn Plantain. (m) 1 kb DNA Fermentas marker was used.

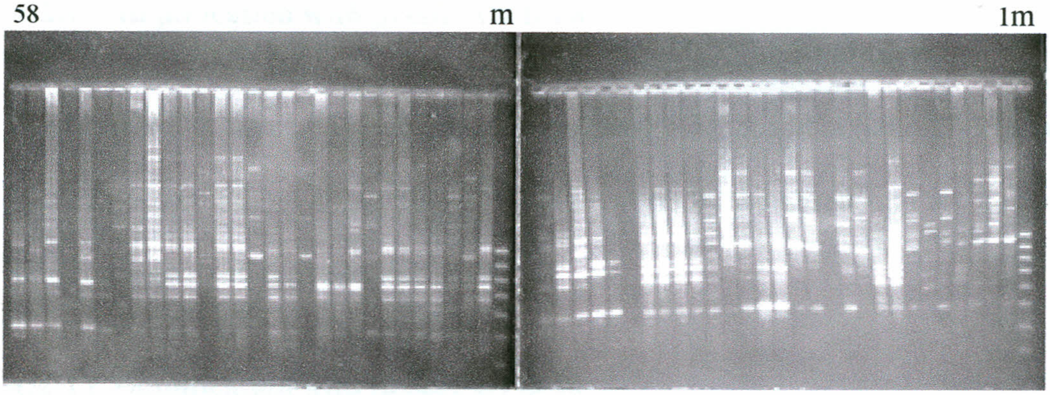

Fig.10a). Amplification with primer OPD-03

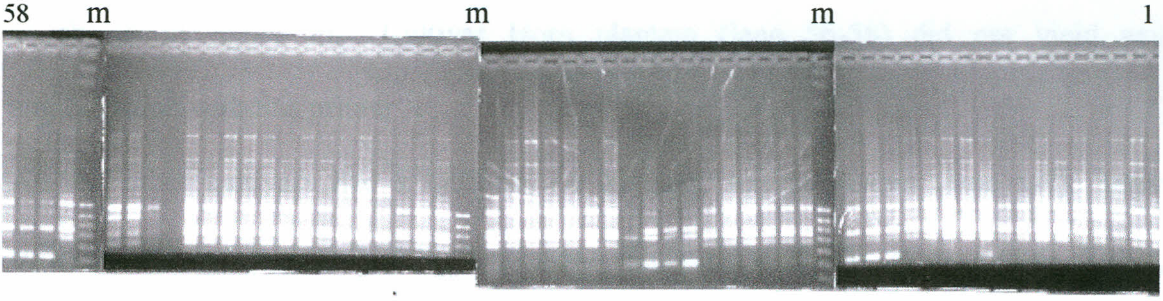

Fig. 10b). Amplification with primer OPD-04

Lanes 1-58 (Right-Left) are: (1- 2) Kimuga, (3-4) Ng'ombe, (5-7) Pekera, (18-10) Mokoya, (11-13) Ntobe,(14-16) Nusu Ng'ombe, (17-19) Spambia, (20-22) Murure, (23-25) Sialamule, (26-28) Matumbo, (29-31) Manyatta, (32-34) Sibusi, (35-37) Bukamba, (38-40) Nyar Sausett, (41-43) kibuzi, (44-46) Mfupi, (47-49) Gitigi, (50-52) Muraru, (53-55) Mtama, (56-58) Horn Plantain. (m) 1 kb DNA Fermentas marker was used.

4:2:12 Amplification with primer OPD-08

All the cultivars had a common 0.5 kb band and a 0.75 kb band except Sibusi (lane 32-34) as shown in figure 11b. Cultivar Pekera (lane 5-7), Spambia (lane 17-19) and Mtama (lane 53-55) could be differentiated from the others by the absence of a 1 kb band which was common to all the other cultivars.

4:2:13 Amplification with primer OPD-10

Primer OPD-10 clearly distinguished cultivar Spambia (lane17-19) from all the other cultivars due to the absence of a 3 kb band which was common to all the other cultivars as shown in figure 11c. Cultivar Horn plantain (lane 56-58) did not yield any amplification with this primer.

All the other cultivars had a uniform 1.5 kb band except Pekera (lane 5-7). Some cultivars that had a clear 0.75 kb band were: Ntobe (lane 11-13), Murure (lane 20-22), Sibusi (lane 32-34) and Kibuzi (lane 41-43).

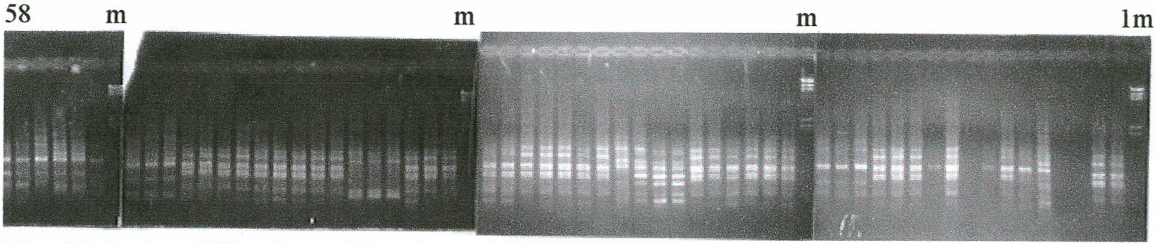

Fig. 11a). Amplification with primer OPD –07

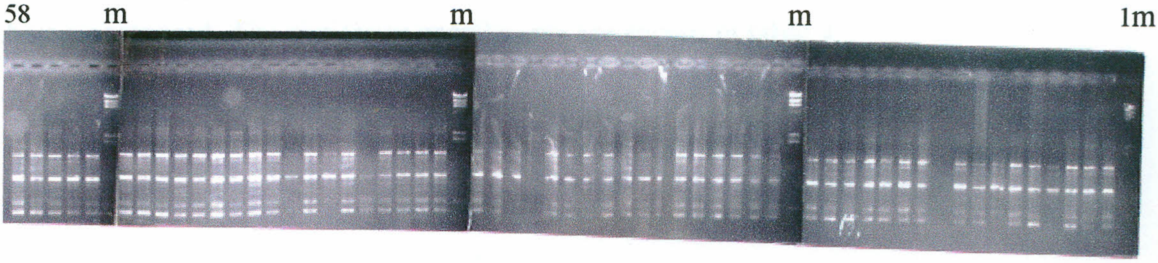

Fig. 11b). Amplification with primer OPD-08

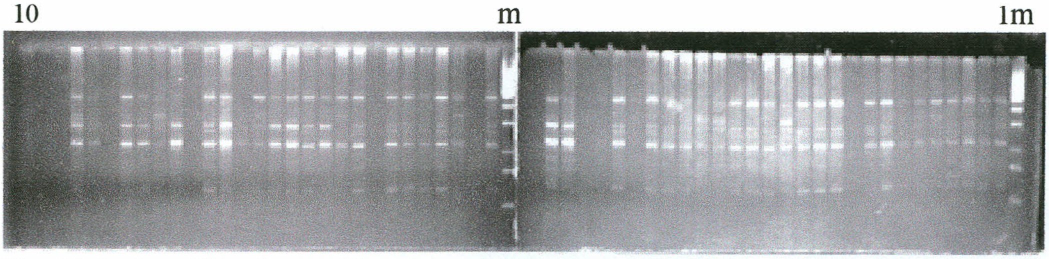

Fig.11c). Amplification with primer OPD-10

Lanes 1-58 (Right-Left) are: (1- 2) Kimuga, (3-4) Ng'ombe, (5-7) Pekera, (18-10) Mokoya, (11-13) Ntobe,(14-16) Nusu Ng'ombe, (17-19) Spambia, (20-22) Murure, (23-25) Sialamule (26-28) Matumbo, (29-31) Manyatta, (32-34) Sibusi, (35-37) Bukamba, (38-40) Nyar Sausett, (41-43) kibuzi, (44-46) Mfupi, (47-49) Gitigi, (50-52) Muraru,(53-55) Mtama, (56-58) Horn Plantain.(m) 1 kb DNA Fermentas marker was used.

The Polymorphisms in PCR amplification products shown in figures 5-10 were scored as discrete variables. A score of '1' showed the presence of bands while a score of '0' showed the absence of bands. The data were used to calculate Jaccard's (1908) similarity coefficients. The formula used was a/n where 'a' was the number of bands in the two lanes being compared, and 'n' was the total number of bands in the two lanes being compared. The similarity coefficients are shown in table 4.

	NGO	PEK	MOK	NTO	NUS	SPA	MUR	SIA	MAT	MAN	KIM	SIB	BUK	NYA	KIB	MFU	GIT	RAR	MTA	HOR
NGO	1																			
PEK	0.849	1																		
MOK	0.671	0.597	1																	
NTO	0.641	0.617	0.806	1																
NUS	0.567	0.516	0.7	0.722	1															
SPA	0.684	0.613	0.507	0.542	0.646	1														
MUR	0.684	0.724	0.542	0.575	0.6	0.716	1													
SIA	0.698	0.62	0.558	0.536	0.481	0.591	0.64	1												
MAT	0.757	0.635	0.579	0.541	0.506	0.616	0.634	0.829	1											
MAN	0.793	0.823	0.557	0.522	0.455	0.651	0.754	0.662	0.685	1										
KIM	0.803	0.721	0.667	0.628	0.543	0.642	0.688	0.821	0.781	0.742	1									
SIB	0.846	0.72	0.632	0.551	0.537	0.651	0.7	0.756	0.881	0.803	0.812	1								
BUK	0.792	0.654	0.571	0.5	0.55	0.657	0.639	0.622	0.698	0.75	0.737	0.826	1							
NYA	0.775	0.759	0.556	0.571	0.545	0.655	0.759	0.71	0.688	0.769	0.779	0.776	0.743	1						
KIB	0.495	0.465	0.518	0.569	0.471	0.489	0.516	0.594	0.593	0.493	0.551	0.535	0.512	0.58	1					
MFU	0.515	0.489	0.58	0.559	0.505	0.495	0.505	0.6	0.639	0.52	0.573	0.589	0.561	0.602	0.871	1				
GIT	0.49	0.446	0.558	0.57	0.52	0.468	0.511	0.538	0.505	0.474	0.531	0.5	0.466	0.553	0.691	0.681	1			
RAR	0.624	0.652	0.529	0.526	0.554	0.589	0.741	0.618	0.616	0.603	0.608	0.608	0.575	0.667	0.491	0.509	0.673	1		
MTA	0.609	0.632	0.482	0.489	0.505	0.614	0.692	0.571	0.613	0.62	0.604	0.604	0.628	0.596	0.43	0.421	0.54	0.805	1	
HOR	0.756	0.631	0.544	0.517	0.485	0.658	0.617	0.765	0.829	0.694	0.741	0.8	0.757	0.67	0.557	0.557	0.475	0.594	0.69	1

Table 4: Similarity coefficients between twenty *Musa* genomes based on DNA fingerprints

The similarity measures were subjected to unweighted pair group method analysis (UPGMA) for classification of the accessions. All data analyses were performed using NTSYS- pc ver. 1.9 (Exeter software, New York, USA). The results were produced in the form of a phenogram as shown in figure 13.

The phenogram linked together samples that were more genetically similar to one another than to samples in the other clusters. The phenogram showed three main clusters. The first cluster consisted of Ng'ombe, Nusu ng'ombe, Ntobe, Sialamule, Kimuga, Kibuzi Sibusi, Bukamba, Mfupi and Gitigi. They had similarity coefficients between 0.68 and 0.83 units as shown on the scale in table 4. The second cluster consisted of Manyatta, Mokoya, Murure and Horn plantain. They had similarity coefficients between 0.60 and 0.68 units. The third cluster had Pekera, Muraru, Spambia, Matumbo, Nyar Sausett and Mtama. They had similarity coefficients between 0.50 and 0.60 units on the scale.

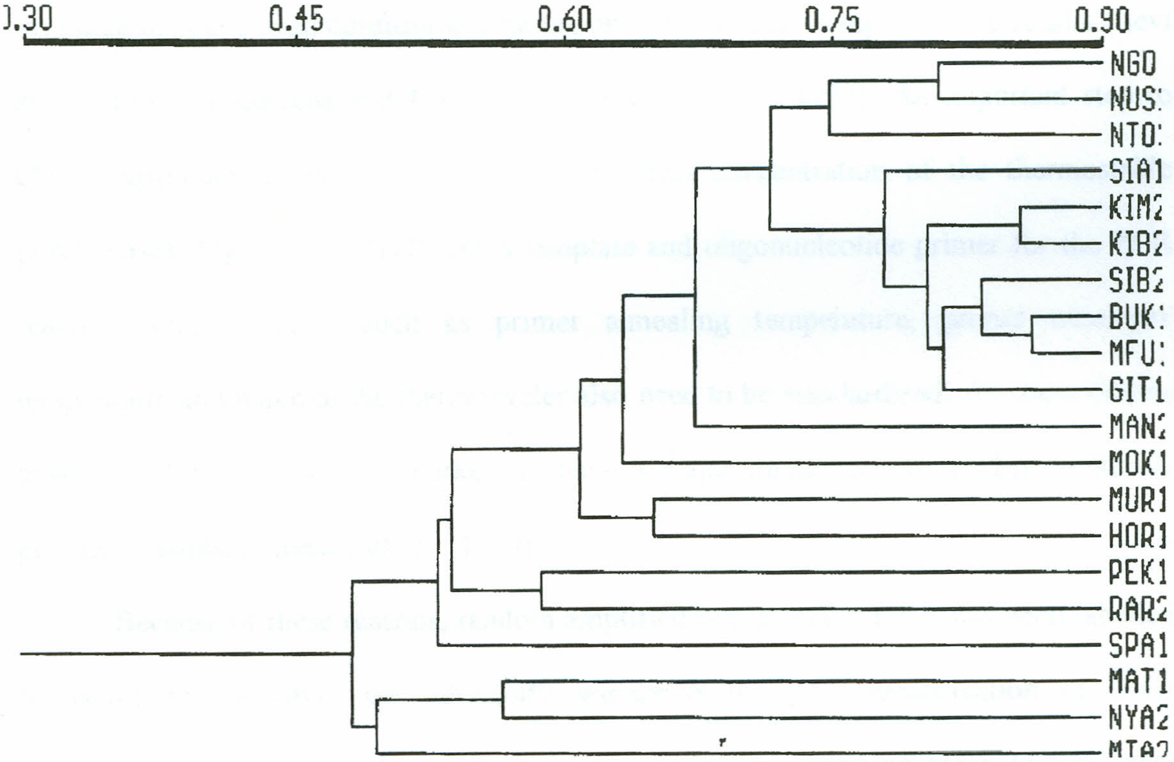

Figure 12: Phenogram derived from the analysis of twenty cultivars using ninenteen primers based on UPGMA

The cultivars analysed were: NGO1- Ng'ombe, NUS2- Nusu Ng'ombe, NTO3- Ntobe, SIA1- Sialamule, KIM2- Kimuga, KIB2- Kibuzi, SIB2- Sibusi, BUK1- Bukamba, MFU1- Mfupi, GIT1- Gitigi, MAN2- Manyatta, MOK1- Mokoya, MUR1- Murure HOR1- Horn Plantain, PEK1-Pekera, RAR2- Muraru, SPA1- Spambia, MAT1- Matumbo, NYA2- Nyar Sausett, MTA2- Mtama.

CHAPTER 5

5:0 DISCUSSION

5:1 Optimization of the reaction conditions

Although the RAPD technology is simple, quick to perform, requires only small amounts of DNA and it does not involve the use of no radioactive consumables, many investigators have been confronted with the problem of lack of reproducible results (Levi *et al.,* 1993; Gogorcena and Parfitt, 1994, Penner *et al.,* 1993). An important step to obtain reproducible results is to define the ideal concentration of the thermostable polymerases, Mg^{2+} ions, dNTP, DNA template and oligonucleotide primer for the PCR reaction. Other factors such as primer annealing temperature, primer extension temperature and make of the thermocycler also need to be standardized. All these factors have been found by several workers to have a major impact on the quality of RAPD profiles obtained (Mneney *et al.,* (1999).

Because of these reasons, random amplified polymorphic DNA has been labelled ‘a useful and sensitive yet inherently precarious tool’ for identification of DNA polymorphisms. To minimize such errors in this study, many of these factors were standardized initially. Most of the RAPD polymorphisms which were obtained using the optimal reaction conditions were observed to be consistent. However, all PCR reactions were replicated twice to confirm the reproducibility of RAPD bands and to resolve inconsistent amplification profiles.

5:1:1 Amplification with different concentrations of Mg^{2+} ions

Results of this study revealed that the amplification product formation was dependent upon the optimum Mg^{2+} ion concentration. The optimum Mg^{2+} ion concentration was 3 mM. This was consistent with other published work such as Bhat *et al., (*1995a) and Howel *et al.,* (1994) who found that the optimum Mg^{2+} ion concentration for the amplification of banana DNA was 2.5 mM. Using a higher concentration could not improve the results but would lead to wastage of the chemical. Damasco *et al.,* (1996) used 2.5-4.0 mM Mg^{2+} ions for the random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated Cavendish (*Musa spp* AAA) bananas. In constructing a molecular marker based linkage map of diploid bananas, Faure *et al.,* (1993) used 2 mM for the RAPD analysis. The $MgCl_2$ concentration in the reaction mixture was important for successful DNA amplification in banana. The number of products amplified varied widely with the concentration of $MgCl_2$. Good amplification was obtained with 3.0-5.0 mM Mg^{2+}. At 1 mM $Mgcl_2$ the number of products amplified was reduced compared to the other concentrations. In scoring for polymorphisms, only products which were reproducibly amplified in different reactions were included.

5:1:2 Amplification with different concentrations of the primer

The optimum primer concentration was found to be 0.4 μM for good results and better utilization of the chemical. Faure *et al.,* (1993) used 0.2 μM of the random primers while Bhat and Jarret (1995) used 0.6 μM of the 10-mer random primer for the random amplified polymorphic DNA and genetic diversity in Indian *Musa* germplasm.

A primer is a short piece of DNA (usually 9-10 base pairs in length) which is added to the template DNA in the presence of an enzyme, dNTPs and cofactors. When the temperature is decreased, the primer anneals to an homologous sequences in the template DNA. Once the temperature is increased, there is synthesis of a complementary strand of DNA by extension of the primer, along its template (Gowen, 1995).

5:1:3 Amplification with different concentrations of Taq DNA polymerase

The amplification with different concentrations of Taq DNA polymerase revealed that the optimum concentration was 0.75U of Taq DNA polymerase. The concentration was exactly the same as the one used by Bhat *et al.,* (1995a) for DNA profiling of banana and plantain cultivars using random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers. However, Faure *et al.,* (1993) used 1 unit of Taq DNA polymerase as earlier stated. Taq DNA polymerase is very important in the reaction because it is an enzyme capable of lengthening a short oligonucleotide primer by attaching additional nucleotides to its 3' end when the primer is hybridized to a longer complementary strand of DNA (the template) (Gowen, 1995)

5:1:4 Amplification with different concentrations of template DNA

The study revealed that the optimum template DNA concentration that yielded consistent results was 20ng. Increasing the template DNA concentration did not improve the results. Wachira (1996), working on the development of molecular markers for tea noted that little amounts of high molecular weight DNA were sufficient for RAPD analysis. He used 20ng of template DNA. Chague *et al.,* (1996) used exactly the same concentration of template DNA for the identification and mapping of chromosome 9 of RAPD markers in tomato by bulked segregant analysis. Bhat and Jarret (1995) used 20ng of template DNA for random amplified polymorphic DNA and genetic diversity in Indian *Musa* germplasm. Kaemmer *et al.*, (1992) used 25ng of template DNA for oligonucleotide and amplification fingerprinting of wild species and cultivars of bananas (*Musa* ssp). Damasco *et al.*, (1996) observed that the DNA concentration in the reaction mixture was important for successful amplification in banana. The DNA concentration influenced the intensity of the products formed. DNA concentrations between 20 to 75 ng resulted in good amplification, and products that were easy to score. More products were amplified at 5 to 10ng of DNA than at higher DNA concentrations; however, bands were less intense and difficult to score (Damasco *et al.*, (1996). A similar observation was made in this study.

5: 2 PCR analysis of all the cultivars using random primers

PCR amplification of total genomic DNA from twenty *Musa* cultivars using twenty random 10-mer primers was carried out. However, only data from the nineteen primers that gave reproducible product formation were included in the statistical analysis. The size range of amplification products also differed with selected primer sequence and ranged from 0.1 to 0.3 kilobases (kb). The data were used to calculate Jaccard's (1908) similarity coefficients.

The similarity measures were subjected to UPGMA for classification of the accessions. The results were produced in the form of a phenogram. The phenogram linked together samples that were more genetically similar to one another than to samples in the other clusters. The phenogram showed three main clusters as shown in figure 12.

The first cluster consisted of Ng'ombe, Nusu ng'ombe, Ntobe, Sialamule, Kimuga, Kibuzi, Sibusi, Bukamba, Mfupi and Gitigi. They had similarity coefficients between 0.675 and 0.85 units. Within this cluster, some cultivars were more closely related with similarity coefficient of 0.8 and above. These were: Ng'ombe and Nusu Ng'ombe with a similarity coefficients of 0.8, Kimuga and Kibuzi with a similarity coefficient of 0.825 and Bukamba and Mfupi with a similarity coefficient of 0.85.

The second cluster consisted of Manyatta, Mokoya, Murure and Horn plantain. They had similarity coefficients between 0.60 and 0.675 units. Within this cluster, Murure and Horn plantain were more closely related with a similarity coefficient of 0.675.

The third cluster consisted of Pekera, Muraru, Spambia, Matumbo, Nyar Sausett and Mtama. They had similarity coefficients between 0.50 and 0.60 units on the scale. Here, cultivar Matumbo and Nyar Sausett were more closely related than the others within the same cluster. This showed that the cultivars that were more closely related had more characters in common than those that were distantly related within the same cluster. Some cultivars like Manyatta, Mokoya and Pekera were on the borderline and would easily fit into either of the adjacent clusters.

The range of similarity coefficients between and within clusters might have been due to somatic mutations that have occurred to the East African highland bananas. Karamura, (1996) observed that environmental effects on the phenotype of the crop or somatic mutations have given rise to several clones of bananas.

Genetic relationship has been used for cultivar identification of many plants

(Shim *et al.,* 1996) and is important in the protection of plant breeders' rights and germplasm maintenance. The use of RAPD technique in potato (Mori *et al.,* 1993), tomato (Williams and St. Clair, 1993), navy bean (Graham *et al.,* 1994), rice (Mackill, 1995) and cranberry (Novy *et al.,* 1996) are some successful examples of cultivar identification.

In the genetic analysis of bananas and plantains cultivated in Kenya, the use of nineteen random primers placed the twenty cultivars into three clusters. The number of RAPD markers (and therefore the number of primers) needed varies with the test materials. When the variation between cultivars is high, the use of a few primers will be sufficient. For instance, 11 navy bean genotypes were satisfactorily distinguished by only two primers (Graham *et al.*, 1994) while Mori *et al.,* (1993) identified 36 Japanese potato cultivars and three introduced cultivars with 15 polymorphic RAPD markers generated by five primers. However, more primers will be needed for more closely related cultivars. The use of 21 primers still could not differentiate a number of cultivars in the template Japonica group of rice (Mackill, 1995). Some vintage and modern tomato cultivars remained undistinguished even after using 215 RAPD markers (24 primers) because of their low diversity (Williams and St Clair, 1993).

Bhat and Jarret (1995) used 49 RAPD primers for studying genetic diversity in Indian *Musa* germplasm. They however, noted that out of the 49 random decammer primers useful in their analysis, as few as 12 were sufficient to distinguish all the 57 accessions included in their study. In the present study, ninenteen primers were used to distinguish twenty banana cultivars.

The application of DNA fingerprinting technique for *Musa* germplasm conservation and breeding will allow the identification of species and cultivars and the determination of the evolutionary relationships between clones. It will help in the identification of duplications among accessions in the field and in tissue culture germplasm banks. They will also be useful in the monitoring of genetic stability of the tissue cultured material (i.e. somaclonal variation) and identification of trait markers for use in cross and mutation breeding programs. DNA profiling would also be important for policing plant patents and for legal protection of new bred cultivars in asexual *Musa* crops (Kaemmer *et al.*, 1997).

Problems associated with clonal classification emphasize the need for complementary keys for identification and characterization of *Musa* cultivars. It demonstrates the usefulness of RAPDs for these purposes (Bhat *et al.*, 1995a). Thus, the genome of the cultivars used in this study could be composed from the clustering pattern they formed with cultivars morphologically classified by Simmonds (1962). According to Simmonds, the cultivars Ntobe and Gitigi had the genome AAA. In this study, they were in the same cluster with Ng'ombe, Nusu Ng'ombe, Sialamule, Kimuga, Kibuzi, Sibusi, Bukamba and Mfupi. This implies that all the cultivars in this cluster could be AAA.

Cultivar Horn plantain was classified by Simmonds (1962) as AAB. In this study, it was in the same cluster with Manyatta, Mokoya and Murure. The other cultivars in the same cluster could also be AAB. However, looking at these cultivars morphologically, they lacked the salient features of true plantains. Thus, they may have been put in the same cluster with AAB because they could be possessing a B genome. It is difficult to separate AAB and ABB (Bhat and Jarret, 1995). Kung'u (1999) indicates cultivar Muraru as AA. It has also been morphologically classified as AA in the KARI, Kisii banana germplasm collection. In this study, it was in the same cluster with Pekera, Spambia, Matumbo, Nyar Sausett and Mtama. The other cultivars in the same cluster could also be AA.

The East African bananas whose names are too numerous to catalogue have evolved to suit the local environment. They can be grouped into three categories: dessert bananas, plantains and East African Highland cooking bananas. The dessert bananas include *Musa* AB (e.g. Kisubi and Sukari), AA (e.g. Sindika and Gatumia) diploids and one AAB triploid (Prata), but the majority are AAA (Karamura, 1998). In this study, half of all the twenty cultivars studied clustered with cultivars Ntobe and Gitigi which were classified morphologically as AAA by Simmonds as stated earlier.

Only four out of the twenty cultivars studied could be having the B genome since they clustered with the ABB Horn plantain. Plantains are a staple crop of many parts of West- Central Africa, as are cooking bananas in East Africa. The plantain is much better suited to the ecological conditions of the rain forest than are root or cereal crops, and it is arguable that the sustenance of relatively large populations there became possible after the introduction of *Musa* (Rossel, 1995). In the past, and in diverse parts of the world, the term 'plantain' has been applied to different groups of cultivars (AAA-East Africa and AAB), but it should be restricted to the very specific group of starchy AAB cultivars. These are grown as a major staple crop in the rainforest areas of Central and West Africa. Banana germplasm in East Africa offers challenges in that, since there is no test for clonal identity, many clones may have been collected several times under different names. This is because there is a long list of different vernacular names attached to each clone (Kyobe, 1981, Karamura and Karamura, 1994). This is one of the principal problems facing researchers, farmers and extension officers alike. Some names may be synonyms due to the many languages spoken, others may just refer to environmental effects on the phenotype of the crop or somatic mutations.

It is generally believed that the first highland bananas introduced in Uganda gave rise to all the present day highland bananas through mutation (Karamura, 1998). The numerous EA-AAA cultivars must have been generated through somatic mutation, and this process must have required more than a millenium of cultivation in the East African highlands (Simmonds, 1966). Clones claimed by farmers to be the oldest in their farms or in their villages have several variants. Some of these variants may appear to be generated by the environment but majority are probably mutants (Karamura, 1998).

The first and probably the most crucial step in the evolution of edible bananas was the development and, subsequently, the selection of parthenocarpy and seed sterility in *M. acuminata,* giving rise to the edible diploid (AA) cultivars. The wild seedy diploid *M. acuminata* has fruits inedible by man but which can be eaten by birds, bats and monkeys. Edible bananas evolved from the seedy wild types by becoming able to produce parthenocarpic fruits, which are seedless so long as the female flowers are not pollinated. Female sterility developed later so that fruits from pollinated flowers were also seedless (Simmonds, 1962). Edibility was improved greatly by parthenocarpy and seed sterility. It is believed that in the process of chromosome restitution in diploid edible *M. acuminata,* there arose AAA triploids which now dominate the world's bananas. There has never been a natural edible parthenocarpic diploid (BB) from *M. balbisiana* nor triploid (BBB) (Simmonds, 1966, Simmonds and Weatherup, 1990; Jarret, 1990; Simmonds, 1959).

Another important step in the evolution of bananas was the crossing of AA (and perhaps AAA too) cultivated bananas with the wild *M. balbisiana* (BB). As *M. acuminata* derivatives spread extending their range in the territories of the wild seeded *M. balbisiana* natural hybridization occurred resulting in several genome combinations. Hybridization probably took place repeatedly as cultivation of edible triploids of *M. acuminata* continued to spread into the territory of the wild diploid *M. balbisiana.* Armed with good edibility (imparted by the diploid AA) and dry environment tolerance (imparted by the diploid BB) the resulting hybrids (AB, AAB and ABB) extended their geographical ranges out from the wetter tropics into the seasonally drier zones. At present, there are many clones of cultivated bananas in existence belonging to different genome groups of which AA, AAA, AAB and ABB are the most numerous (Karamura, 1998). Since bananas and plantains are clonally propagated, their spread is entirely a result of human intervention (Rossel, 1995). They produce no seeds, and genetic variation arises from mutations over time. Subsequently, this variation has been subjected to all kinds of selection pressures. Besides chance, various selection mechanisms – botanical, ecological or human-may have influenced the pattern of spread of certain cultivars or cultivar types.

The East African highland bananas are extensively pigmented black or brown with glossy pseudostems and robust green leaves deeply split along veins. For quite some time, no highland bananas have been reported to occur along the East African Coast (Simmonds, 1959). Together with the African Plantains, the East African highland bananas are believed to have increased their number and diversity through somatic mutations.

It was possible that the AAA banana was introduced to the L. Victoria region and through mutation it diversified. East African is a land of contrast both in its physical features and socially so that the different climate, the physical nature of East Africa and social background of the region played part in the diversification of the different clones (Karamura, 1998). Assessment of genetic diversity is an essential component in germplasm characterization and conservation in order to effectively establish rational breeding programs.

PCR methods offer several advantages when compared to hybridization based techniques, especially if large numbers of samples have to be processed. Random priming of DNA has also been exploited for the generation of a theoretically unlimited number of DNA markers for *Musa* germplasm identification as well as mapping (Howell *et al.*, 1994; Faure *et al.*, 1993; Bhat *et al.*, 1995a). Nevertheless, the reproducibility of this technique is questionable (e.g. Schierwater and Ender, 1993). An alternative class of PCR markers coined sequence tagged microsatellites (STMS), based on variable number tandem repeats (VNTR) of simple sequence repeats (SSR) employs locus specific primers to amplify polymorphic DNA regions. Daniells (1990) described 28 spots of the Cavendish (AAA) subgroup derived from a single clone. Distinguishing such closely related cultivars would require a wide range of molecular markers such as isozymes, VNTR, RAPDs and RFLPs. Use of highly discriminating markers based on simple sequence repeats (SSR) would help greatly in distinguishing relationship within more narrowly defined AAB and ABB group cultivars (Bhat and Jarret, 1995)

SSRs also called microsatellites are ubiquitous components of eucaryotic genomes (Delseny *et al.*, 1983; Tautz and Renz, 1984). However, the first evidence of their abundance in plant genomes and their polymorphic character was reported only a few years ago (e.g. Condit and Hubbell, 1991; Largercrantz *et al.*, 1993; Morgante and Olivieri, 1993). SSRs have been shown to detect more polymorphic alleles per locus than allozymes (e.g. Terauchi and Konuma, 1994) or RFLPs (e.g. Roder *et al.*, 1994) and a number of laboratories are exploiting the potential of these markers for genome analysis and mapping in plants.

SSR markers have already been developed and applied for Soybean (Rongwen *et al.*, 1995; Manghan *et al.*, 1995, Akkaya *et al.*, 1995), rice (e.g. Wu and Tanksley, 1993; Yang *et al.*, 1994; Zhang *et al.*, 1995) and maize (Senior and Heun, 1993). They have also been used in wheat (Roder *et al.*, 1994; Plaschke *et al.*, 1995), barley (Saghai Maroof *et al.*, 1994; Becker and Heun, 1995) and rapeseed (Kresovich *et al.*, 1995). Microsatellite analysis has also been employed with tropical plants e.g. trees (Condit and Hubbell, 1991), Avocado (Lavi *et al.*, 1994) citrus (Kijas *et al.*, 1995) and kiwifruit (Weising *et al.*, 1996).

CHAPTER 6

6:0 CONCLUSION AND RECOMMENDATION

The general aim of this research was to study the diversity of bananas and plantains cultivated in Kenya. The specific objective was to characterize the locally cultivated bananas and plantains and to determine the relationship among them.

The preliminary study aimed at optimizing procedures for isolating DNA from bananas and plantain leaves which was of sufficiently high purity for PCR and other DNA –based analyses. It also aimed to devise PCR/RAPD-based techniques to assess genetic similarities and differences in twenty banana and plantain cultivars grown in different regions of the country.

The major achievements of this study and observations are listed and elaborated below:

-The optimum reaction conditions for PCR were established. The RAPD methodology is particularly appealing as it can be used to analyze DNA from genetically uncharacterized organisms, does not require radioisotopes and can be adapted for processing large numbers of samples. However, the RAPD profiles are known to be influenced by a number of factors including annealing temperatures, template DNA concentration, dNTP concentration, $Mgcl_2$ concentration, polymerase concentration and supplier, thermocycler used and duration of melting and annealing temperatures (Mneney *et al.*, 1999). To minimize such errors, many of these factors were standardized initially as mentioned earlier. Most of the RAPD polymorphisms which were obtained using optimal reaction conditions were observed to be consistent. Hence, these optimal reaction conditions may be useful in future routine work involving *Musa* identification and characterization.

-PCR analysis of banana and plantain DNA using random –10mer primers was extensively done and revealed similarities and differences in the twenty cultivars used. This helped to group the cultivars into three clusters of closely related cultivars. Some cultivars were more closely related within clusters yet others were on the borderline between adjacent clusters. More research work needs to be done to establish the true genomic constitution of the cultivars that have been grouped together. These will be possible by running the sample cultivars along with genetically characterized reference cultivars.

-Further research needs to be done using more specific genetic markers such as restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs) or amplified fragment length polymorphisms (AFLP). The use of alternative PCR-based markers such as SSRs may reveal additional unique relationships within more narrowly defined groups (e.g. ABB group). SSRs have been shown to be effective genetic markers even within highly inbred species such as *Glicine max* (L) mer. Akkaya *et al.,* (1992). They may be useful in cultivar identification and for germplasm classification.

-It is recommended that the sample size for future studies be increased to at least thirty and the scope expanded to include more banana growing areas.

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