MOLECULAR CHARACTERIZATION OF BANANA (*Musa* AAB PLANTAIN SUBGROUP) CLONES

17985

BY

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DECLARATION

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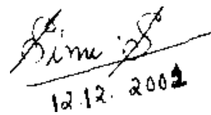

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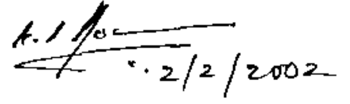

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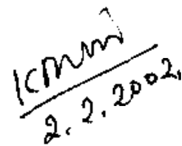

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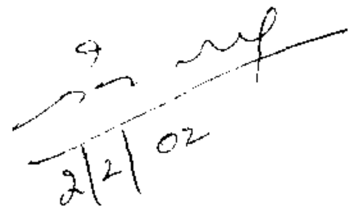

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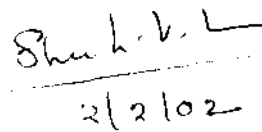

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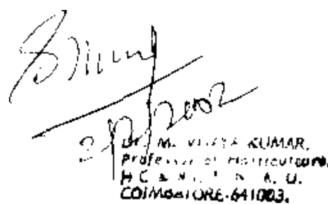

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LIST OF ABBREVIATIONS

µl	microlitre
µM	micromolar
AFLP	amplified fragment length polymorphic DNA
AN	Attu Nendran
bp	base pair
BRS	Banana Research Station
CATIC	Agronomic Centre for Research and Training
CK	Changazhikodan
CN	Changanasseri Nendran
CORBANA	National Banana Corporation of Costa Rica
DNA	deoxy ribonucleic acid
dNTPs	deoxy nucleotides
EDTA	ethylene diamino tetra acetic acid disodium salt
IITA	Intenational Institute of Tropical Agriculture
ISSR	Inter simple sequence repeats
KA	Kaliethan
KO	Koonoor Ethan
ME	Mysore Ethan
mM	millimolar
MN	Manjeri Nendran
MY	Myndoli
ng	nanogram
PCR	polymerase chain reaction
pM	picomolar
PM	Padalamurian
PVP	poly vinyl pyrrollidone
QB	Quintal Banana
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
SCAR	sequence characterized amplified region
SDS	sodium dodecyl sulphate
SSR	simple sequence repeats
SSRLP	Single sequence repeat length polymorphism
STMS	sequence tagged microsatellite sites
Tris_HCl	tris (hydroxy methyl) aminomethane hydrochloride
TSS	Total Soluble Solids
VNTR	varible number of tandem repeats
ZA	Zanzibar

Introduction

1. INTRODUCTION

Banana (*Musa* sp.) is one of the most important fruit crops of India, next only to mango. It is grown in an area of 4,64,300 ha, with an annual production of 1,50,72,700 t. In Kerala also it is the leading fruit crop, being cultivated in an area of 77,450 ha with an annual production of 6,47,890 t (Anon., 2000).

The centre of origin of banana is South East Asia, especially Assam and Myanmar. India has been considered as one of the important centres of diversity for banana. It has a number of banana cultivars ranging in diversity from delicate diploid acuminata (AA) types, grown in humid environments, to hardy hybrid triploid (AAB) types, capable of tolerating seasonally arid climates.

Robusta (AAA), Poovan (AAB), Red banana (AAA), Monthan (ABB), Palayankodan (AAB), Nendran (AAB) and Njalipoovan (AB) are some of the important varieties of banana grown in Kerala. 'Nendran' belonging to *Musa* AAB plantain subgroup is the leading banana cultivar of the state. It comes under the French Plantain group. It is well known for its multi farious uses. Nendran fruits can be consumed raw, cooked or after processing. It is extensively used for the preparation of chips, halwa and sweets. It is also used for preparing baby foods. Horn Plantain (eg. Zanzibar) and Giant Plantain (eg. Quintal Banana) are also important groups of banana under commercial cultivation in Kerala.

'Nendran', in spite of being propagated through suckers, exhibits clonal / ecotype variation with respect to growth, yield and reaction to biotic and abiotic stresses. In different parts of the state, different names are used to describe 'Nendran' banana *viz.*, Attu Nendran, Changanasseri Nendran, Changazhikodan, Manjeri Nendran, Mysore Ethan, Kaliethan, Koonoor Ethan, Padalamurian etc. Such naming is based on fruit and plant traits as well as the locality of cultivation. This type of naming of 'Nendran' can be confusing, creating problems in the precise identification and classification of cultivars.

Characterization of varieties is generally being done based on morphological and agronomic traits. Isozyme analysis has also been resorted to. However, these approaches are subject to environmental influences and hence their effectiveness is debatable. DNA-based molecular markers have been widely accepted as ideal for genetic characterization. They are not affected by environmental conditions. Several molecular markers, namely, RAPD (Random Amplified Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism) etc. are being utilized for this purpose. Among these, RAPD marker technique is quick, reliable, and widely applicable. It is used for characterization of genetic variability, determination of somaclonal variants and hybrids, taxonomic studies, sex determination etc.

The present study was undertaken for characterizing some of the important clones of banana (*Musa* AAB Plantain subgroup) grown in Kerala, using RAPD markers.

Review of Literature

2. REVIEW OF LITERATURE

India has a number of clonal banana cultivars ranging in diversity from the delicate edible diploid acuminata types that can be cultivated only in sheltered and humid environments to hardy hybrid triploids which can tolerate seasonally arid monsoon climate prevailing in most parts of the country. Several synonyms of the local names of banana clones make it difficult to identify them. It is estimated that more than 300 banana cultivars are available in India.

It is difficult to confidently distinguish *Musa* species and cultivars. Clone identifications are traditionally based upon various combinations of morphological, phenological and floral criteria. Fifteen different criteria are used to recognize participation of the A (*acuminata*) and B (*balb isiana*) genomes in the hybrid constitution of a given cultivar (Simmonds and Shepherd, 1955). A close genetic relationship among cultivars, somatic mutations, subjective interpretation of morphological characters and changes due to environmental modification create problems in correct identification of germplasm.

'Nendran' belonging to the French plantain group is the leading commercial banana variety in Kerala. Nayar (1962) reported several ecotypes of 'Nendran' in different parts of Kerala. These ecotypes showed slight difference in their growth and yield characters (KAU, 1990). 'Nendran' in different parts of Kerala exhibited variation with respect to yield and

vegetative characters (Sunilkumar, 1977). Iyer (1987) suggested isolation of intraclonal variants as a tool for better productivity in banana.

2.1. Variation in growth characters

The 'Nendran' fruit is known in all parts of the world as plantain (Rao, 1998). This is because the fruit is always cooked to make it more palatable. There are a number of clones / ecotypes in ' Nendran'. 'Velathen' (Wine Plantain), a type from southern Kerala has a very conspicuous pink colouration over psuedostem, petiole and midrib. Kaliethan from the same place has hard flesh. 'Moongil' (Horn Plantain) is a sport of 'Nendran' with only one or two hands in the branch. Another type of this group is 'Anil Vazhai', a variegated mutant with young leaves and fruits showing different degrees of variegation. In 'Tiger Plantain', the fruit rind is spotted with round black circles resembling the spots on a leopard. In white plantain, the flesh colour is nearly white and not yellowish.

Biswas and Hussain (1982) based on the studies on the performance of five exotic cultivars of plantain obtained from IITA, Nigeria found significant variation in their pseudostem height.

Valsalakumari and Nair (1990) reported that the cultivars with in each genomic group were highly variable with respect to vegetative characters. Studies on the evaluation of 'Nendran' ecotypes revealed that height of plants varied significantly under the influence of various treatments and during different stages of growth. Changazhikodan, Poovanchira and

Puthur types recorded the lowest plant height during early and late vegetative phase, while Pandaloor, Muttathukonam, Kothala and Kaliethan recorded the highest values. However at flowering, plant height was the lowest in Pandaloor, Muttathukonam, Changazhikodan and Kaliethan and the highest in Kothala, Poovanchira and Puthur types (Devi, 1996). Kaliethan had significantly higher Leaf Area Duration followed by Puthur type. Sunilkumar (1997) reported that there was significant difference among various accessions of Kaliethan with respect to time taken for flowering and bunch maturity and total crop duration.

According to Gonzalez *et al.* (1990) all the seven plantation clones *viz.* plantano Enano, Dominician Dwarf, Harton and Maricongo of Horn type and Congo Enano, Congo 300 and Lacknan of French type were harvested on an average of 107 days after flowering.

An experiment conducted at Banana Research Station, Kannara (KAU, 1984 a) showed that in Nendran clones, the crop duration varied from 332.30 (clone No.100) to 359.30 days (clone No 134). For the same Nendran clones, the crop duration varied from 207.33 to 309.33 days and 291.30 to 327.60 days in the subsequent seasons (KAU, 1987). Devi (1996) reported that the total crop duration was the shortest in Muttathukonam, followed by Pandalloor, Kaliethan and Poovanchira types and the longest in Changazhikodan, followed by Puthur and Kothala types. Rajamony *et al.* (1984) observed a variation of 327.10 to 437.71 days among the 21 banana clones of AAB group, 327.10 days for 'Malaikali' and 437.71 days for

'Myndoli'. The duration of most of the types, except 'Myndoli', was about 11 to 12 months after planting. 'Myndoli' takes about 18 months and produces very large bunches and bigger fruits (Rao, 1998).

2.2. Variation in yield charcters

Results of an experiment carried out at Kerala Agricultural University (KAU, 1982) revealed that the Nendran clones from Pampady and Meenadom area in Kottayam district recorded the highest mean bunch weight of 12.5 kg. Rajeevan (1985) reported significant variation in bunch weight among *Palayankodan accessions.An evaluation of 194 Nendran clones on* Banana Research Station (BRS), Kannara (KAU, 1989) revealed a bunch weight variation of 9.13 (local variety) to 10.54 kg (clone 123). Shanmughavelu *et al.* (1992) reported that Attu Nendran, Nana Nendran, Myndoli, Moongil and Nendrapadathi yielded 12, 8, 25, 8 and 10 kg bunch per plant, respectively.

Rao and Edmunds (1985) observed that the number of hands per bunch in plantain cultivars varied from 6.71 to 9.29. Among the seven types evaluated, Changazhikodan had significantly higher number of hands per bunch, followed by Kothala, Poovanchira and Pandalloor. The lowest number of hands per bunch was recorded in Muttathukonam and Kaliethan (Devi, 1996). Clonal variation studies in 'Nendran' at BRS, Kannara showed that number of hands per bunch did not differ significantly (KAU, 1984 a; KAU, 1987).

Studies in 'Nendran' conducted at BRS, Kannara showed that the number of fingers varied between 49.7 and 66.4 per bunch (KAU, 1987).

Rao and Edmunds (1985) observed that among the four plantain cultivars, the number of finger per bunch was maximum in 'Dominique' and minimum in 'Horn' (14.7 and 35.7, respectively). Number of fingers per bunch was significantly higher in Changazhikodan and Poovanchira (Devi, 1996).

Clonal variation studies in 'Nendran' at BRS, Kannara showed that the clone No.123 (Puthur – Trichur) showed significantly higher pulp / peel ratio by weight of green finger (1.82) and was significantly superior to clone No.134 (Chengallore – Trichur) and clone No. 35 from Muttathukonam – Quilon (KAU, 1984 a and b).

2.3. Variation in quality characters

Significant variation in the quality aspects such as TSS (28.7 – 34.3 per cent), acidity (0.27 – 0.34 per cent), total sugars and sugar / acid ratio were reported in 'Nendran' (KAU, 1984 b). Studies on evaluation of 'Nendran' ecotypes (Devi, 1996) indicated significant difference in fruit quality of different ecotypes. Changazhikodan, followed by Kothala, Muttathukonam and Pandalur types had higher TSS content, compared to other types. Changazhikodan, Kothala and Poovanchira types had significantly lower acidity. Total sugar, reducing sugar and non-reducing

sugar content of the fruit were significantly higher in Kothala, Changazhikodan and Puthur types. The overall assessment of quality aspects projected the superiority of Kothala and Changazhikodan over the other types evaluated. Changazhikodan fruits were the tastiest. The thickness of rind and keeping quality also varied with the types (Rao, 1998).

Rajeevan and Mohanakumaran (1993) studied intraclonal variation in *Musa* (AAB) 'Mysore', and reported that there was no significant variation in quality characters like acidity and non-reducing sugars. However, there was variation in TSS, reducing sugar and total sugar.

Devi (1996) reported that dry matter production in vegetative parts such as pseudostem, corm and leaf was high in Kaliethan and Muttathukonam, low in Changazhikodan and Pandalur and the other types were intermediary. In fruits, the dry matter content was the highest in Kaliethan, followed by Puthur and Kothala. The total dry matter production was higher in Kaliethan and Muttathukonam, low in Pandaloor and Changazhikodan, while the other types were intermediary.

2.4. Variation in reaction to biotic and abiotic stress

Babylatha *et al.* (1990) observed that Pisanglilin, Sannachenkadali and Tongat were highly tolerant to leaf spot diseases. Elavazhai, Karpooravally, Njalipoovan, Dudhsagar, Mottapoovan, Dakshin Sagar, Bodles Altafort and Mysore Ethan were tolerant, and Matti, Nendran,

Myndoli and Zanzibar were highly susceptible. Even within Kaliethan, there was significant variation in the incidence of Sigatoka leaf spot among various accessions (Sunilkumar, 1997).

It was seen that cultivars varied widely in their susceptibility or tolerance to the rhizome weevil (Babylatha *et al.*, 1990). Out of the 84 banana cultivars screened, Matti, Nendran, Myndoli and Zanzibar were highly susceptible to rhizome weevil. In the studies by Devi (1996) it was observed that the extend of incidence of rhizome weevil was lower in Puthur, Kothala and Kaliethan and higher in Changazhikodan and Pandaloor types.

Attu Nendran thrives even under rainfed conditions. 'Nana Nendran' is always grown under irrigation.

'Nendran' exhibits wide variation in growth and yield characteristics throughout the country. The genetic system of *Musa* is extremely complicated. The complexity of *Musa* genetics illustrates the need for a more sophisticated system to support conventional breeding programmes (Novak, 1992). There is great potential for biotechnology in this crop. Several research priorities for the application of biotechnology to *Musa* taxonomy and germplasm conservation has been formulated (Jarret, 1990). One such priority is the use of molecular markers.

2.5. MOLECULAR MARKERS

Molecular markers are genotypic markers (Bretting and Widrlechner, 1995). They are used to study the differences among strains at molecular level. Molecular markers constitute biochemical constituents (secondary metabolites in plants) and macromolecules (protein, DNA). Biochemical markers have been used since long for the characterization of variation in a plant, now considered to be inappropriate as universal markers (Cooke, 1984).

Molecular markers have been shown to be useful for diversity assessment in a number of plant species (Waugh and Powell, 1992) Molecular markers are direct manifestations of genetic content (Weising *et al.*, 1995). They serve as reliable indices of genetic variation. In the past decade, molecular markers have very rapidly complemented the classical strategies.

The genetic markers are used for clonal identification, linkage mapping, population diversity, taxonomy, evolutionary studies, determining the genetic fidelity during micropropagation, germplasm conservation etc. (Bretting and Widrlechner, 1995).

2.5.1. ISOZYMES

Numerous attempts have been made to use isozyme polymorphism as genetic markers in *Musa* (Bonner *et al.*, 1974 ; Rivera, 1983; Jarret and Litz, 1986 a, b, c ; Bhat *et al.*, 1992). Esterase has been found specific for distinguishing 'French' plantain (AAB) from 'Bluggoe' (ABB) (Horry, 1985). No difference was observed among the different AAA triploid clones using

different isozymes (Novak, 1992). The enzyme coding loci do not constitute a random sample of genes and they are not randomly dispersed throughout the genome. Electrophoresis will detect only portion of the actual variability present in amino acid sequences (Hillis and Moritz, 1990). Some isozyme variants are not selectively neutral (De Michele *et al.*, 1991). Moreover, isozymes, are unstable markers during plant development and standardization of sampling procedures is sometimes difficult. Therefore, the isozymes have been replaced by DNA based molecular markers (Anolles and Trigiano, 1997).

2.5.2. DNA MARKERS

With the advent of molecular biology techniques, DNA based markers have replaced enzyme markers in germplasm identification and characterization as well as in gene tagging. Because of its plasticity, ubiquity and stability, DNA is the ideal molecule for such analysis (Caetano-Anollees *et al.*, 1991). Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization based markers and polymerase chain reaction (PCR) based markers (Joshi *et al.*, 1999).

2.5.2.1. Hybridisation based DNA Markers

The hybridization based DNA marker techniques utilize labeled nucleic acid molecules as hybridization probes (Anolles *et al.*, 1991). Probe molecules range from synthetic oligonucleotides to cloned DNA. Some of the important hybridization based DNA techniques are restriction fragment length polymorphism (RFLP), hypervariable sequences and variable number of tandem repeats (VNTRs).

2.5.2.1.1. Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism analysis involves digesting the genome with restriction enzymes, separating the fragments electrophoretically and then preferentially visualizing fragments containing particular homologous sequences by hybridizing them to a specific DNA probe (Deverna and Alpert, 1990 ; Walton, 1990).

Genetic diversity in *Musa* was documented using RFLPs (Gawel *et al.*, 1992). Examination of 20 *Musa* species and subspecies using total DNA was done via RFLP. The result shows that there are two clear groupings among these species, one containing species from sections Musa and Rhodoclamys and the other containing species from sections Australimusa and Callimusa.

Chloroplast DNA RFLPs were used to study cytoplasmic genetic diversity in various *Musa* species and subspecies (Gawel and Jarret, 1991). Bhat *et al.* (1994) examined nuclear and chloroplast DNA RFLP variability

within 57 *Musa* germplasm collections in order to evaluate the ability of RFLPs to identify and classify the Indian bananas.

Restriction fragment length polymorphism was used as a marker to determine the transmission of cytoplasmic DNA in diploid banana crosses. Progenies from controlled crosses were studied with heterozygous cytoplasmic probes. This analysis provided evidence for a strong bias towards maternal transmission of chloroplast DNA and paternal transmission of mitochondrial DNA in *Musa acuminata* (Faure *et al.*, 1994). Knowledge of the organelle mode of inheritance constitutes an important point for phylogeny analyses in banana and may offer a powerful tool to confirm hybrid origins.

2.5.2.1.2. Hypervariable sequences and variable number of tandom repeats (VNTR)

Kaemmer *et al.* (1993) used oligonucleotide probes to differentiate *Musa* cultivars in various genomic groups. Bhat *et al.* (1995) found that DNA fingerprinting using oligonucleotide probes was useful for cultivar identification and for overall genome analysis to establish relatedness among the various accessions of *Musa* germplasm. The presence of hypervariable sequences was confirmed in plants and animals by Gupta *et al.* (1996). Studies by Crouch *et al.* (1999) to compare different PCR – based marker systems [Random Amplified Polymorphic DNA (RAPD), Variable number of Tandom Repeats (VNTR) and Amplified Fragment Length Polymorphism

(AFLP)] for the analysis of breeding populations of *Musa* showed that VNTR analysis detected the highest levels of polymorphism.

2.5.2.2. Polymerase Chain Reaction (PCR) based DNA marker techniques

These are fingerprinting techniques that use an *in vitro* enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules (Anolles and Trigiano, 1997 ; Michelli and Bova, 1996).

Among the PCR based marker techniques, the important ones are amplified fragment length polymorphism, microsatellites, sequence characterized amplified region and random amplified polymorphic DNA.

2.5.2.2.1. Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism is based on PCR amplification of restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of few neucleotide bases (Vos *et al.*, 1995). RAPD, Inter Simple Sequence Repeats (ISSR) and AFLP markers were used to fingerprint and to examine genetic diversity among 12 genotypes of the gooseberry (*Ribes grossularia*). AFLP generated unique profiles for each genotype. Studies conducted for identification of some accessions of Musaceae at the *Musa* germplasm bank established at EI Agardo, Columbo using AFLP showed highest similarity index within the Cavendish group (Sanchez *et al.*, 1998). AFLP has been used to detect genetic differences and

somaclonal variants in *Musa* spp. (Engelborgh *et al.*, 1998). Nine accessions of *Musa* were evaluated by Amplified Fragment Length Polymorphism (AFLP). Screening of Curare enano and its medium sized off-type somaclone for polymorphisms correlated with the dwarf genotype revealed twelve differences among 104 generated fragments. Differences were also found between very closely related accessions. Crouch *et al.* (1999) while working with *Musa* breeding population found that, of the different PCR-based marker systems (RAPD, VNTR and AFLP), AFLP assays had by far the highest multiplex ratio.

2.5.2.2.2. Micro satellites

Micro satellites consist of tandemly arranged di-tri-tetra nucleotide repeats, which are hypervariable and ubiquitously distributed throughout eukaryotic genomes. Micro satellite DNA markers, which can be directly amplified by PCR, have been developed using the unique sequences that flank micro satellites (Litt and Luty, 1989; Tantz, 1989; Weber and May, 1989). Jarret *et al.* (1994) presented information on the isolation of a number of short tandemly repeated sequences or simple sequence repeats (SSRs), also known as variable number tandem repreats (VNTRs) or microsatellites, in *Musa*. Crouch *et al.* (1997) stressed the use of simple sequence repeat length polymorphism (SSRLP) assays for finger printing of *Musa* hybrids. Data on segregation at microsatellite loci in haploid and diploid gametes of *Musa* indicated the suitability of microsatellite markers for marker – assisted selection systems in *Musa*. Candidate markers for such complex and

important characters as parthenocarpy, earliness and regulated suckering are also being tested (Vuylsteke *et al.*, 1998).

Two size selected genomic libraries from banana were screened for the presence of simple sequence repeats (SSR) by Kaemmer *et al.* (1997). They demonstrated that SSR are readily applicable to the study of *Musa* genetics. Comprehensive analysis of a significant number of banana sequence tagged microsatellite sites (STMS) would add to the knowledge on the structure and phylogeny of genomes of the *Musa* species and suggested the use of microsatellites as anchor markers for a banana genetic core map.

2.5.2.2.3. Sequence Characterized Amplified Region (SCAR)

Sequence Characterized Amplified Region DNA analysis was developed to produce reliable PCR–based results. Parent and page (1998) used this technique to identify raspberry cultivars. Damasco *et al.* (1998) used markers based on SCAR to detect dwarf off-types of *in vitro* grown Cavendish bananas.

2.5.2.2.4. Random amplified polymorphic DNA (RAPD)

Polymerase chain reaction in conjunction with random primers, was used for finger printing genomes (Welsh and Mc Clelland, 1990), for population biology studies (Astley, 1992), identification of genome specific markers and other uses (Williams *et al.*, 1990 and Erlich *et al.*, 1991).

Several authors have applied the RAPD technique to investigate genetic variability and found the technique very efficient and reliable (Brown *et al.*, 1993; Munthali *et al.*, 1996).

Analysis of RAPDs offers several advantages, compared to RFLP. The most important advantage is that RAPD is not a labour intensive procedure. It is not necessary to construct or maintain a genomic library. RAPD requires smaller quantities of genomic DNA than RFLP analysis. Also it is less costly compared to RFLP. Generation of RAPD is quicker than RFLP and can be used to detect even single gene mutations (Williams *et al.*, 1990)

2.5.2.2.4.1. RAPD and linkage maps

RAPD assay has been used by several groups as an efficient tool for identification of markers linked to agronomically important traits which are introgressed during the development of near isogenic lines. Traits of interests studied include jointless pedicel in tomato (Wing *et al.*, 1994), disease resistance [*Pseudomonas* resistance (Martin *et al.*, 1991) and spotted wilt virus resistance (Chaque *et al.*, 1996) in tomato, Anthracnose resistance in mango (Subramanian *et al.*, 1996), scab resistance in apple (Hong *et al.*, 1997 and Tartarini, 1996) and Lettuce infectious yellows virus resistance in melon (*Cucumis melo*) (Mc Creight, 2000)], leaf miner resistance (Moriera *et al.*, 1999) etc.

The trait associated with the seed oil content in Indian mustard was identified with three RAPD markers *viz.* OPH -11, OPJ - 06 and OPL -15 (Sharma *et al.*, 1999). It was revealed that there was significant association of oil content with these markers.

Genetic linkage maps have been created in banana (Faure *et al.*, 1993), sweet cherry (Stockinger *et al.*, 1996), citrus (Christofani *et al.*, 1999), rose (Debener and Mattiesch, 1999) and in oilpalm (Moretzsolm *et al.*, 2000) using RAPD.

In an effort to map the loci affecting the cooking quality traits in basmati rice, a doubled haploid population from the basmati indica (Hasan Serai) x non – basmati japonica (Xiang Nuo 4) hybrid generated earlier was genotyped using 121 RAPD markers and a linkage map was constructed. Single factor analysis of variance revealed significant association between some of the markers and cooking quality traits (I.A.R.I., 1999).

2.5.2.2.4.2. RAPD and Taxonomic Studies

RAPD markers have been widely used for taxonomic and related studes. Demeke *et al.* (1992) investigated the potential use of RAPDs for taxonomic studies using *Brassica, Sinapis* and *Raphanus* taxa. Analysis of the RAPD bands revealed the relationship between diploid and amphidiploid *Brassica* taxa. Results showed that the *Raphanus sativus* and *Sinapis alba* were distinct from t*he Brassica* taxa.

Dunemann *et al.* (1994) investigated the use of RAPD markers for taxonomic studies in *Malus*. Eighteen accessions of wild species and twenty seven apple cultivars were tested with 29 pre-selected primers. The analysis of the bands using unweighted pair group arithmetic average showed the relationship among the cultivars which was in agreement with the known lineage. A dendrogram generated for wild species gave relationships that were in accordance with the known phylogenetic information.

The technical simplicity of the RAPD technique has facilitated its use in the analysis of phylogenetic relationships in several plant genera, e.g. roses (Debener *et al.*, 1996), blue berry (Levi and Rowland, 1997), barley (Noli *et al.*, 1997), *Cymbidium* (Obara – Okeyo and Kako, 1998) etc.

The genetic closeness of various species of *Vanda* was determined using RAPD markers. Strip-leaved *Vanda* sp (including *Vanda sanderiana*) and *Ascocentrum miniatum* were more closely related to each other than to the terete leaved *Vanda* species studied. RAPD analysis supported the suggestion that terete leaved *Vanda* trees and *V. hookeriana* be classified in the separate genus *Papilionanthe* and that *V. sanderiana* should remain in the genus *Vanda* (Lim *et al.*, 1999).

2.5.2.2.4.3. RAPD and somaclones

RAPD analysis was used to detect genetic variation in micropropogated Cavendish bananas (Damasco *et al.*, 1996). A RAPD marker specific to the

dwarf off-type from micropropagation of Cavendish group cultivars. New Guinea Cavendish and Williams was identified following an analysis of 57 normal and 59 dwarf plants generated from several different micropropagation events. Of the 66 random decamer primers used in the initial screen, 28.8 per cent revealed polymorphisms between normal and dwarf plants. Use of this marker could facilitate early detection and elimination of dwarfs from batches of micropropagated bananas. Results of studies by Hammerschlag *et al.* (1996) showed the feasibility of using tissue culture methods to generate fruit trees with increased level of disease resistance. RAPD was used to study genetic variation at the DNA level among somaclonal variants. Four different types of somaclonal variants were identified and characterized in banana plants generated by meristem culture (Walther *et al.*, 1997). Tissue cultured off types did not display any visual differences during *in vitro* culture. But after six weeks of hardening in a commercial nursery, the field established plants showed significant phenotypic difference. RAPD analysis of somaclonal variants revealed the presence of polymorphic bands with at least one set of primers. This enabled early detection of somaclonal variants. This allows the elimination of off-types before planting of micropropagated plants in the field. Randomly amplified polymorphic DNA markers were found to be useful for confirmation of genetic fidelity in micropropagated plants (Gupta *et al.*, 1999). Somaclonal variant CIEN BTA – 03 resistant to yellow sigatoka was obtained from a susceptible banana clone (Williams clone) by increasing the production of adventitious buds using 6-belicilapurine at high concentration (Vidal and Garcia, 2000).

According to Lu *et al.* (1996), RAPDs are useful for establishing a genetic basis for somaclonal variation in rice. The results of RAPD analysis in cultured rice showed that somaclonal variation might have occurred in transfer RNA, ribosomal protein and other genes during cell culture. Also somaclonal variation was found to increase with culture age (Yang *et al.*, 1999).

Random amplified polymorphic DNA technology was applied to monitor the genetic fidelity of micropropagated, meadow fescue *viz.*, *Festuca pratensis* (Valles *et al.*, 1993), Norway spruce (Heinze and Schemidt, 1995) and Strawberries (Kumar *et al.*, 1995).

Somaclonal variants were reported in *Triticum aestivum* (Brown *et al.*, 1993) *Populus* (Rani *et al.*, 1995), beet (Munthali *et al.*, 1996) peach (Hashmi *et al.*, 1997) tomato (Hong *et al.*, 1999), grapes (Verdisson *et al.*, 1999) and pigeon pea (Prasannalatha *et al.*, 1999) using RAPDs.

Plants regenerated by somatic embryogenesis from long term callus cultures derived from five garlic cultivars were subjected to RAPD analysis (Al-Zahim *et al.*, 1999). Certain changes were observed in the RAPD profiles of the regenerants of different cultivars, suggesting the existence of somaclonal variants.

RAPD analysis was done by Babu (2000) to access the genetic stability in tissue culture derived black pepper plants. Monomorphic banding pattern was observed for the tissue culture regenerants, compared with their respective source plants. Uniformity was confirmed at both stages of development studied. Thus genetic stability and clonal fidelity was ensured for the tissue culture regenerants and the viability of the protocol was confirmed.

2.5.2.2.4.4. RAPD and hybrids

RAPD technique has been used for the identification of hybrids and their parent determination as well. Wang *et al.* (1994) proposed RAPD fingerprinting as a convenient tool for the identification, protection and parentage determination of plant hybrids. In their study, DNA from three families of rice plants selected in Northern China (each comprising the male sterile, the restorer, the hybrid F1 and the maintainer lines) was extracted and amplified by RAPD technique. The results obtained were useful for identification of each single plant line.

Truksa and Prochazka (1996) reported different banding pattern based on the DNA polymerase used for testing three lines of cucumber used for the production of hybrid seeds. Low level of polymorphism was obtained which indicated that RAPD was not suitable for verifying the hybridity of seeds.

RAPD markers have been successfully used to test the paternity of Japanese pear hybrid (Banno *et al.*, 2000).

2.5.2.2.4.5. RAPD for identification of somatic hybrids.

One of the limiting factors for the efficient exploitation of protoplast fusion is the difficulty of unequivocally identifying nuclear hybrids. RAPDs have been used to characterize molecularly both interspecific and intraspecific somatic hybrids. Baird *et al.* (1992) proposed RAPDs for the identification of hybrids at an early stage following fusion in potato. Inter and intra–specific somatic hybrids of potato were characterized by using RAPD along with sexual hybrids.

Xu *et al.*, (1993) used RAPD assay for the identification of somatic hybrids between *Solanum tuberosum* and *Solanum brevidens*. Somatic hybrids showed a combination of the parental banding pattern with four of the five primers screened whereas regenerants from one of the parents had a similar banding pattern as that of the parent.

2.5.2.2.4.6. RAPD in sex determination

Early identification of sex in dioecious plants like papaya (Somri, 1998) and nutmeg (Shibu *et al.*, 2000) was possible with the help of RAPD markers.

Genotypic and morphogenetic differences among three female varieties of *P. longum*, one variety each from Assam & Calicut and one variety released from Kerala, were investigated using RAPD analysis and it was revealed that these varieties were genetically different. In *Piper longum*, RAPD technique was used to investigate the molecular basis of genotypic differentiation between the male and female parents (Banerjee *et al.*, 1999). As a result male sex associated RAPD markers were identified for the first time in *Piper longum*.

2.5.2.2.4.7. RAPD detection of genetic variability

RAPD markers have been used successfully to detect genetic variation among lowland and upland rice cultivars and the genetic characterization and classification of Japonica cultivars into temperate and tropical groups for analysis of genetic variability in rice populations (Yu and Nguyen, 1994). In general, a higher level of polymorphism was found between upland and lowland cultivars within the *indica* subspecies. Thus among rice cultivars, genetic variation was easily detected using RAPDs. According to Verma *et al. (1999)*, RAPD analysis allows the identification and discrimination of the individual genotypes of Basmati rice including the identification of duplicates in genetic resource collections. Random amplified polymorphic DNA technique was used to detect the genetic variation at the level of DNA among aromatic and non-aromatic cultivars by Baishya *et al.* (2000). With the objective of identifying and classifying 48 aromatic rice genotypes, RAPD profiling was employed using 58 random decamer primers. Most of these primers (96.5 per cent) detected polymorphism among the genotypes. Of the

465 amplified bands, 314 were polymorphic. All the rice genotypes included in the study could be distinguished from each other at the level of 19 to 186 polymorphic bands between individuals in pair wise comparison over all the 58 primers (Choudhary *et al.*, 2001).

Lashermes *et al.* (1996) have successfully employed RAPD markers to analyse genetic diversity among cultivated and sub spontaneous accessions of *Coffea arabica.* The narrow genetic base of commercial cultivars were confirmed by their study. On the other hand, a relatively large genetic diversity was observed within the germplasm collection. Results suggested an East-West differentiation in Ethiopia, the primary centre of diversification of *Coffea arabica.*

Machado *et al.* (1996) carried out a study at Brazil to evaluate polymorphism and genetic similarity between 39 Mediterranean mandarin genotypes, using RAPD analysis. One hundred and eleven amplification products were identified using 21 random primers. Cluster analysis revealed a low level of genetic variation between accessions of Mediterranean mandarins, whereas their hybrids with other citrus species showed greater genetic dissimilarity.

Duran *et al.* (1997) analysed 48 coconut types belonging to East African Tall types by different DNA marker techniques including RAPDs, microsatellite primed PCR and Inter Specific Tandem Repeats (ISTR) analysis. All three approaches detected large number of DNA polymorphism among the set of genotypes and allowed the identification of single genotypes

by individual-specific fingerprints. The cluster and principal co-ordinate analysis were done and the observed clustering and association of individuals corroborated the expectations based on the known geographical origin and parental relationships.

Varghese *et al.*, (1997) evaluated the applicability of RAPD markers in the cultivated rubber tree, *Hevea*, using 43 primers in a set of 24 clones selected from different south-east Asian countries. Out of the total 220 fragments amplified, 111 were polymorphic. The statistical analysis indicated the absence of a distinct geographical grouping because of the breeding history of *Hevea*.

Keil and Griffin (1994) assessed the reproducibility of the RAPD technique and its ability to discriminate between individual genotypes for verification of clonal identities in four separate studies, using samples of *Eucalyptus* supplied by several organizations. RAPD profiles unique to a genotype were generated reliably and simply and even closely related genotypes were distinguished.

Random amplified polymorphic DNA markers were used for fingerprinting genotypes within and between *Annona* species. (Renning *et al.*, 1995). The use of RAPD analysis for *Mangifera* germplasm classification and clonal identification was reported by Schnell *et al.* (1995). Graham and Mc Nicol (1995) generated RAPD markers from different *Rubus* species in order to access the degree of similarity between species. Inter and intra specific /

varietal variations were observed in the RAPD analysis of 42 accessions of *Vitis*, representing 13 species (Wang *et al.*, 1999). According to Lanham and Brennen (1999), RAPD markers were used to fingerprint and to examine genetic diversity among 12 genotypes of gooseberry. Six hazelnut (*Corylus avellana*) cultivars were identified using RAPD markers (Galderisi *et al.*, 1999). An RAPD analysis was carried out by Egashira *et al.* (2000) to investigate genetic diversity of 'peruvianum-complex' (PC) species of highly polymorphic wild tomato relatives and the genetic relationship between the PC and the 'esculentum – complex' (EC) species including the cultivated species. A total of 435 RAPDs were obtained from 50 accessions of all the 9 *Lycopersicon* species using only 16 random primers.

RAPD markers can be used to detect genetic variation at the intra as well as interspecific level (Aboelwafa *et al.*, 1995).

Randomly amplified polymorphic DNA analysis was done to determine intra-specific variability in *Andrographis paniculata*. The results indicated that RAPD could be effectively used for genetic diversity analysis in wild species of prospective value as it is reliable, rapid and superior to those based on pedigree information (Padmesh *et al.*, 1999).

According to Hu and Quiros (1992), RAPD markers provided a quick and reliable alternative to identify broccoli and cauliflower cultivars. RAPD markers generated by four arbitrary-10-mer primers, discriminated 14

broccoli (*Brassica oleraceae italica*) and 12 cauliflower (*B. oleraceae botrytis*) cultivars by banding profiles.

Iqbal *et al.* (1995) used RAPD markers to establish polymorphisms among local sugarcane varieties. The amplification profiles of the varieties L118, L116, BL4, BF162, CO144 and CO154 were compared and polymorphisms were detected.

The RAPD technique was used for cultivar identification of 11 aubergine cultivars. Twelve 10-mer primers were used, 9 of which revealed polymorphism in cultivars (Kochieva *et al.*, 1999).

Randomly amplified polymorphic DNA markers were extensively used for the molecular characterization of various crop species. RAPD markers have been used to characterize germplasm in several important crop species including *Carica papaya* L. (Stiles *et al.*, 1993), rice (Fukoka *et al.*, 1992), apple (Koller *et al.*, 1993) and pigeon pea (Ratnaparkhe *et al.*, 1995).

RAPD markers have been used to characterize cocoa clones representing the three main cultivated subpopulations *viz.* Criollo, Forestro and Trinitario (Wilde *et. al.*, (1992). The use of single primers of arbitrary nucleotide sequence resulted in the selective amplification of DNA fragments which were unique to the individual cocoa clones studied. Mulcahy *et al.* (1993) characterized twenty five accessions of apple, representing eight cultivars (Golden Delicious, Delicious, Gala, Jonathan, Jonagold, Florina,

Fior di Cassia and Imperate Dallago) with RAPD. Using separate ten base pair primers, it was possible to obtain a distinctive fingerprint for each of the cultivars. Thus RAPD provided a simple and reliable method for cultivar identification in apple. Efforts were done in *Piper longum* to find out the genetic difference among the varieties using RAPD analysis by Phillip *et al.*(2000).

RAPD and SSR markers were used to characterize genetic relationship among 46 accessions in two *Cucumis melo* L. subsp. (*Cantaloupensis lodorus*) and subsp. *agrestis* (Conomon and Flexuosus) groups (Jack *et al.*, 2000). Empirical estimation of variances associated with each marker type in the accessions examined indicated that per band, lower coefficients of variation can be attained in the estimation of genetic diversity when using RAPDs compared to SSRs. Result of RAPD marker analysis suggest that 80 marker bands were adequate for assessing the genetic variations present in the accessions examined.

Evaluation of the genetic diversity among 27 superior tea accessions (*Camellia sinensis* var. *sinensis*) accessions from Korea, Japan and Taiwan was done by Kaundun *et al.* (2000) using RAPD-PCR markers. Out of the 50 primers screened, 17 primers generated 58 polymorphic and reproducible bands. A minimum of three primers was sufficient to distinguish all the 27 accessions studied.

In order to assess genetic diversity of clones of a subset collection of wild apple which were collected at forest sites in the federal state of Herse, PCR based methods were applied by Vornam and Gebhardt (2000). RAPD analysis allowed them to distinguish clones and single tree progenies.

Using the technique of RAPD, Howell *et al.* (1994) identified 116 amplification products in *Musa* germplasm using nine primers. This enabled them to identify RAPD markers that are specific to each of the nine genotypes of *Musa* representing AA, AAA, AAB, ABB, and BB genotypes. Fifty seven accessions of *Musa* including cultivated clones of six genomic groups (AA, AB, AAA, AAB, ABB, ABBB), *Musa balbisiana* Colla (BB), *Musa acuminata* Colla ssp. Banksii F. Muell. (AA), *Musa acuminata* Colla ssp. *malaccensis* Ridl. (AA) and *M. velutina* Wendl. and Drude were examined by Bhat and Jarret (1995) for RAPD genetic markers using PCR with sixty 10-mer random primers which generated 605 polymorphic amplification products. RAPD analysis was performed on several clones of the variety Williams by Iqbal *et al.* (1995). Results of the studies by Bhat *et al.* (1995) on DNA profiling of banana and plantain using RAPD and RFLP markers showed that the use of different kinds of molecular markers in gene banks is essential for characterization and classification of germplasm collections.

RAPD using operon primers, was used to evaluate genetic variability of 66 *Musa* spp accessions in the germplasm collections of the National Banana Corporation of Costa Rica (CORBANA) and the Agronomic Centre for Research and Training (CATIE) also in Costa Rica. PCR products were

separated electrophoretically in agarose gels which were then analysed on a binary basis [presence (1) or absence (0) of bands]. The data obtained were used to generate a similarity matrix according to Jaccard's criteria, and a phenogram was built. High variability among the AA clones and a low variability among the AAA, AAB, ABB triploids were observed (Cabrera *et al.*, 1998).

With the aim of identifying RAPD markers for the A and B genomes, Pillay *et al.* (2000) used eighty 10-mer operon primers to amplify DNA from *Musa acuminata* subsp. *burmanicoides* clone Calcutta 4 (AA genomes) and *M. balbisiana* clone Honduras (BB genomes). Three primers (A-17, A-18 and D-16) that produced unique genome specific fragments in the two species were identified. The results showed that RAPD analysis can provide a quick and reliable system for genome identification in *Musa* that could facilitate genome characterization and manipulations in breeding lines.

Genetic diversity among 76 plantain land races has been studied by Crouch *et al.* (2000) using RAPD analysis at two levels of intensity and compared with groupings based on phenotypic indices and morphotypes. There was also a poor correlation between RAPD analyses and morphotype do not provide a true reflection of overall genetic divergence.

RAPD analysis of fifteen African plantain land races by Newbury *et al.* (2000) revealed a very low proportion of polymorphic bands (13 of 276). However, further examination of these thirteen marker bands demonstrated that they varied within land races and could not be used to distinguish between land races.

Materials and Methods

3. MATERIALS AND METHODS

Investigations were carried out at the Department of Pomology and Floriculture and the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani during January 2000 to October 2001 for molecular characterization of banana (*Musa* AAB Plantain subgroup) clones. The materials and methods tried for the research work are described in this chapter.

Two suckers each from the following clones were maintained in the Instructional farm, College of Agriculture, Vellayani for collecting plant tissues for the study.

Attunendran

Changanasseri Nendran

Changazhikodan

Manjeri Nendran

Mysore Ethan

Myndoli

Padalamurian

Zanzibar

Kaliethan

Koonoor Ethan

Quintal Banana

Suckers of Kaliethan, Koonoor Ethan and Quintal Banana were collected from the Instructional farm, College of Agriculture, Vellayani and the rest of the clones were collected from Banana Research Station, Kannara. Planting was done in October 2000. Suckers of about three to four months age were used for planting. Spacing adopted was 2x2 m. The plants were grown under uniform conditions following the package of practices recommendations of Kerala Agricultural University (KAU, 1996).

3.1. Isolation of genomic DNA

For the isolation of genomic DNA, the tissues from the emerging leaves of all the eleven clones of plantain before they fully unfurl were used. The method of isolation followed was a modified method given by Walbot (1988). This method is less expensive and it has been found from earlier studies conducted at PMBBC, College of Agriculture, Vellayani by Nayar (2001) that this method yielded more quantity of DNA in red banana, compared to the other methods [Methods of Aljanabi and Martinez (1997), Walbot(1988) and Rogers and Benedich (1994)].

Modified method given by Walbot (1988) is as follows :-

About 10 g of emerging leaves of Nendran banana before they fully unfurl was used for DNA extraction. The leaves were collected in the

morning (around 8.00 am) and first washed in running tap water and later in distilled water two or three times after chopping the leaves coarsely. After wiping off the water using tissue paper, the chopped leaf was placed in a cool dry porcelain container and liquid nitrogen was poured over the leaf material. The leaves were ground well to a fine powder and transferred to the extraction buffer (168 g urea, 28 ml 5 M NaCl, 20 ml 1M tris HCl, pH 8.0, 16 ml 0.5 M EDTA, 20 ml phenol, made up to 400 ml with sterile water) placed in the water bath at a temperature of 55°C. A volume of 2.5 ml of 20 per cent SDS and a pinch of polyvinyl pyrollidone (PVP) were added and mixed gently. The temperature of the water bath was set at 50°C and the lysis buffer was allowed to equilibriate at that temperature. The solution was kept at 50°C for 10 minutes. The lysate was then squeezed through four layers of sterile muslin cloth. Then 25 ml of phenol : chloroform : isoamyl alcohol (25: 24: 1) solution was added and kept at room temperature for 15 minutes. The same was then centrifuged at 10000 rpm for 10 minutes at 4.0 °C. After collecting the upper phase, the phenol: chloroform : isoamyl alcohol (25: 24: 1) extraction was repeated until the interphase disappeared. After that, to the aqueous phase collected, equal volume of chloroform : isoamyl alcohol (24: 1) solution was added and the two phases were mixed gently. Centrifugation was done at 10000 rpm for 10 minutes at 4.0°C. To the upper phase collected $1/10^{th}$ volume of 3.0 M sodium acetate and two volumes of cold absolute ethanol were added. It was mixed gently and DNA strings were spooled out with a glass rod. The DNA was washed with 70 per cent cold ethanol .

The pellet was allowed to dry and dissolved in 100 µl to 200 µl TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, pH 8.0) and stored at 4.0°C.

All the materials used in the preparation and storage of reagents including reagent bottles, conical flasks, centrifuge tubes, spatula, glassrods, funnels and tips of micro pipettes were washed with Labolin solution and rinsed with distilled water and autoclaved for 45 minutes before use. Phenol used was saturated and equilibriated using Tris buffer and pH adjusted to 8.0.

3.2. Quantification of DNA

Reliable quantification of DNA concentration is important for many applications in molecular biology including amplification of target DNA by polymerase chain reaction. DNA quantification was carried out with the help of UV- Vis spectrophotometer (Spectronic Genesis 5).

The spectrophotometer was calibrated at 260 nm and 280 nm wave length using TE buffer, in which DNA was dissolved. The optical density (O.D) of the DNA sample dissolved in the buffer was recorded at both 260 nm and 280 nm.

Since an O.D of 1.0 at 260 nm represent 50 μgml^{-1} of DNA, the quantity of DNA in the sample was estimated by employing the following formula :

$$\text{Amount of DNA } (\mu gml^{-1}) = A_{260} \times 50 \times \text{dilution factor}$$

(where, A_{260} = absorbance at 260nm)

The quality of DNA could be judged from the ratio of the O.D values recorded at 260 nm and 280 nm. A ratio between 1.8 and 2.0 indicates good quality of DNA.

3.3. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit supplied by the Bangalore Genei. Various conditions required for carrying out the gel electrophoresis were standardized. These include concentration of agarose, voltage and type of buffer. The concentrations of agarose tried were 0.7 per cent, 0.8 per cent, 0.9 per cent and 1.0 per cent for visualizing the genomic DNA. For RAPD analysis, 1.2 per cent, 1.3 per cent, 1.4 per cent and 1.5 per cent concentrations were tried. The voltage levels tried were 50 V, 60 V, 75 V and 100V. The types of buffer used were 1x TAE buffer (0.04 M Tris acetate, 0.01 M EDTA, pH 8.0) and 0.5 x TBE buffer (0.045 M Tris borate, 0.001 M EDTA, pH 8.0).

The required amount of agarose was weighed out (0.9 per cent for visualizing the genomic DNA and 1.4 per cent for visualizing the amplified products) and added to 0.5x TBE buffer. Agarose was dissolved by boiling. After cooling to about 50°C, ethidium bromide was added to a final concentration of 0.5 $\mu g ml^{-1}$. The mixture was poured immediately to a preset template with appropriate comb. After solidification, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank filled with 0.5x TBE running buffer. The gel was completely covered on the surface by the buffer. The DNA sample was mixed with required volume of gel loading buffer (6.0x loading dye *viz.* 40 per cent sucrose, 0.25 per cent bromophenol blue). Each well was loaded with 20 µl of sample. One of the wells was loaded with 5.0 µl of molecular weight marker along with required volume of gel loading buffer. Electrophoresis was performed at 75 volts until the loading dye reached $3/4^{th}$ of the length of the gel. The gel was visualized using a ultravisible (UV - Vis) transilluminator (Appligene oncor, France).

3.4. Random Amplified Polymorphic DNA (RAPD) analysis

Random Amplified Polymorphic DNA analyses were performed, following the protocol standardized by Nayar (2001) for red banana. Forty arbitrarily designed decamer primers supplied by Operon Inc., CA, USA were used.

Genomic DNA (20 ng) was amplified *in vitro* in a reaction mixture containing 2.5 μl 10x buffer (10 mM Tris HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01 per cent gelatin), 5 pM primer, 200 μM each of deoxy nucleotides (dNTPs) and 0.6 units of Taq DNA polymerase (Bangalore Genei. Pvt. Ltd., Bangalore). Amplifications were carried out in a Programmable Thermal Controller(MJ Research, Inc.) set for the following programme. An initial denaturation at 95°C for 3.0 minutes, followed by 45 cycles of denaturation at 95°C for 1.0 minute, annealing at 36°C for 1.0 minute 30 seconds and extension at 72°C for 2.0 minutes. The synthesis step of the final cycle was extended further by 6.0 minutes. Finally the products of amplification were cooled to 4.0°C until attended. A negative control containing water, instead of template DNA was included in each reaction set.

Amplified products along with *DNA* molecular weight marker supplied by US Biochemicals were separated by electrophoresis using 1.4 per cent agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

The number of monomorphic bands, number of polymorphic bands and intensity of bands were recorded. Those primers which when used for amplification produced the maximum number of bands were used to amplify the DNA of all the eleven plantain clones. The photographs of the amplification profile obtained in all the plantain clones amplified *in vitro*

using two of the primers, were taken with the help of gel documentation system. The RAPD bands were represented as '+' (for presence) and '-' (for absence) and recorded. The PCR was repeated at least twice in order to check the reproducibility. The amplification products of those primers alone, which could produce amplification for most of the clones were used for further analysis.

3.5. Data analysis

The reproducible bands were scored for their presence (+) or absence (-) for all the plantain clones studied. A genetic similarity matrix was constructed using the Jaccard's coefficient method (Jaccard, 1908).

$$Sj = a / (a+b+c)$$

where,

a : number of bands present in both the clones in a pair.

b : number of bands present in the first clone but not in the second one.

c : number of bands present in the second clone but not in the first.

Based on the similarity co-efficient, the distance between the clones was computed with the help of the software package SYSTAT (version 9). Using these values of distances between clones, a dendrogram was constructed by following the nearest neighbour (single link) method (Krzanowski, 1988). Association between the various clones was found out from the dendrogram.

Results

4. RESULTS

The results of the investigations carried out for characterizing the banana (*Musa* AAB Plantain subgroup) clones using RAPD markers are presented in this chapter.

4.1. DNA isolation

Modified Walbot method standardized for red banana (Nayar, 2001) was followed for DNA isolation. Emerging leaves before fully unfurling were the best with respect to DNA yield and purity.

The leaves collected were either used fresh for extraction or stored in an ultra freezer (Sanyo ultra low) at –85°C. It was shown that this storage did not interfere with the yield of DNA (Table 1). When fresh leaves were used, the yield of DNA was 280 μg ml^{-1}. The leaves after storage in the ultra freezer for one week yielded 390 μg ml^{-1}. Purity ratio was also not affected by storage. The purity ratio of the sample used fresh was 1.69 while that of sample used after storage was 1.86.

Table 1. **Effect of storage of leaves on the quantity and quality of the DNA of Padalamurian**

	A260	A280	A260/ A280	DNA yield (µg/ml)
Fresh leaves	0.056	0.033	1.69	280
Leaves stored at -85°C	0.078	0.042	1.86	390

A260 - Absorbance at 260 nm

A280 - Absorbance at 280 nm

A260/ A280 - Optical Densiity (O.D ratio)

4.2. Purification of DNA

DNA pellets obtained were brown in colour. Addition of PVP and β-mercaptoethanol to the extraction buffer along with other reagents reduced the browning of the pellet.

The DNA yield of various clones ranged from 280μg ml^{-1} (Padalamurian) to 3220μg ml^{-1} (Changazhikodan) . The O.D. ratios (Table 2) ranged from 1.57 (Myndoli) to 2.07 (Quintal Banana).

4.3. Gel Electrophoresis

The quality of DNA was assessed by gel electrophoresis also. When agarose gel electrophoresis was carried out using genomic DNA, in some of the samples, a continuous or smearing band was obtained. RNA was observed as a thick band below the genomic DNA. For the rest of the clones, the DNA was observed as a crisp single band.

Various conditions required for carrying out the gel electrophoresis were standardized. These include the concentration of agarose, voltage and the type of buffer used. For visualizing genomic DNA, 0.9 per cent agarose was the best among the concentrations (0.7 per cent, 0.8 per cent, 0.9 per cent and 1.0 per cent) tried. For RAPD analysis, 1.2 per cent, 1.3 per cent, 1.4 per cent, 1.5 per cent and 1.6 per cent agarose were tried. Of these, 1.4 per cent was the best.

Table 2 **Quality and quantity of DNA isolated from different clones of banana (*Musa* AAB plantain subgroup) using modified Walbot's method**

Sl.No.	Clone	A260	A280	A260/ A280	DNA yield (µg/ml)
1	Attu Nendran	0.173	0.092	1.88	865
2	Changanasseri Nendran	0.166	0.086	1.93	830
3	Changazhikodan	0.644	0.327	1.97	3220
4	ManjeriNendran	0.106	0.066	1.6	530
5	Mysore Ethan	0.171	0.094	1.8	855
6	Myndoli	0.145	0.092	1.57	725
7	Padalamurian	0.056	0.033	1.69	280
8	Zanzibar	0.106	0.063	1.68	530
9	Kaliethan	0.093	0.046	2.02	465
10	Koonoor Ethan	0.285	0.146	1.95	1425
11	Quintal Banana	0.122	0.059	2.07	610

Among the various voltage levels (50, 60, 75 and 100V) tried, 75 V was the best. Melting of agarose gel was noticed at higher voltage which rendered the gel sticky, causing difficulty in handling of the gel. The bands were brighter when thin gels were used, compared to thick ones. Out of the two types of buffer used (1x TAE buffer : 0.04 M Tris acetate, 0.01 M EDTA, pH 8.0 and 0.5x TBE buffer : 0.045 M Tris borate, 0.001 M EDTA, pH 8.0), TBE buffer was better for the separation of bands. Agarose gels and the buffer could be re-used several times.

4.4. Polymerase chain reaction (PCR)

Polymerase chain reaction conditions, standardized for the amplification of the DNA from Red banana plant (Nayar, 2001), were used for the plantain clones. The same conditions could produce good amplification for plantain clones also. Twenty nanogram of DNA, 200 µM each of dNTPs, 0.6 units Taq DNA polymerase and 5.0 pM primer in presence of 1x assay buffer gave good amplification. The programme consisted of an initial denaturation at 95°C for 3.0 minutes, followed by 45 cycles of denaturation at 95°C for 1.0 minute, annealing at 36^0C for 1.0 minute 30 seconds and extension at 72°C for 2.0 minutes. The synthesis step of the final cycle was extended further by 6.0 minutes. The amplification products were cooled to 4.0°C after the reaction.

Out of the 40 primers used, all the primers except OPA-04, OPA-05, OPA-06, OPA-08, OPA-09, OPA-10, OPA-11, OPA-12 and OPB-09 yielded amplification products with the DNA from Attu Nendran. The total number of bands, number of faint bands and the number of intense bands produced by the primers are given in Table 3.

A total of 106 RAPDs (average of 2.65 bands per primer) were generated, of which 94.43 per cent (100 bands) were polymorphic. This accounts to an average of 2.5 bands per primer. Primers varied in their ability to yield banding patterns with the template DNA (Fig.1 to 4). Monomorphic bands were produced by the primers OPA-14, OPA-15, OPB-03, OPB-08, OPB-11 and OPB-13.

The highest number of RAPDs (six each) were produced by the primers OPA-03, OPB-04, OPB-05, OPB-10 and OPB-18. Of these primers, the highest number of intense bands (four bands each) were produced by OPA-03 and OPB-10. OPB-18 produced three intense and three faint bands. OPB-18 produced two intense and four faint bands. The least number of intense bands (one band) was obtained when OPB-05 was used for amplification. The highest number of faint bands (five) was given by OPB-05.

OPA-01, OPA-02, OPA-20, OPB-01, OPB-06, OPB-12 and OPB-15 when tried for RAPD analysis produced five bands each. Among these primers, the highest number of intense bands (four) was obtained for OPB-12. OPA-01 and OPB-06 produced three intense bands. Two intense bands were obtained when OPA-20 and OPB-01 were tried.

Table–3 Primer associated banding patterns with the DNA of Attu Nendran using 40 primers (belonging to kit A and kit B) supplied by the Operon Inc.,CA, USA.

Sl.No	Primers	No.of faint bands	No.of intense bands	Total no.of bands
1	OPA-01	2	3	5
2	OPA-02	5	0	5
3	OPA-03	2	4	6
4	OPA-04	0	0	0
5	OPA-05	0	0	0
6	OPA-06	0	0	0
7	OPA-07	0	2	2
8	OPA-08	0	0	0
9	OPA-09	0	0	0
10	OPA-10	0	0	0
11	OPA-11	0	0	0
12	OPA-12	0	0	0
13	OPA-13	1	3	4
14	OPA-14	1	0	1
15	OPA-15	1	0	1
16	OPA-16	1	1	2
17	OPA-17	1	2	3
18	OPA-18	1	3	4
19	OPA-19	2	1	3
20	OPA-20	3	2	5
21	OPB-01	2	3	5
22	OPB-02	0	2	2
23	OPB-03	1	0	1
24	OPB-04	4	2	6
25	OPB-05	5	1	6
26	OPB-06	2	3	5
27	OPB-07	2	1	3
28	OPB-08	1	0	1
29	OPB-09	0	0	0
30	OPB-10	2	4	6
31	OPB-11	0	1	1
32	OPB-12	1	4	5
33	OPB-13	0	1	1
34	OPB-14	0	2	2
35	OPB-15	5	0	5
36	OPB-16	2	0	2
37	OPB-17	1	1	2
38	OPB-18	3	3	6
39	OPB-19	3	0	3
40	OPB-20	2	1	3

Fig.1. Amplification profiles (total bands) of the DNA of Attu Nendran using 20 primers (belonging to kit A)

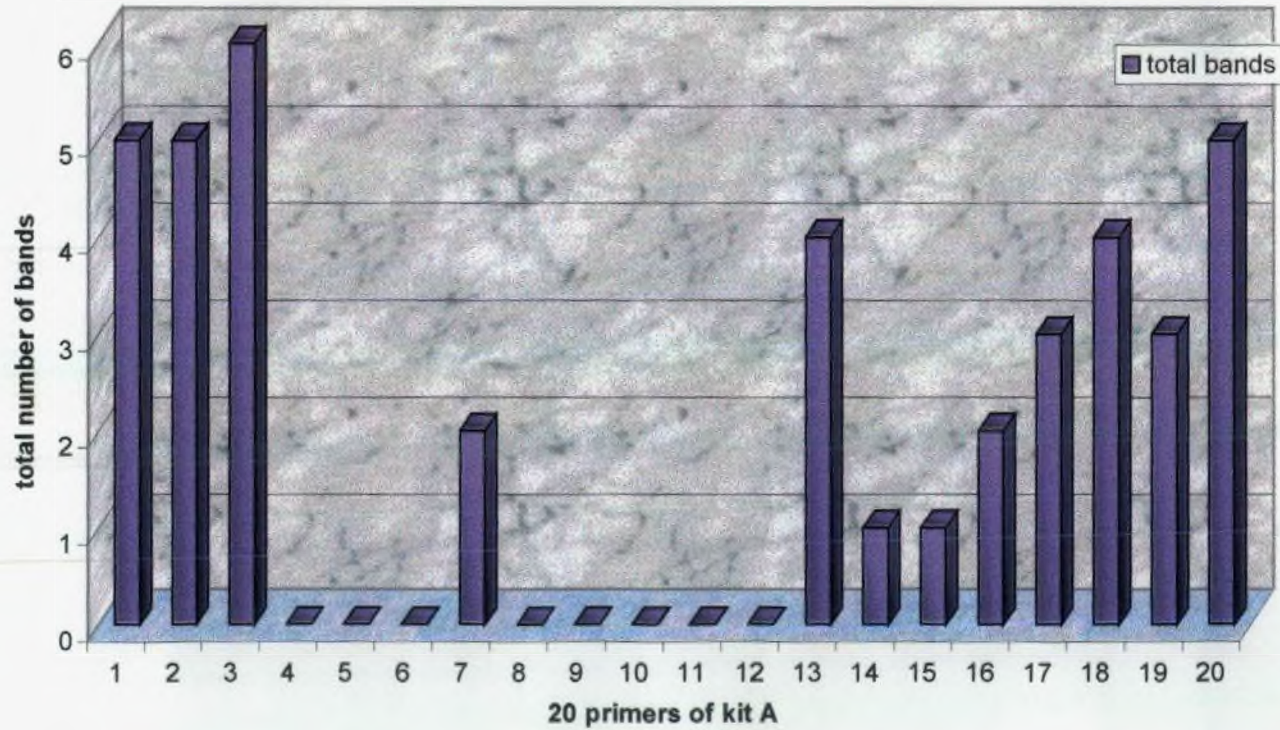


Fig. 2 Amplification profiles (total bands) of the DNA of Attu Nendran using 20 primers (belonging to kit B) from Operon Technologies Inc., CA, USA

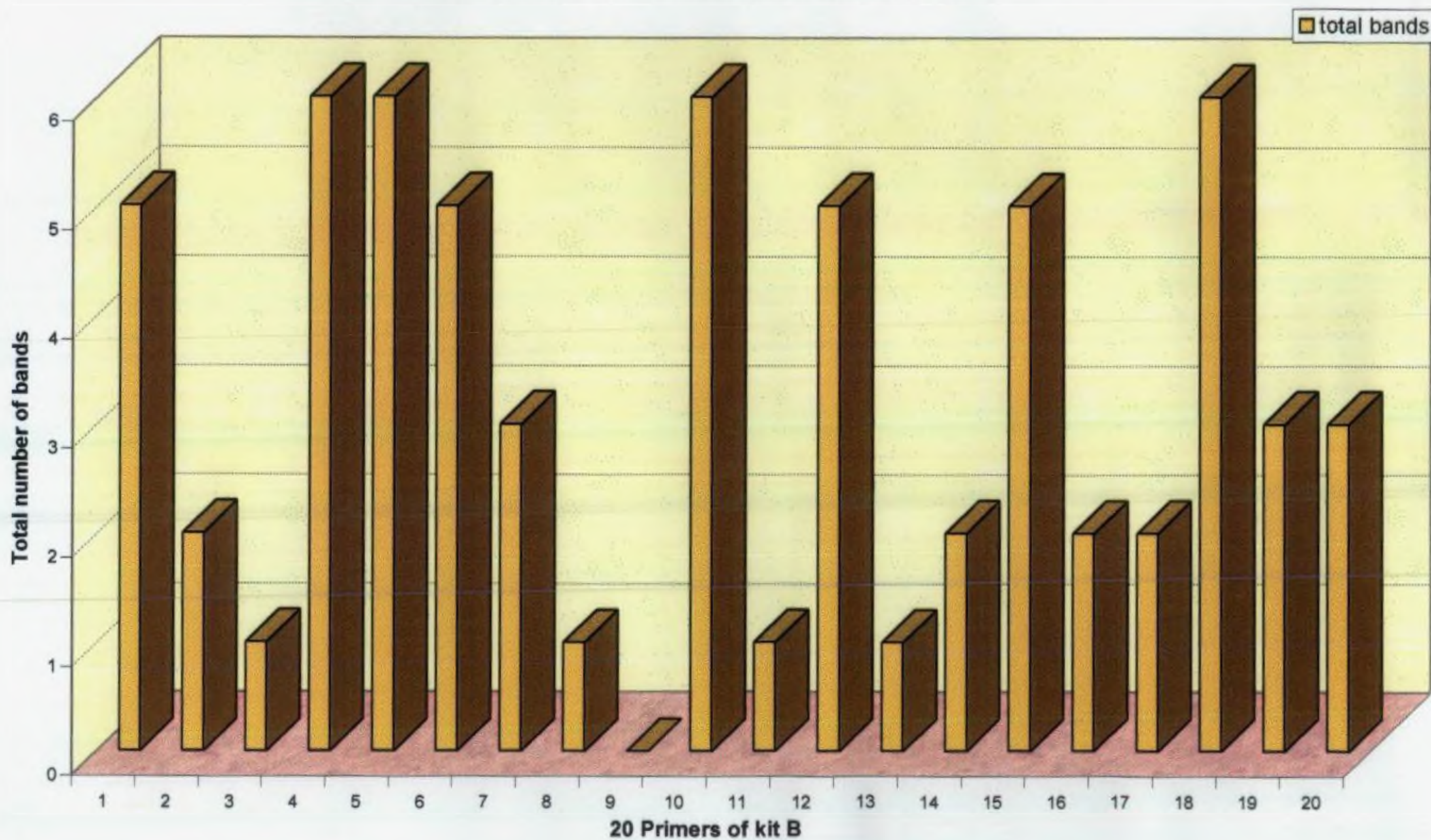


Fig. 3 Amplification profiles (intense and faint bands) of the DNA of Attu Nendran using 20 primers(belonging to kit A)

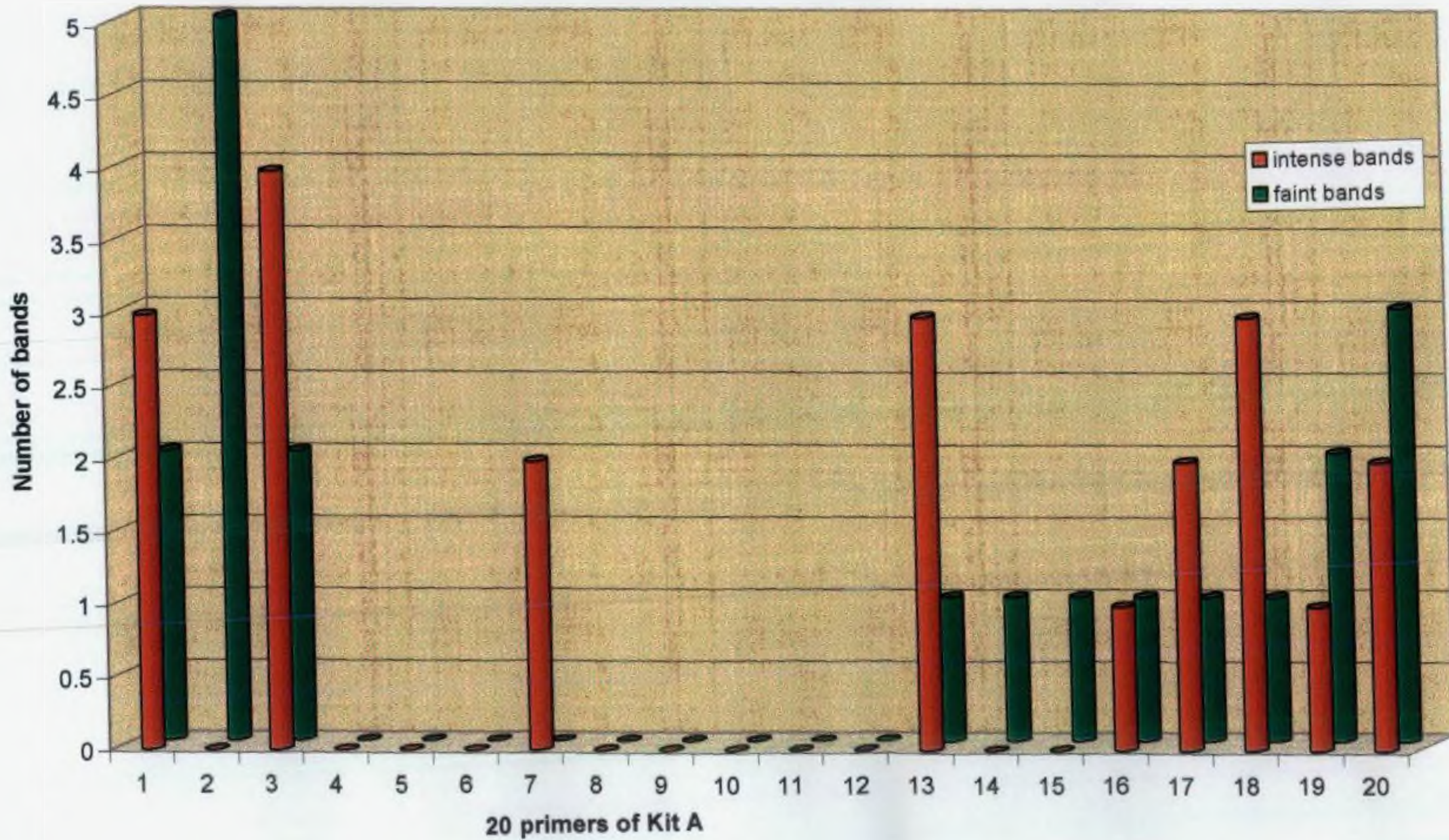
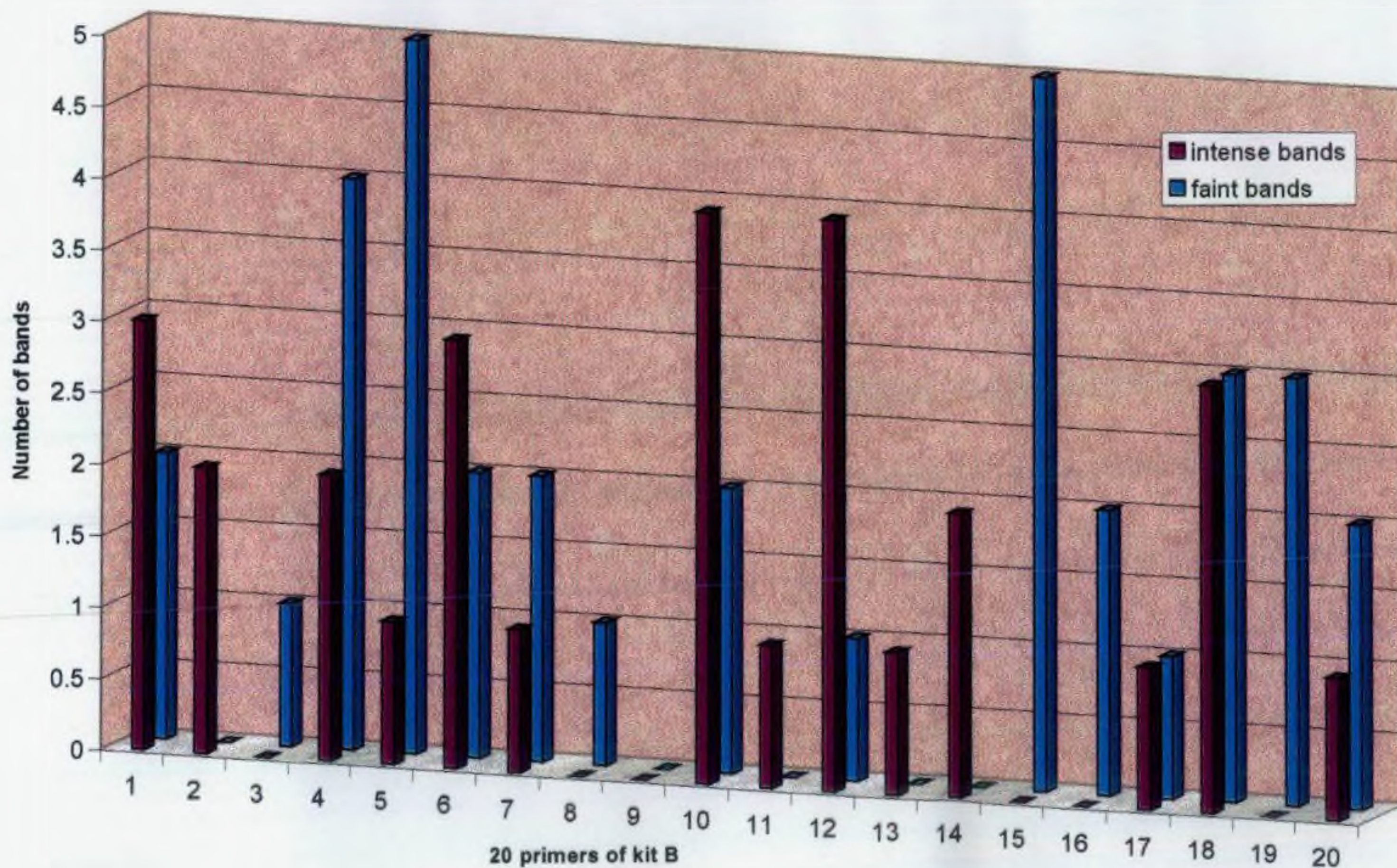


Fig.4. Amplification profiles (intense and faint bands) of the DNA of Attu Nendran using 20 primers (belonging to kit B)



The highest number of faint bands (five) was produced by OPA-02 and OPB-15. These two primers did not produce any intense band. OPA-20 and OPB -01 produced three faint bands while OPA-01 and OPB-06 produced two faint bands. Only one faint band was obtained for OPB-12.

Four bands each were obtained when OPA-13 and OPA-18 were used for the amplification reaction. Of these three bands were intense and one faint in each case.

OPA-17, OPA-19, OPB-07, OPB-19 and OPB-20 gave three bands each with the DNA in the amplification reactions. Among these primers, the highest number of intense bands (two) was obtained when OPA-17 was used. OPA-19, OPB-07 and OPB-20 gave one intense band each. Highest number of faint bands (three) was given by OPB-19.

Only two bands each were produced in a reaction when OPA-07, OPA-16, OPB-02, OPB-14, OPB-16 and OPB-17 were used. Among these primers, the highest number of intense bands (two) was obtained with the primers OPA-07, OPB-02 and OPB-14. Highest number of faint bands (two) was given by OPB-16.

The eleven primers which produced the highest number of bands as well as the highest number of intense bands were selected for amplifying DNA from all the plantain clones. The PCR reaction was repeated at least twice in order to check the reproducibility. Data from eight primers that gave reproducible product formation on at least two runs alone were included in statistical analysis.

The eight primers used in this analysis (OPA-01, OPA-03, OPA-13, OPB-01. OPB-06, OPB-10, OPB-12 and OPB-18) yielded 42 scorable bands with an average of 5.25 bands per primer. The amplification products ranged in size from 400 to 1500 base pairs. The number of bands resolved per amplification was primer dependent and varied from a minimum of three to a maximum of nine (Table 4) (Fig.5). The nucleotide sequence of these eight primers and number of informative RAPD markers given by each primer are shown in table 4. The GC content of the primers used varied from 60 to 70 per cent.

The highest number of scorable bands (nine bands) was given by the primer OPB-10. But this primer could not produce amplification with DNA of the clone, Zanzibar. Changazhikodan, Manjeri Nendran, Padalamurian, Kaliethan and Koonoor Ethan gave six bands each when OPB-10 was used for amplification. Mysore Ethan, Myndoli and Quintal Banana gave five bands each. Four bands were produced by Attu Nendran and two bands by Changanasseri Nendran (Plate 1 and Fig.6).

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Table 4 **Nucleotide sequences of primers and total number of informative RAPD markers amplified with them in the plantain clones used in this study**

Primer	Sequence	Number of informative RAPD markers
OPA - 01	CAGGCCC TTC	3
OPA - 03	AGTCAGCCAC	7
OPA - 13	CAGCACCCAC	7
OPB - 01	GTTTCGCTCC	4
OPB - 06	TGCTCTGCCC	3
OPB - 10	CTGCTGGGAC	9
OPB - 12	CCTTGACGCA	5
OPB - 18	CCACAGCAGT	4

Fig. 5 Total number of informative RAPD markers amplified with the eight primers in the plantain clones

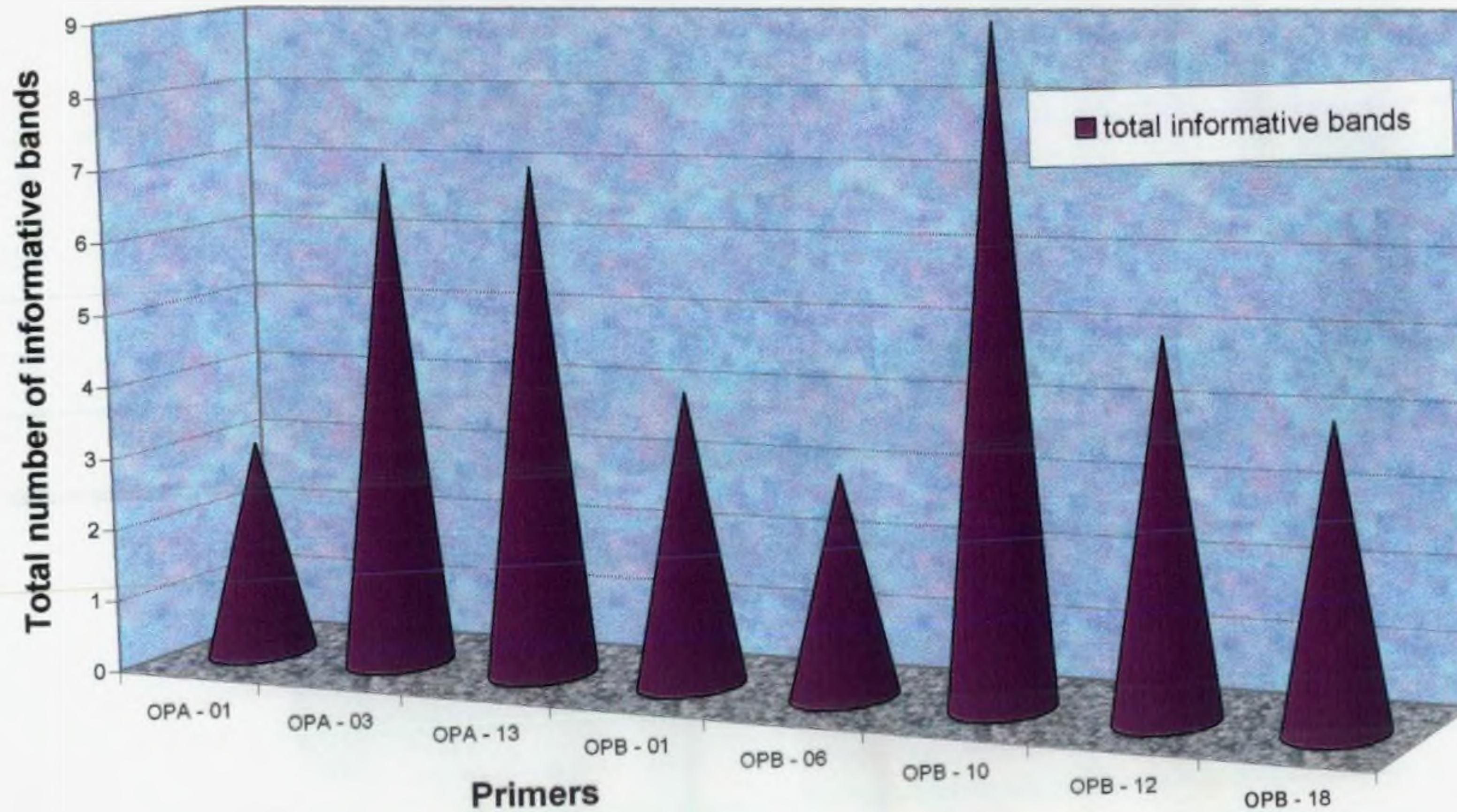


Plate 1

Amplification profiles of the DNA of eleven plantain clones using the primer OPB-10

Lane 1 .... Attu Nendran

Lane 2 .... Changanasseri Nendran

Lane 3 .... Changazhikodan

Lane 4 .... Manjeri Nendran

Lane 5 .... Mysore Ethan

Lane 6 .... Myndoli

Lane 7 .... Padalamurian

Lane 8 .... Zanzibar

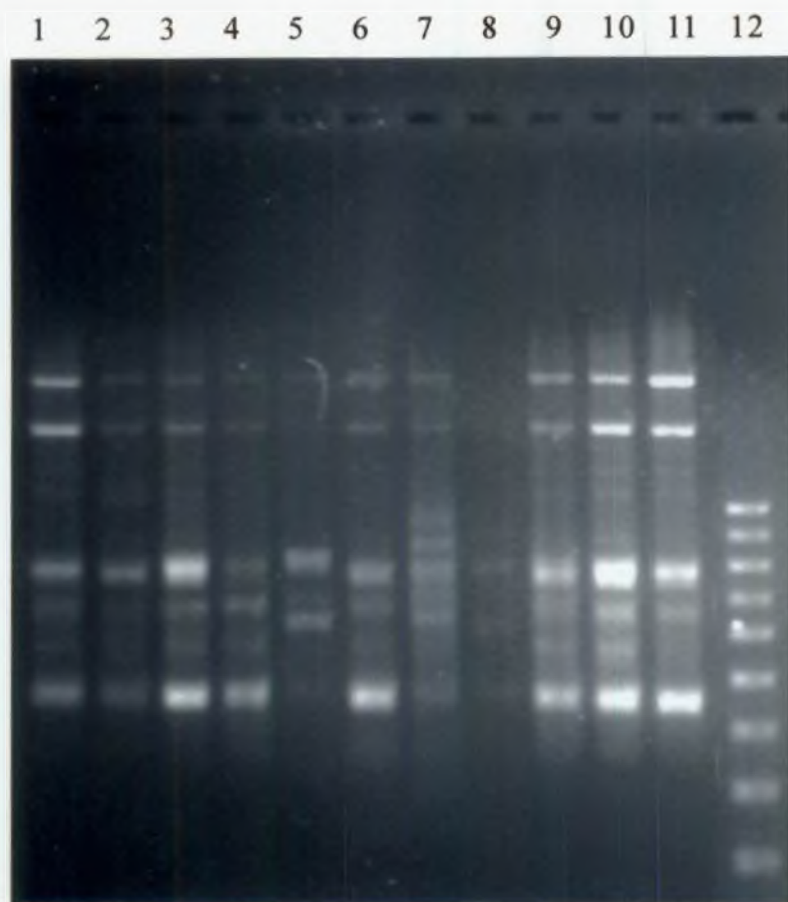
Lane 9 .... Kaliethan

Lane 10 .... Koonoor Ethan

Lane 11 .... Quintal Banana

Lane 12 .... DNA molecular weight marker (U.S. Biochemicals)

PLATE 1



Seven bands each were obtained on amplification using the primers, OPA-03 and OPA-13. With OPA-03, three bands were monomorphic for all the clones. The highest number of scorable bands (six) was given by the clones, Attu Nendran, Changanansseri Nendran and Changazhikodan. Mysore Ethan gave five bands. Four bands each were given by Padalamurian, Zanzibar, Kaliethan, Koonoor Ethan, Quintal Banana, Manjeri Nendran and Myndoli (Fig. 7).

When OPA-13 was used for amplification, two bands were monomorphic in all the clones. Changazhikodan and Kaliethan yielded six bands each. Five bands were obtained for Mysore Ethan. Attu Nendran, Manjeri Nendran and Myndoli gave four bands each. Koonoor Ethan and Quintal Banana gave three bands each. Two bands were obtained when Changanasseri Nendran and Zanzibar were subjected to amplification reaction with OPA-13 (Fig. 8).

A total of five scorable bands were obtained on *in vitro* amplification of the plantain clones using the primer OPB-12. Of these, two bands were monomorphic for all the clones. Manjeri Nendran and Zanzibar yielded three bands each. All the other clones gave four bands each (Plate 2 and Fig. 9).

Four scorable bands were obtained on amplification with the primers OPB-01 and OPB-18. In the primer, OPB-01, one band was monomorphic for all the clones studied. Four bands were given by the clones, Attu Nendran, Changanasseri Nendran, Koonoor Ethan and Quintal Banana. Zanzibar yielded

AN	Attu Nendran
CN	Changanasseri Nendran
CK	Changazhikodan
MN	Manjeri Nendran
ME	Mysore Ethan
MY	Myndoli
PM	Padalamurian
ZA	Zanzibar
KA	Kaliethan
KO	Koonoor Ethan
QB	Quintal Banana

Fig. 6 **Representation of the amplification profile of the DNA of eleven plantain clones using the primer OPB-10**

AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
+		+	+	+	+	+	-	+	+	+
+	-	+	+	+	+	+	-	+	+	+
-	-	-	-	-	-	+	-	-	-	-
-	-	-	-	-	-	+	-	-	-	-
+	+	+	+	+	+	+	-	+	+	+
-	-	+	+	+	+	-	-	+	+	+
-	-	-	-	+	-	+	-	-	-	-
-	-	+	+	-	-	-	-	+	+	-
+	+	+	+	-	+	-	-	+	+	+

Fig. 7 **Representation of the amplification profile of the DNA of eleven plantain clones using the primer OPA-03**

AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
+	+	+	-	+	-	+	+	+	+	+
+	+	+	-	-	-	-	-	-	-	-
+	+	+	-	-	-	-	-	-	-	-
+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+
-	-	-	-	+	-	-	-	-	-	-

Fig. 8 **Representation of the amplification profile of the DNA of eleven plantain clones using the primer OPA-13**

AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
+	-	+	-	+	-	-	-	+	+	+
+	-	+	-	-	-	-	-	+	-	-
-	-	-	-	-	-	-	-	+	-	-
+	+	+	+	+	+	+	+	+	+	+
-	-	+	+	+	+	-	-	+	-	-
-	-	+	+	+	+	-	-	-	-	-
+	+	+	+	+	+	+	+	+	+	+

+ = presence of band - = absence of band

Plate 2

Amplification profiles of the DNA of eleven plantain clones using the primer OPB-12

Lane 1 .... Attu Nendran

Lane 2 .... Changanasseri Nendran

Lane 3 .... Changazhikodan

Lane 4 .... Manjeri Nendran

Lane 5 .... Mysore Ethan

Lane 6 .... Myndoli

Lane 7 .... Padalamurian

Lane 8 .... Zanzibar

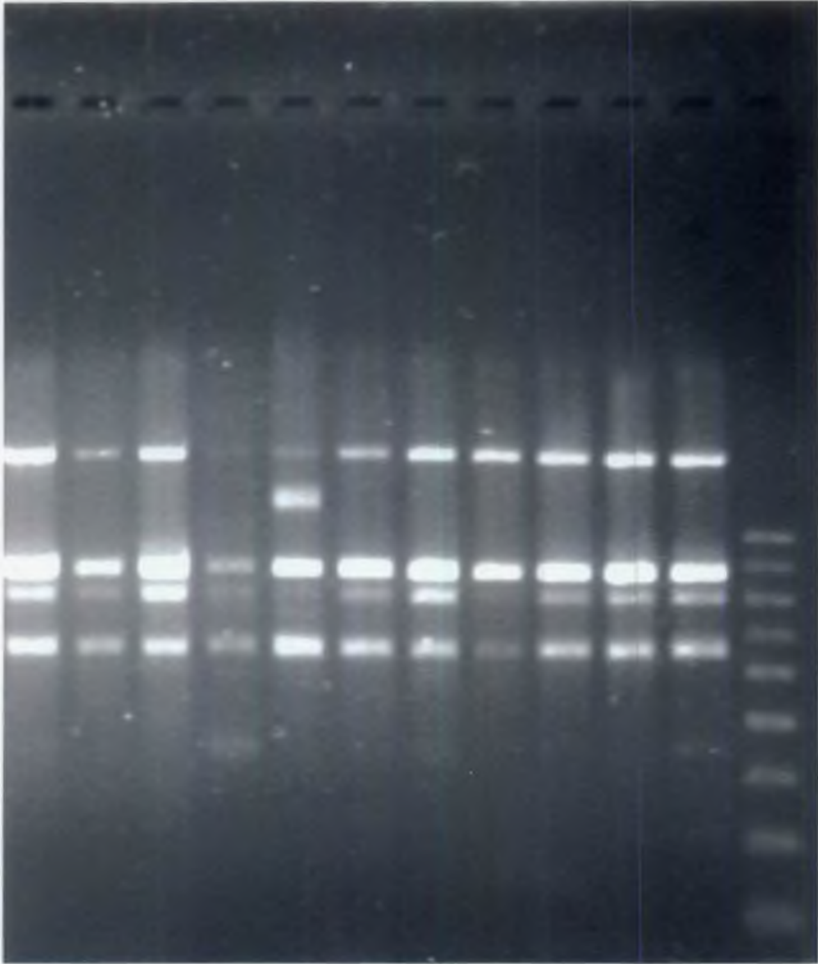
Lane 9 .... Kaliethan

Lane 10 .... Koonoor Ethan

Lane 11 .... Quintal Banana

Lane 12 .... DNA molecular weight marker (U.S. Biochemicals)

PLATE 2



only one band. All the other clones gave three bands each (Fig. 10). With the primer, OPB-18, no amplification was found in Zanzibar. Two bands were monomorphic for all the clones, except Zanzibar. Kaliethan, Koonoor Ethan and Quintal Banana gave four bands each. Three bands were given by Myndoli. Two bands alone were produced by the rest of the clones (Fig. 11).

The primer OPA-01 and OPB-06 yielded a total of three scorable bands each when used for amplification. With OPA-01, Attu Nendran, Changazhikodan and Koonoor Ethan gave three bands each. Zanzibar gave only one band. The rest of the clones yielded two bands each (Fig. 12).

When OPB-06 was used, three bands were given by Attu Nendran, Changanasseri Nendran, Changazhikodan, Kaliethan, Koonoor Ethan and Quintal Banana. No amplification was produced in Zanzibar with this primer. All the other clones gave two bands each (Fig. 13).

4.5 Data analysis

Reproducible bands were scored for their presence (+) or absence (-) for all the plantain clones studied. A genetic similarity matrix was constructed using the Jaccard's coeffecient method (Table 5). The pairwise coefficient values varied between 0.3333 and 0.9355. The least similarity coefficient values were those of Zanzibar with Changazhikodan and Manjeri Nendran (0.3333). The next higher value is that between Zanzibar and Kaliethan (0.3438), followed by that

Fig. 9 **Representation of the amplification profile of the DNA of eleven plantain clones using the primer OPB-12**

AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
+	+	+	-	+	+	+	+	+	+	+
-	-	-	-	+	-	-	-	-	-	-
+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	-	+	+	-	+	+	+
+	+	+	+	+	+	+	+	+	+	+

Fig. 10 **Representation of the amplification profile of the DNA of eleven plantain clones using the primer OPB-01**

AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	-	+	+	+
+	+	-	-	-	-	-	-	-	+	+
+	+	+	+	+	+	+	-	+	+	+

Fig. 11 **Representation of the amplification profile of the DNA of eleven plantain clones using the primer OPB-18**

AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
+	+	+	+	+	+	+	-	+	+	+
-	-	-	-	-	-	-	-	+	+	+
+	+	+	+	+	+	+	-	+	+	+
-	-	-	-	-	+	-	-	+	+	+

\+ = presence of band   - = absence of band

Fig.12 **Representation of the amplification profile of the DNA of eleven plantain clones using the primer OPA-01**

AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
+	-	+	-	-	-	-	-	-	+	-
+	+	+	+	-	+	+	+	+	+	+
+	+	+	+	-	+	+	-	+	+	+

Fig.13 **Representation of the amplification profile of the DNA of eleven plantain clones using the primer OPB-06**

AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
+	+	+	+	+	+	+	-	+	+	+
+	+	+	-	-	-	-	-	+	+	+
+	+	+	+	+	+	+	-	+	+	+

+ = presence of band - = absence of band

Table 5 Similarity matrix of eleven plantain clones based on the Jaccard's similarity index

	AN	CN	CK	MN	ME	MY	PM	ZA	KA	KO	QB
Attu Nendran (AN)	1										
Changanasseri Nendran (CN)	0.8333	1									
Changazhikodan (CK)	0.8529	0.7059	1								
Manjeri Nendran (MN)	0.6176	0.6129	0.7576	1							
Mysore Ethan (ME)	0.5556	0.5000	0.6389	0.6452	1						
Myndoli (MY)	0.6471	0.6452	0.7353	0.8889	0.6774	1					
Padalamurian (PM)	0.6667	0.6667	0.6111	0.6667	0.6452	0.7000	1				
Zanzibar (ZA)	0.3667	0.4400	0.3333	0.3333	0.3704	0.3704	0.4400	1			
Kaliethan (KE)	0.7222	0.6286	0.8056	0.7576	0.6111	0.7576	0.6286	0.3438	1		
Koonoor Ethan (KO)	0.7941	0.6970	0.7778	0.6970	0.5833	0.7273	0.6471	0.3548	0.8529	1	
Quintal Banana (QB)	0.7879	0.7419	0.7222	0.6875	0.6176	0.7742	0.6875	0.3793	0.8485	0.9355	1

of Zanzibar with Koonoor Ethan (0.3548). The average similarity coefficient value for the French Plantain clones was 0.6616.

The highest value for similarity index was obtained for Koonoor Ethan – Quintal Banana pair (0.9355) followed by Manjeri Nendran – Myndoli pair (0.8889). The next value was for Kaliethan – Koonoor Ethan pair (0.8529).

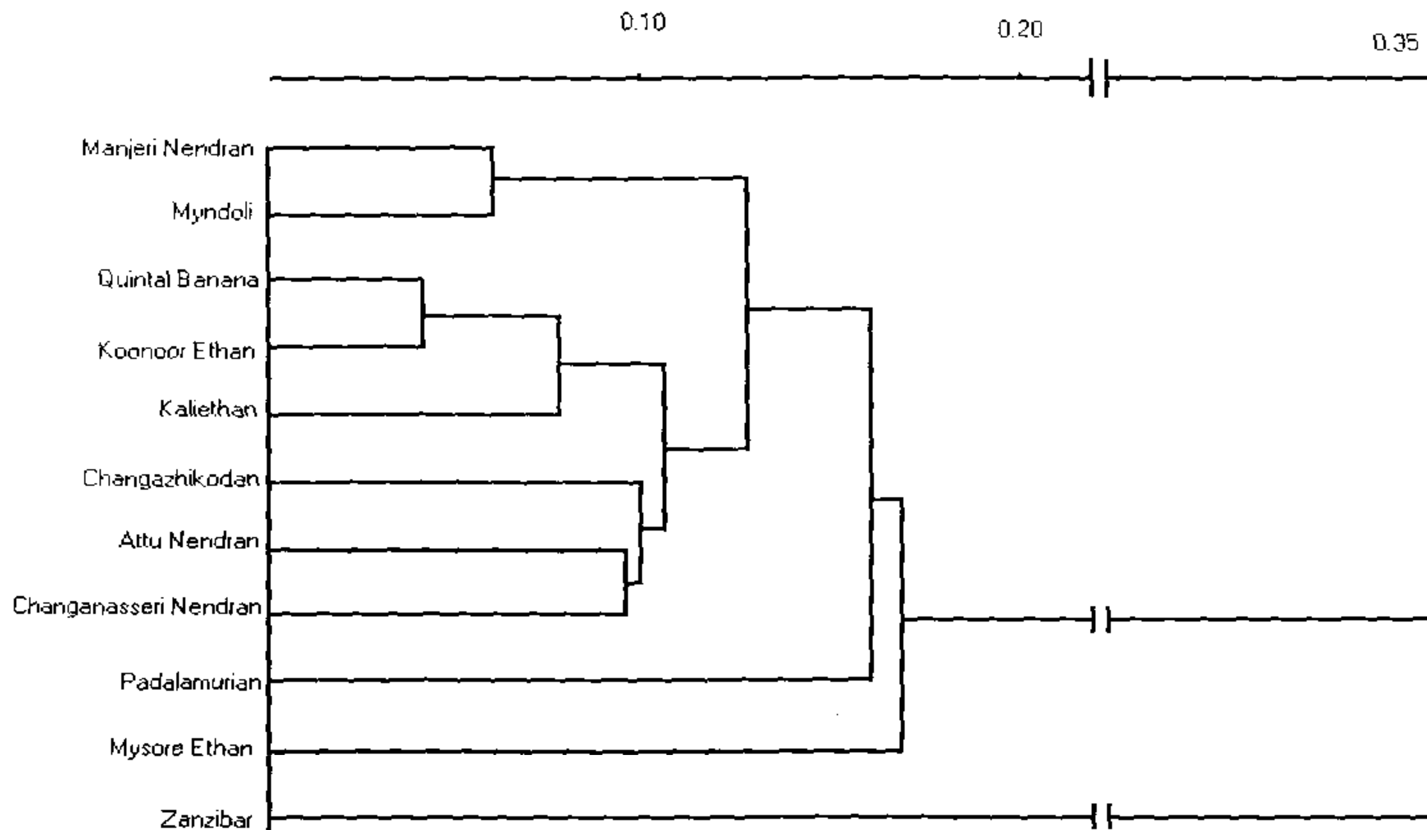
Based on similarity coefficients, distances between the clones was computed using SYSTAT (version-9) software package. The distance between pairs of clones and number of clusters formed corresponding to each distance were tabulated (Table 6). The distance was least between Koonoor Ethan and Quintal Banana (0.042), followed by Manjeri Nendran and Myndoli (0.060). Manjeri Nendran and Myndoli showed a distance of 0.060 between them. Zanzibar and Mysore Ethan showed the greatest distance (0.349) followed by Mysore Ethan and Padalamurian (0.167).

On drawing a vertical line in the dendrogram (Fig. 14), along the point corresponding to a distance of 0.125, all the eleven clones got divided into five clusters. Attu Nendran, Changanasseri Nendran, Changazhikodan, Kaliethan, Koonoor Ethan and Quintal Banana together formed the largest cluster. Within this cluster, Quintal Banana and Koonoor Ethan were more close to each other. Manjeri Nendran and Myndoli formed another cluster. Padalamurian, Mysore Ethan and Zanzibar formed three separate clusters.

Table 6 **Genetic distance calculated using the nearest neighbour (single-link) method**

Clones	Genetic distance	No.of clusters formed
QB and KO	0.042	2
MY and MN	0.060	2
QB and KA	0.078	3
CN and AN	0.098	2
CK and CN	0.100	3
CK and QB	0.105	6
CK and MY	0.127	8
PM and CK	0.160	9
ME and PM	0.167	10
ZA and ME	0.349	11

Fig. 14 **Dendrogram obtained from RAPD analysis using nearest neighbour (single-link) method**



Discussion

5. DISCUSSION

Banana (*Musa* sp.) is the most important fruit crop of Kerala. A number of banana cultivars are being cultivated in the varied agroclimatic regions of the state. 'Nendran', Red banana, Palayancodan, Njalipoovan, Poovan and Monthan are some of the important varieties grown in Kerala. 'Nendran' is an important plantain variety, well known for its multi farious use. In spite of the wide variability, the *Musa* germplasm in this region has not been systematically characterized so far.

'Nendran' exhibits clonal and ecotype variation with respect to growth and yield and reaction to biotic and abiotic stresses (Sunil k umar, 1997). There are several types of Nendran cultivated in different parts of Kerala, known in different local names. Synonyms of clones / ecotypes in different localities are confusing. This necessitates precise identification methods. Morphological and biochemical traits usually form the basis for characterization of cultivars and varieties. However, it fails to provide reliable information at genetic level. The use of molecular markers for genotyping becomes important in this context. Molecular markers like RAPD are widely used to characterize plant genetic resources. RAPD analysis is fast and easy. It is comparatively cheap and free from environmental influences.

The present study was undertaken to characterize eleven clones of banana (*Musa* AAB Plantain subgroup) using RAPD markers. The results obtained are discussed in detail in this chapter.

5.1. DNA isolation

Banana contains large amount of phenols, tannins, pigments and polysaccharides. These interfere with the isolation of DNA (Babu, 2000). They impair the quantity and purity of isolated DNA and also inhibit the activity of most of DNA synthesizing and modifying enzymes which may lead to difficulties during the RAPD analysis. Hence selection of a suitable protocol is important in overcoming these difficulties.

Nayar (2001) standardized a protocol for the isolation of DNA from Red banana using modified Walbot's method. This method was found suitable for the DNA isolation of the plantain clones used in this study.

The quantity and quality of the isolated DNA depends on the source of tissue as well as efficient disruption of the plant cell wall (Babu, 2000). Emerging leaves of mature plant, before unfurling, was used for the isolation of DNA from plantain clones. As observed by Nayar (2001) emerging leaves of mother plant yielded more quantity of DNA, compared to mature leaves in Red banana. This may be because of the easy disruption of tender tissues during

isolation steps. Moreover tender leaves contain actively dividing cells with lesser intensity of extranuclear materials like proteins, carbohydrates and other metabolites that interfere with isolation of nucleic acids which in turn improve the quality of DNA (Mondal *et al.*, 2000).

The leaves collected for DNA isolation were used either fresh or after storing in an ultrafreezer at –85 °C. The storage did not interfere with the yield and purity of DNA as observed in Padalamurian (Table 1). When fresh leaves were used, the yield of DNA was 280 μg ml^{-1}. The leaves after storage in ultra freezer for one week yielded 390 μg ml^{-1}. The purity ratio of the fresh and stored samples were 1.69 and 1.86 respectively. This observation has an implication that the unfurled leaves once collected can be stored without any damage to DNA for a few days in ultrafreezer in cases where there is dearth of samples. Ram and Sreenath (1999) have made a similar observation in coffee where stored samples permitted batch processing.

The yield of DNA and its purity varied with clones. The yield ranged from 280 μg ml^{-1} (Padalamurian) to 3220 μg ml^{-1} (Changazhikodan). The purity (A_{260} / A_{280}) ranged from 1.57 (Myndoli) to 2.07 (Quintal Banana). This could be due to the interference of various compounds in the plant tissue during the procedure.

5.2. Purification of DNA

The DNA isolated using modified Walbot method was slightly brown in colour. The same observation was made by Nayar (2001) in Red banana and this problem was overcome by including 0.1 per cent PVP in extraction buffer in addition to the other reagents. But in plantain, the use of PVP could not prevent browning of the extract. However, the inclusion of the antioxidant β-mercaptoethanol along with PVP during extraction improved the quality of DNA as observed by the reduction of browning. Mondel *et al.* (2000) reported similar observation in tea. Weising *et al.* (1995) reported that high phenolic oxidation in coffee tissues to brown coloured quinonic compounds damaged DNA and proteins. They suggested that this could be effectively counteracted by the use of certain ingredients in the extraction medium. Reduction of browning could be due to the binding of PVP to phenolic compounds and its co-precipitation as well as inhibition of the action of polyphenol oxidase.

5.3. Gel electrophoresis

Agarose gel electrophoresis was used for analyzing the genomic DNA isolated from different clones as well as for the RAPD products. The concentration of gel is an important factor for the separation of DNA fragments. A low concentration of agarose is ideal for the separation of genomic DNA which are of high molecular weight while small DNA fragments give better

separation in a high concentration agarose gel. In the present study, among the various concentrations of agarose tried, 0.9 per cent was found optimum for genomic DNA and 1.4 per cent for RAPD analysis. Mulcahy *et al.* (1993) and Yu and Nguyen (1994) used 0.9 per cent agarose for visualizing the genomic DNA. Prasannalatha *et al.* (1999) reported the separation of amplified products through 1.4 per cent agarose gel. Separation of bands was more when 0.5x TBE buffer was used instead of TAE buffer. The optimum voltage was 75 V. When higher voltage was applied, it resulted in melting of the gel rendering a sticky nature to the gel.

Most of the DNA samples isolated were obtained as single crisp band and a few samples showed smearing indicating shearing. Ribonucleic acid (RNA) was observed as a thick band below the genomic DNA in gel electrophoresis. But the presence of RNA did not interfere in amplification. Mondal *et al.* (2000) reported that RNA will not interfere in RAPD analysis.

5.4. RAPD analysis

The PCR amplification (as per the procedure mentioned in 3.4 materials and methods) was carried out using forty decamer primers (Operon Inc., CA, USA) of Kit A and Kit B with the DNA of Attu Nendran. The procedure standardized by Nayar (2001) for Red banana was used for amplification. Thirty one primers out of the 40 used, yielded amplification products. The total number

of bands ranged from 1.0 to 6.0. The primers, OPA-04, OPA-05, OPA-06, OPA-08, OPA-09, OPA-10, OPA-11, OPA-12 and OPB-09 did not yield any bands. This indicated that there is no sequence complementary to the sequence of these primers in the DNA of Attu Nendran.

A total of 106 RAPDs (average of 2.65 bands per primer) were generated by the 31 primers, of which 94.43 per cent (100 bands) were polymorphic. This accounts to an average of 2.5 bands per primer (Fig.8 to 11). Twenty five primers showed high level of polymorphism. This could be explained by the capability of individual primers to amplify the less conserved and highly repeated regions of the genomic DNA (Prasannalatha *et al.*, 1999). There is a high probability for the amplified fragments to contain repeated sequences.

In the present study, eleven promising primers for the RAPD analysis were identified, based on the number of polymorphic bands obtained. They were OPA-01, OPA-03, OPA-13, OPA-20, OPB-01, OPB-04, OPB-05, OPB-06, OPB-10, OPB-12 and OPB-18. These primers were used for amplifying DNA from all the plantain clones. The PCR reaction was repeated at least twice in order to check the reproducibility. Out of the eleven primers used, three primers (OPB-20, OPB-04 and OPB-05) could produce amplification only in three to five clones. So, only the data from the rest of the primers (eight primers) that gave reproducible results on at least two runs for most of the clones were included in statistical analysis. The GC content of these primers varied from 60 to 70 per

cent. It is well known that primers with a GC content of at least 50 per cent are generally used (Weising *et al.*, 1995). A previous report in *Brassica* L. (Demeke *et al.*, 1992) indicated that a minimum of 17 primers (284 polymorphisms) were necessary to obtain a stable classification of related species. How ever, Bhat and Jarret (1995) suggested that the number of polymorphisms may be more important than the number of primers for the generation of a stable phenogram. They also suggested that the number of polymorphisms required to generate a stable phenetic analysis will vary with the plant material under investigation and the sequences that are amplified.

The eight primers used in this analysis yielded 42 scorable bands with an average of 5.25 bands per primer. The amplification products ranged in size from 400 to 1500 bp. The number of bands resolved per amplification was primer dependent and varied from 4.0 to 9.0. A similar study (Bhat and Jarret, 1995) was carried out on the assessment of genetic diversity in Indian *Musa* germplasm. The number of polymorphic bands amplified per primer was more than twice that found here (1.0 to 24). This may be explained as due to the differences in the RAPD conditions used in the two studies. Primer sequence, cycling temperatures, source of polymerase, DNA quality and magnesium concentration are known to affect PCR products (Howell *et al.*,1994). The number of fragments per primer is largely independent of the genome complexity of the investigated organism (Weising *et al.*, 1995).

The highest number of scorable bands (nine) was given by the primer OPB-10. But this primer could not produce amplification with DNA of the clone Zanzibar. In all the other clones, one of the bands produced was monomorphic.

The primers OPA-01 and OPB-06 yielded the least number (three each) of scorable bands, under the conditions studied. Attu Nendran, Changazhikodan and Koonoor Ethan gave three bands with OPA-01. When OPB-06 was used, a maximum of three bands was given by Attu Nendran, Changazhikodan, Kaliethan, Koonoor Ethan and Quintal Banana. Zanzibar was not amplified by OPB-06.

5.5. Data analysis

Jaccard's similarity coefficient values ranged from 0.3333 to 0.9355. The genetic distance between the clones ranged from 0.042 (between Manjeri Nendran and Myndoli) to 0.349 (between Zanzibar and Mysore Ethan). The average similarity coefficient value for 'French Plantain' clones was 0.6616. This substantiates the moderately broad distribution of genetic variability which can be attributed to the broad genetic base in their origin.

The estimation of similarity coefficients and construction of dendrogram by using the nearest neighbour (single-link) method revealed the presence and extent of genetic similarities among the eleven plantain clones examined. All the eleven clones got divided into five clusters. Attu Nendran, Changanasseri

Nendran, Changazhikodan, Kaliethan, Koonoor Ethan and Quintal Banana together formed a large cluster. All the three clones collected from Vellayani (Kaliethan, Koonoor Ethan and Quintal Banana) got grouped together. The dendrogram illustrates a close relationship between the clones, Koonoor Ethan and Quintal Banana. Also it is clearly evident from the figure that Zanzibar is entirely different from the rest of the clones and it formed a separate cluster. It is well known that Zanzibar unlike the rest of the clones belong to 'Horn Plantain'. Myndoli and Quintal banana are considered as identical in many places. They belong to Giant Plantain group. The male bud degenerates after some development in 'Horn Plantains'. All the other clones included in the study belong to 'French Plantain'.

Manjeri Nendran and Myndoli are grouped together. Myndoli differs in crop duration from the other clones like Kaliethan, Koonoor Ethan, Changanasseri Nendran and Attu Nendran. The crop duration of Myndoli is 18 months while those of the other clones varied from 11 to 12 months (Rao, 1998). Thus the clustering of Myndoli in a cluster different from these clones is in confirmation with earlier known facts. Also it was found out in an earlier study (Babylatha *et al.*, 1990) that Myndoli differed in its susceptibility to the attack of rhizome weevil. Myndoli is susceptible whereas Kaliethan and Changazhikodan showed less incidence of rhizome weevil attack. Manjeri Nendran is more drought tolerant than Kaliethan and Changazhikodan. Grouping of Manjeri

Nendran and Myndoli together could be due to the drought tolerant character of these two clones.

In the present study, two clones, Myndoli and Quintal Banana did not cluster as expected. These two clones were considered as identical. This is an interesting observation. However, further studies are necessary for confirmation. Padalamurian and Mysore Ethan formed two separate clusters.

Not much literature is available about the morphological and agronomic characterization of all the plantain clones included in the present study. So proper comparison of the results of this study with those of earlier studies is not possible. The polymorphism obtained in the present study will be useful in fingerprinting and in determining the genetic diversity among the plantain clones. Further studies on morphological and agronomic traits and analysis of the clones with more number of reliable DNA markers may be helpful in confirming the results. Knowledge of the degree of genetic relationship between these clones will be important for the development of new accessions and to establish a core collection as part of the germplasm collection and management.

Summary

6. SUMMARY

Attempts were made for the molecular characterization of banana (*Musa* AAB Plantain subgroup) clones using RAPD technique during January 2000 to October 2001 at the Department of Pomology and Floriculture and the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani. The salient features of the studies are summarized below.

1. Modified Walbot's method was found suitable for the isolation of DNA from the plantain clones used in this study.
2. The storage of leaves in ultra freezer at -85°C for up to one week did not affect either the yield of DNA or the purity ratio.
3. Addition of PVP and β mercaptoethanol to the extraction buffer helped in preventing the browning of the DNA pellet.
4. Agarose concentrations of 0.9 per cent was the best for visualizing the genomic DNA, as compared to 0.7 per cent, 0.8 per cent and 1.0 per cent. For visualizing the RAPD pattern, 1.4 per cent agarose concentration was better than 1.2 per cent, 1.3 per cent and 1.5 per cent. Among the various voltage levels (50 V, 60 V, 75 V and 100 V) tried, 75 V was the optimum. When TBE buffer was used, separation of bands was better compared to that of TAE buffer. The bands were brighter when gel thickness was less.
5. Twenty nanogram of DNA, 200 μM each of dNTPs, 0.6 units Taq DNA polymerase and 5 pM primer in presence of the assay buffer gave good

PCR amplification results. The programme consisted of an initial denaturation at 95°C for 3.0 minutes, followed by 45 cycles of denaturation at 95°C for 1.0 minute, annealing at 36°C for 1.0 minute 30 seconds and extension at 72°C for 2.0 minutes. The synthesis step of the final cycle was extended further by 6.0 minutes. The products of amplification were kept at 4.0°C until attended.

6. A total of 106 RAPDs were generated when PCR amplification was carried out using 40 decamer primers (Operon Inc., CA, USA) of kit A and kit B. of these 100 bands were polymorphic which accounted to an average of 2.5 polymorphic bands per primer. Eight primers (OPA–01. OPA–03. OPA–13, OPB–01, OPB–06, OPB–10, OPB–12 and OPB–18) produced reproducible banding patterns on at least two runs. These primers yielded 42 scorable bands with an average of 5.25 bands per primer. The amplification products ranged in size from 400 to 1500 bp. The number of bands resolved per amplification was primer dependent and varied from a minimum of three to a maximum of nine.
7. The similarity coefficient values ranged from 0.3333 to 0.9355 and the genetic distance varied from 0.042 to 0.349. From the dendrogram, it was studied that the eleven plantain clones clustered into five groups. The largest group consisted of Attu Nendran, Changanasseri Nendran, Changazhikodan, Koonoor Ethan, Kaliethan and Quintal Banana. All the clones collected from Vellayani (Kaliethan, Koonoor Ethan and Quintal Banana) were grouped together. Manjeri Nendran and Myndoli formed

another group. Padalamurian, Mysore Ethan and Zanzibar formed three separate groups. Zanzibar, which belongs to Horn Plantain group, is entirely different from the rest of the clones. Quintal Banana and Myndoly, which were considered to be identical, got grouped under two different clusters.

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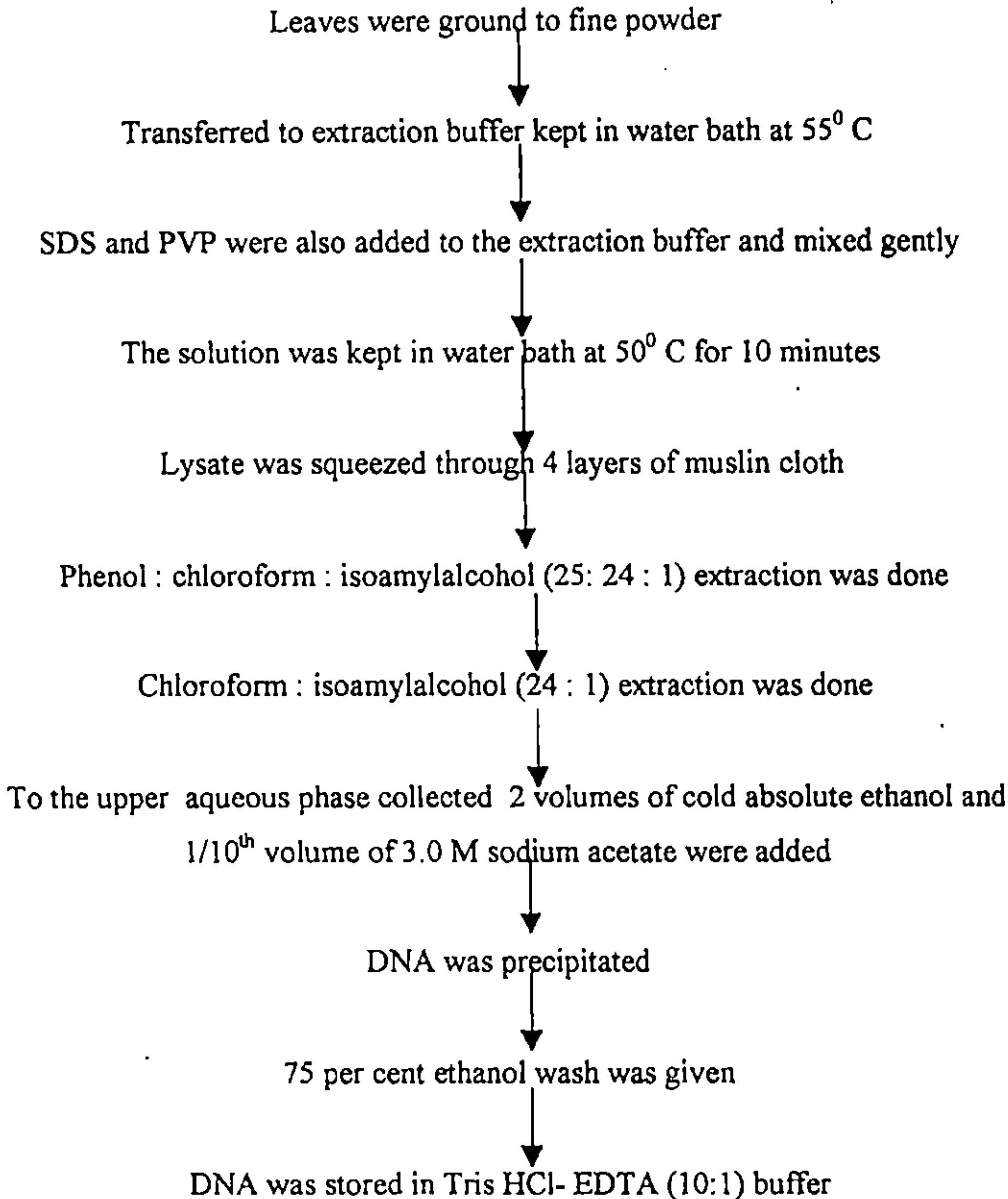
Appendix

APPENDIX

MODIFIED WALBOT'S METHOD



MOLECULAR CHARACTERIZATION OF BANANA (*Musa* AAB PLANTAIN SUBGROUP) CLONES

BY

SIMI. S.

ABSTRACT OF THE THESIS
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ABSTRACT

Attempts were made for characterizing eleven banana (*Musa* AAB Plantain subgroup) clones at molecular level during January 2000 to October 2001 at the Department of Pomology and Floriculture and the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani. Tissues from fully unfurled leaves of the clones, were used for isolating DNA, using modified Walbot's method. Storage of leaves at -85°C did not affect either the DNA yield or purity ratio. Gel elctrophoresis using agarose concentrations of 0.09 per cent and 1.4 per cent were the best for visualizing the genomic DNA and RAPD pattern, respectively. The best voltage level was 75 V. TBE buffer could produce better separation of bands compared to TAE buffer. Twenty nanogram of DNA, 200 μM each of dNTPs, 0.6 units Taq DNA polymerase and 5 pM primer in presence of the assay buffer gave good PCR amplification results. The programme consisted of an initial denaturation at 95°C for 3.0 minutes, followed by 45 cycles of denaturation at 95°C for 1.0 minute, annealing at 36°C for 1.0 minute 30 seconds and extension at 72°C for 2.0 minutes. The synthesis step of the final cycle was extended further by 6.0 minutes. The products of amplification were kept at 4.0°C until attended. One hundred and six RAPDs were generated when PCR amplification was carried out using forty decamer primers (Operon Inc., CA, USA) of kit A and kit B. Of these, 100 bands were polymorphic which accounted to an average of 2.5 polymorphic bands per primer. Eight primers (OPA-01, OPA-03, OPA-13, OPB-01, OPB-06, OPB-10, OPB-12 and OPB-18) produced reproducible banding patterns on at least two runs. These primers yielded 42 scorable bands with an average of 5.25 bands per primer. The amplification

products ranged in size from 400 to 1500 bp. The number of bands resolved per amplification was primer dependent and varied from a minimum of three to a maximum of nine. Reproducible bands were scored for their presence (+) or absence (-) for all the plantain clones studied. A genetic similarity matrix was constructed using the Jaccard's coeffecient method. The pairwise coefficient values varied between 0.3333 and 0.9355. The least similarity coefficient values were those of Zanzibar with Changazhikodan and Manjeri Nendran (0.3333). The highest value for similarity index was obtained for Koonoor Ethan – Quintal Banana pair (0.9355), followed by Manjeri Nendran – Myndoli pair (0.8889). The next value was for the Kaliethan – Koonoor Ethan pair (0.8529). Based on the similarity coefficients, distances between the clones were computed using SYSTAT software package. The distance was the least between Koonoor Ethan and Quintal Banana (0.042), followed by Manjeri Nendran and Myndoli (0.06). Zanzibar and Mysore Ethan showed the greatest distance (0.349), followed by Mysore Ethan and Padalamurian (0.167). In the dendrogram constructed by the nearest neighbour (single–link) method (Krzanowski, 1988), all the eleven plantain clones were found grouped under five clusters. Attu Nendran, Changanasseri Nendran, Changazhikodan, Kaliethan, Koonoor Ethan and Quintal Banana formed the largest cluster. Manjeri Nendran and Myndoli formed the second cluster. Padalamurian, Mysore Ethan and Zanzibar formed three separate clusters. Zanzibar, belonging to 'Horn Plantain', was different from the rest of the clones. Quintal Banana and Myndoli, which were considered to be identical, got grouped under two different clusters.