Genetic diversity of East African Highland bananas using AFLP

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Originally from Southeast Asia (Simmonds 1966, Valmayor *et al.* 1981), bananas are believed to have entered the East African highland region through multiple introductions between the first and sixth century AD (Price 1995). A wide range of unique varieties belonging to the East African Highland bananas (AAA-EA) now exist in the region, having evolved locally. The East African Highland region has been called a secondary centre of *Musa* diversity (Stover and Simmonds 1987, Swennen and Vuylsteke 1988), with Uganda showing the highest level of diversity of AAA-EA genotypes (Kyobe 1981, Rubaihayo and Mukasa 1993).

Knowing the degree of genetic relatedness between clones and the range of diversity present in *Musa* germplasm is important for conservation and the selection of parents for breeding programmes (Garwel and Jarret 1992, Ortiz *et al.* 1995, Lagoda *et al.* 1999). Morphological traits have been widely used in clone identification and taxonomic studies (Brewbaker and Umali 1956, Allen 1965, Stover and Simmonds 1987, Sebasigari 1990).

Karamura (1998) used 73 morphological traits to classify the East African Highland bananas of Uganda into five clone sets: *Mbidde* (Beer), *Musakala*, *Nakabululu*, *Nfuuka* and *Nakitembe*. However, the complexity of interactions between genes and the environment (Shanmugavelu *et al.* 1992) creates problems as elaborate field-testing is required for a classification to be effective (De Langhe 1990, Bhat *et al.* 1997, Oliviera *et al.* 2000, Valmayor *et al.* 2000). The narrower the genetic base, the less discriminating morphotaxonomy becomes (Jarret and Garwel 1995).

Molecular techniques have the potential of revealing stable genetic information on which to base classification. Amplified Fragment Length Polymorphism (AFLP) has been shown to be a powerful molecular tool (Donini e*t al.* 1997) capable of detecting genetic differences between related *Musa* accessions (Engelborghs *et al.* 1998) and closely related individuals (Jones *et al.* 1998). This paper reports the results of studies undertaken to assess the genetic relationships among East African Highland bananas using the AFLP technique.

Materials and methods

Young cigar leaf tissues from 115 East African Highland bananas were collected from the Uganda banana germplasm resource center at Kawanda Agricultural Research Institute, and Makerere University Agricultural Research Institute in Kabanyolo. The accessions were chosen on the basis of their expected low levels of polyphenols (Maliyakale 1992, Pich and Schubert 1993).

DNA was isolated from fresh leaf material (0.7 g) according to the protocol described by Vroh *et al.* (1996) but with some modifications. After the first chloroform extraction, a second extraction using 10% N-Cetyl-N,N,N-trimethyl-ammonium bromide (CTAB), followed by repeated chloroform extractions, was added to ensure effective precipitation and elimination of proteins and carbohydrates (Rowland and Nguyen 1993). The composition of the CTAB buffer was modified by increasing polyvinylpyrrolidone (PVP-40) from 2% to 4%, and ß-Mercaptoethanol from 5% to 8%. The problem of polyphenols (Maliyakale 1992, Pich and Schubert 1993) was counteracted by raising the concentrations of PVP-40 (polyvinylpyrrolidone) and ß-Mercaptoethanol in the original CTAB buffer. The DNA yield was estimated by spectrophotometry in a *SmartSpect^{TM} 3000 Version* 1.00.39 (BIORAD), as described by Linacero *et al.* (1998). Spectrophotometry and electrophoresis (Linacero *et al.* 1998) were used to assess the quality of DNA.

The molecular biology grade reagents for AFLP analysis were AFLP analysis system I kits (AFLP Core reagent kit and AFLP Starter primer kit) from Life Technologies (GIBCO BRL®). This system which has been designed for use in plants having genomes raging in size from 0.5×10^9 - 6×10^9 bp was used under license by Keygene N.V. Restriction digestion was carried out using 2.5 U of *Eco*R I and 2.5 U of *Mse* I restriction enzymes on 500 ng DNA as described in the AFLP Analysis system I manual. Assessment of the efficiency of digestion was carried out as recommended by Scott *et al.* (1998). Ligation of oligonucleotide adapters (*Eco*R I and *Mse* I adapters) was performed according to AFLP Analysis system I manual, and the adapters used (Table 1) were those described by Vos *et al.* (1995) for the restriction enzymes *Eco*R I and *Mse* I and were not phosphorylated.

Pre-selective PCR (Polymerase chain reaction) amplification of target sequences in DNA was performed as described by Vos *et al.* (1995) in a *PerKin Elmer® Model 2400 Thermocycler* using 2 pre-amplification primers without selective nucleotides:

1. *Eco*R I+0: 5'-GACTGCGTACCAATTC-3' and
2. Mse I+0: 5'-GATGAGTCCTGAGTAA-3'

Selective PCR amplification was performed using 2 ologinucleotide primers, one correspoding to *Eco*R I ends and the other to *Mse* I ends, each with 3 selective nucleotides (*Eco*R I+3 and Mse I+3).

Four selective primer pairs were used:

*Eco*RI+3
E1 5'-GACTGCGTACCAATTC**aac**-3'
E2 5'-GACTGCGTACCAATTC**acc**-3'
E3 5'-GACTGCGTACCAATTC**act**-3'
E4 5'-GACTGCGTACCAATTC**agc**-3
Mse I+3
M1 5'-ATGAGTCCTGAGTAA**ctt**-3'
M2 5'-GATGAGTCCTGAGTAA**caa**-3'
M3 5'-GATGAGTCCTGAGTAA**ctg**-3'
M4 5'-GATGAGTCCTGAGTAA**cag**-3'

One of the primers in each pair (*Eco*R I+3 primer) was radio-labelled with 2000 Ci/mmol [γ^{33}P]ATP (*Amersham Pharmacia Biotech*) using T_4 Polynucleotide Kinase by phosphorylating at the 5' end using the protocol of the AFLP Analysis system I instruction manual. The pre-selective PCR amplification products were diluted 100X with 1XTE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA [Ethylene diamine tetrachloro acetic acid]) to be used for selective PCR amplification, and the reaction was a 36-cycle event performed in an *iCycler* (*BIO-RAD*), according to Vos *et al.* (1995).

Following selective PCR, reaction products were mixed with equal volumes (20 µl) of formamide loading dye (98% Formamide, 10 mM EDTA pH 8.0; 0.1% Bromophenol and 0.1% Xylene cyanol FF

as tracking dyes). The resulting mixtures were heated for 4 minutes at 95ºC and then quickly chilled on ice. Four microlitres of each sample were loaded on 0.4 mm 6% denaturing (sequencing) polyacrylamide gels. The gel matrix was prepared using 6% Acrylamide, 0.3% N,N'-Methylene bisacrylamide, 7.5 M Urea in 1XTBE buffer pH 8.0 (100 mM Tris, 90mM Boric acid, 1 mM EDTA). To 75 ml of the gel solution, 250 µl of freshly prepared 10% APS (ammonium persulphate) and 50 µl of TEMED (N,N,N'N'-Tetramethylene diamine) were added and the solution was mixed gently but quickly with a syringe. Electrophoresed was performed at 1800 V (constant), 37 mA, 65 W for 1 hour and 10 min, using 1XTBE buffer pH 8.0 as a running buffer. The gels were later fixed for 30 minutes in a fixing solution (5% glacial acetic acid, 4.8% ethanol) to drain off the urea and the blue dyes, dried and exposed to an X-ray film 35 X 43 cm (Biomax MR Kodak) for 48 hours at room temperature.

Selective PCR amplification products (amplified fragments) on the X-ray film were scored: '1' for presence and '0' for absence of a homologous fragment (band). Genetic distance data matrices were constructed using the method of Nei and Li (1979) and group average clustering were performed by the un-weighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal 1973). The entire analysis and drawing of the phenogram were performed using TREECON Version 1.3b phylogenetic program for Window-based environment (Van de Peer and De Wachter 1994).

Results and discussion

The AFLP technique produced amplified fragments in the range of 20-350bp. The variation noted in the fragment size was attributed to the variation in the selective sequence of the *EcoR* I and *Mse* I primers (van Treuren 2001).

The results of UPGMA clustering of the 115 AAA-EA genotypes tested are shown in Figure 1. Low bootstrap values (0 – 85%) suggested absence of clusters and close genetic relationships among the cultivars. The majority of accessions were in the range of 0.1 – 0.4 Nei's genetic distance from each other, which also shows close genetic relatedness.

The majority of the AAA-EA accessions tested ended up in the same clone set identified by Karamura (1998) (*Musakala*, *Nfuuka*, *Nakitembe,* and *Nakabululu*) but some AAA-EA accessions fell into a different group (Table 1). With the exception of the beer clone set, which did not show up as a distinct group in our analysis, the clone sets proposed by Karamura (1998) were subclusters under the AFLP method. The unrooted analysis (Figure 2) further showed that each subcluster was subdivided into sub-subclusters.

Musakala, which the unrooted data analysis classified as the most distinct subcluster, was grouped into 4 sub-subclusters and separated from the other subclusters at 0.72 Nei's genetic distance (Figure 2). Karamura (1998) also reported *Musakala* to be the most distinct cluster and indicated that its accessions are characterized by uniquely giant, lax and long bunches and fingers.

Results also showed that *Nfuuka* was very closely related to *Nakitembe* and *Nakabululu,* as was also reported by Karamura (1998). Although *Nfuuka* was reported to be the most heterogeneous and largest cluster by Karamura (1998), it was the smallest subcluster in our analysis. The most distinguishing feature of *Nfuuka* subcluster is the ability of its accessions to alter phenotypes over time (Karamura 1998), hence its name, *Nfuuka*; which literally means "I am changing", "I am going to change", or " I keep changing". The natural structural rearrangements that frequently occur within and between banana chromosomes may make any attempts to develop realistic classification difficult (Faure *et al.* 1993). The accessions in the *Nfuuka* subcluster could be undergoing such a process that makes it able to alter phenotypes over time. The central topological position of *Nfuuka* subcluster on the unrooted phenogram (Figure 2) and the "keep changing" nature of *Nfuuka* reported by Karamura (1998), from which *Musakala*, *Nakitembe* and *Nakabululu* subclusters branch off (Figure 1) suggests that it could be responsible for the generation of other subcluster accessions.

Nakabululu and *Nakitembe* very closely related, according to our analysis. Karamura (1998) also reported that *Nakabululu* and *Nakitembe* were closely related, a closeness reflected by the early maturation of their accessions. Results showed that *Nakabululu* and *Nakitembe* were separated by negligible bootstrap support at the branch point and majority of accessions separated by less than 0.5 Nei's genetic distance from each other (Figure 1) indicating close genetic relationship. Although Karamura (1998) reported that *Musakala* and *Beer* were the two most distinct clusters, our analysis put *Nakabululu* and *Musakala* as the most distinct subclusters, grouping them at the opposite ends of the phenogram (Figure 1). This is further reflected by the extreme bunch compactness and short fingers of the *Nakabululu* accessions versus the large luxuriant bunches with long fingers of the *Musakala* accessions (Karamura 1998).

Our analysis did not put the beer bananas in a distinct cluster but mixed them with the cooking types. The beer bananas were found to be versions of the cooking bananas but different at a locus (loci) responsible for astringent sap (synthesis of tannins and anthocyanins).

The obligate vegetative reproduction of bananas have led them to maintain the features which accompanied their ancestors when introduced in the region (Simmonds 1966). Somatic mutations, with preferential cultivation of mutants by man, has resulted in the low levels of genetic diversity seen in the germplasm. According to Karamura (1998), a wide diversity of the physical features, climates and social backgrounds of the East African region have played a significant role in the diversification of different clones. It is possible that the high frequency of translocations and (retro)transposons and irregularities in meiosis and methylation (Lagoda *et al.* 1999) have been responsible for the narrow genetic diversity observed in East African Highland bananas in Uganda.

The low levels of DNA diversity in this study contrasts with the high level of morphological diversity present in these genotypes reported by Karamura (1998), probably due to the influence of genotype X environment interaction on morphotaxonomic techniques (Shanmugavelu *et al.* 1992). The discordance between the AFLP classification and the morphological classification could also be explained by the primers used in our analysis.

The selective sequence of a primer is one of the determining factors of the multiplex ratio (the number of different loci that can be simultaneously analyzed per experiment) (Bryene *et al.* 1997). Results indicated (data not shown) that individual primer combinations could not reveal enough polymorphism upon which to draw conclusions, because some parts of the genome were left unscreened for polymorphism (van Treuren 2001).

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Table 1. Classification of 115 East African Highland banana accessions based on rooted and unrooted analyses of AFLP data, and compared to a classification based on morphological characters (Karamura and Pickersgill, 1999).

AFLP classification Code*	Name	Morphological classification
***Subcluster* Musakala**		
Sub subcluster 1 (MS1)		
F6	Mutiliti	*Musakala*
F5	Oruhuna	*Beer*
G3	Kifuba	*Nfuuka*
F9	Kisaabo	*Nfuuka*
F8	Lwandungu	*Nfuuka*
F7	Shombobureku	*Beer*
F4	Entazidukwa	*Nfuuka*
G4	Bareka	*No record*
G2	Mwanga	*Beer*
I7	Mugisu-agenda	*Musakala*
I6	Kisansa	*Musakala*
I5	Rwabakongo	*Musakala*
D6	Enjogabakazi	*Musakala*
D8	Mukazi-alanda	*Musakala*
D7	Nalweunzika	*Musakala*
F3	Enyabakazi	*Musakala*
I8	Mpologoma	*Musakala*
J6	Bitambi	*Nfuuka*
Sub subcluster 2 (MS2)		
J8	Enzirabushera	*Nfuuka*
J7	Ndibwabalangira	*Nfuuka*
J9	Luwata	*Musakala*
K1	Mayovu	*Nfuuka*
J5	Enjagata	*Musakala*
I9	Nandigobe	*Nakitembe*
J4	Bikowekowe	*Nakitembe*
J3	Enyarutere	*Nakitembe*
J2	Nakibinyi	*Nfuuka*
J1	Kigerekyanjovu	*No record*
Sub subcluster 3 (MS3)		
G5	Muvubo	*Musakala*
G1	Nalugolima	*Musakala*
L3	Tulatwogere	*Nfuuka*
B3	Lwezinga	*Nfuuka*
A9	Keitaluganda	*Beer*
E5	Enzirabahima	*Nfuuka*
E1	Kifuba	*Nfuuka*
D9	Enyeru	*Nfuuka*
E2	Namafura	*Nfuuka*
E8	Kasitaza	*Nfuuka*
A8	Likhako	*Nfuuka*
Sub subcluster 4 (MS4)		
B4	Enyoya	*Musakala*
B1	Mudwale	*Beer*
K2	Lumenyamagali	*Musakala*
B2	Namunwe	*Musakala*
D2	Kabusi	*No record*
M5	Musakala	*Musakala*
M7	Bandagyeya	*Musakala*
M4	Nalukira	*Beer*
***Subcluster* Nfuuka**		
Sub subcluster 1 (NF1)		
C1	Namadhi	*Beer*
B5	Nasala	*Nakitembe*
A7	Lisandalo	*Nakitembe*
A6	Nambi	*Nfuuka*
A1	Nyamashari	*Nfuuka*
E3	Njeriadet	*Nfuuka*
C2	Nabusa	*Nfuuka*
L4	Nfuuka	*Nfuuka*
K3	Namwezi	*Nfuuka*
H5	Atwalira-Nyina	*Nfuuka*
Sub subcluster 2 (NF2)		
K9	Entukura	*Beer*
K8	Nakayonga	*Nakabululu*
K7	Katalimbwambuzi	*Beer*
L2	Nante	*Nfuuka*
L1	Siira white	*Nakitembe*
M6	Siira red	*Nfuuka*
H2	Nassaba	*Nfuuka*
E7	Kulwoni	*Nfuuka*
***Subcluster* Nakitembe**		
Sub subcluster 1 (NT1)		
H7	Nakitembe	*Nakitembe*
G9	Entaragaza	*Nakitembe*
I2	Enkonera	*Nfuuka*
H9	Mbwazirume	*Nakitembe*
H1	Salalugazi	*Nakitembe*
G8	Entundu	*Beer*
I1	Imbululu	*Beer*
H4	Enkara	*Beer*
G7	Luvuta	*Nakitembe*
H3	Kibidebide	*No record*
H8	Kibagampera	*Beer*
G6	Nakaangu	*Nakitembe*
Sub subcluster 2 (NT2)		
I4	Ekirama	*No record*
I3	Nakawere	*Nfuuka*
M3	Nalwera	*Nakitembe*
L7	Kafunze	*Nakabululu*
L8	Nakakongo	*No record*
H6	Mende	*Beer*
M2	Entanga	*Beer*
K6	Nakyetengu	*Nakitembe*
K5	Lwefusa	*Nfuuka*
K4	Namaliga	*Nakitembe*
M1	Bagandeseza	*Beer*
L9	Bifusi	*Nakabululu*
L5	Engumba	*Beer*
***Subcluster* Nakabululu**		
Sub subcluster 1 (NB1)		
E9	Bwara	*Beer*
E6	Tereza	*Nfuuka*
D4	Namulondo	*Nakitembe*
F1	Nakinyika	*Nfuuka*
Sub subcluster 2 (NB2)		
B9	Endembezi	*Beer*
B8	Enyambo	*Nfuuka*
B7	Namesti	*Beer*
C4	Kazirakwe	*Nakabululu*
B6	Wekanga	*Nakabululu*
F2	Kibuzi	*Nakabululu*
E4	Nakhaki	*Nfuuka*
C3	Nambogo	*Nfuuka*
A2	Nakasabira	*Nfuuka*
Sub subcluster 3 (NB3)		
D5	Namamuka	*Nfuuka*
C6	Nkobe	*Nfuuka*
L6	Ensasa	*Beer*
C5	Namunyere	*Nakabululu*
A3	Keitabunyonyi	*Nakabululu*
A5	Nalusi	*Beer*
A4	Ensika	*No record*
C7	Engambani	*Beer*
C9	Kabucuragye	*Nakabululu*
C8	Enshenyuka	*Beer*
D1	Butobe	*Nakabululu*
D3	Nakibuule	*No record*

*Codes are for identification purposes only.

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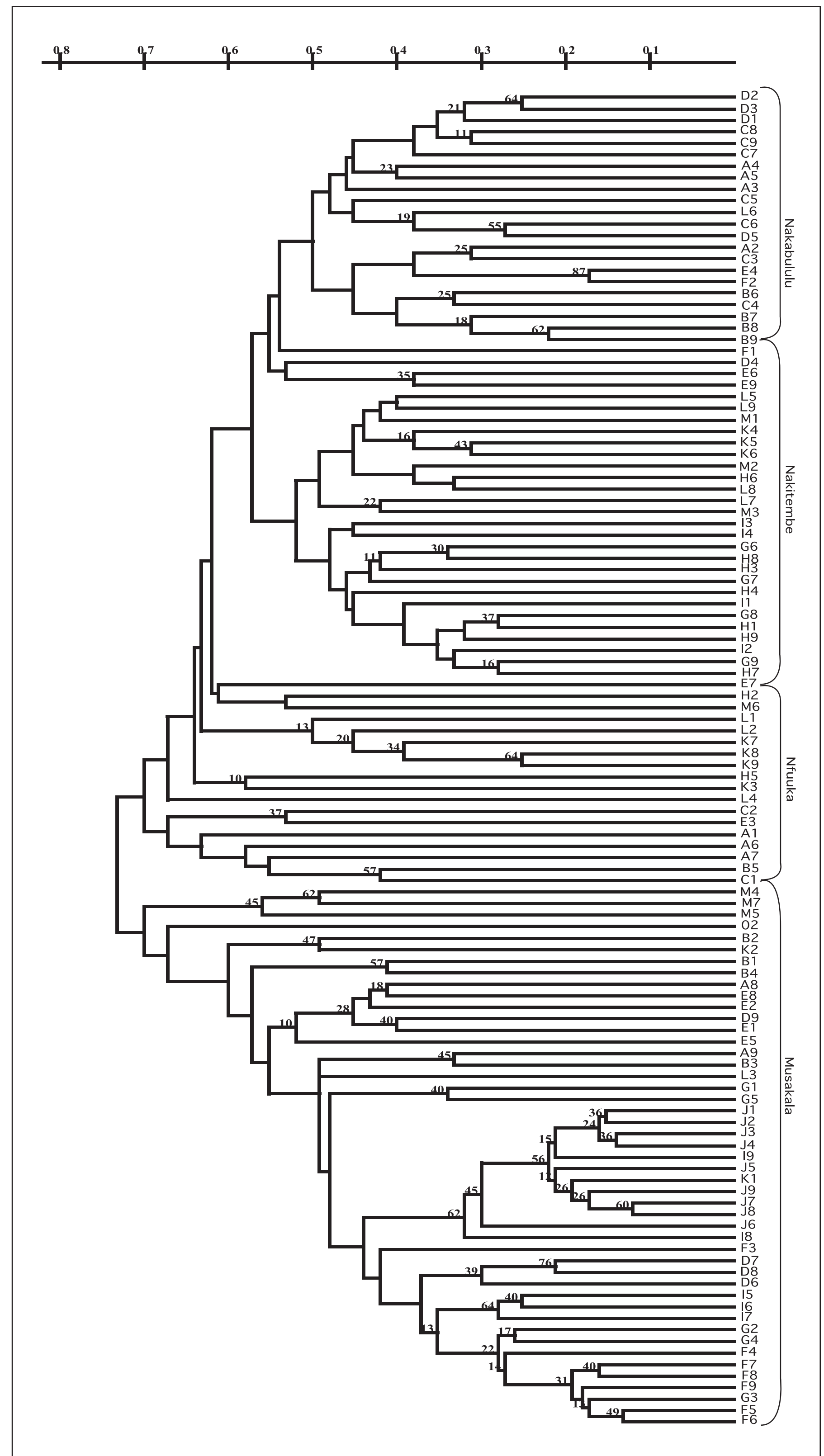
Figure 1. *Phenogram of 115 East African Highland banana accessions from Uganda using Nei's genetic distance on AFLP data. The scaled bar on top of the phenogram refers to Nei's genetic distance and the numbers in the phenogram are bootstrap support values. The codes at the base of the phenogram refer to the accessions tested, which are presented in Table 1.*



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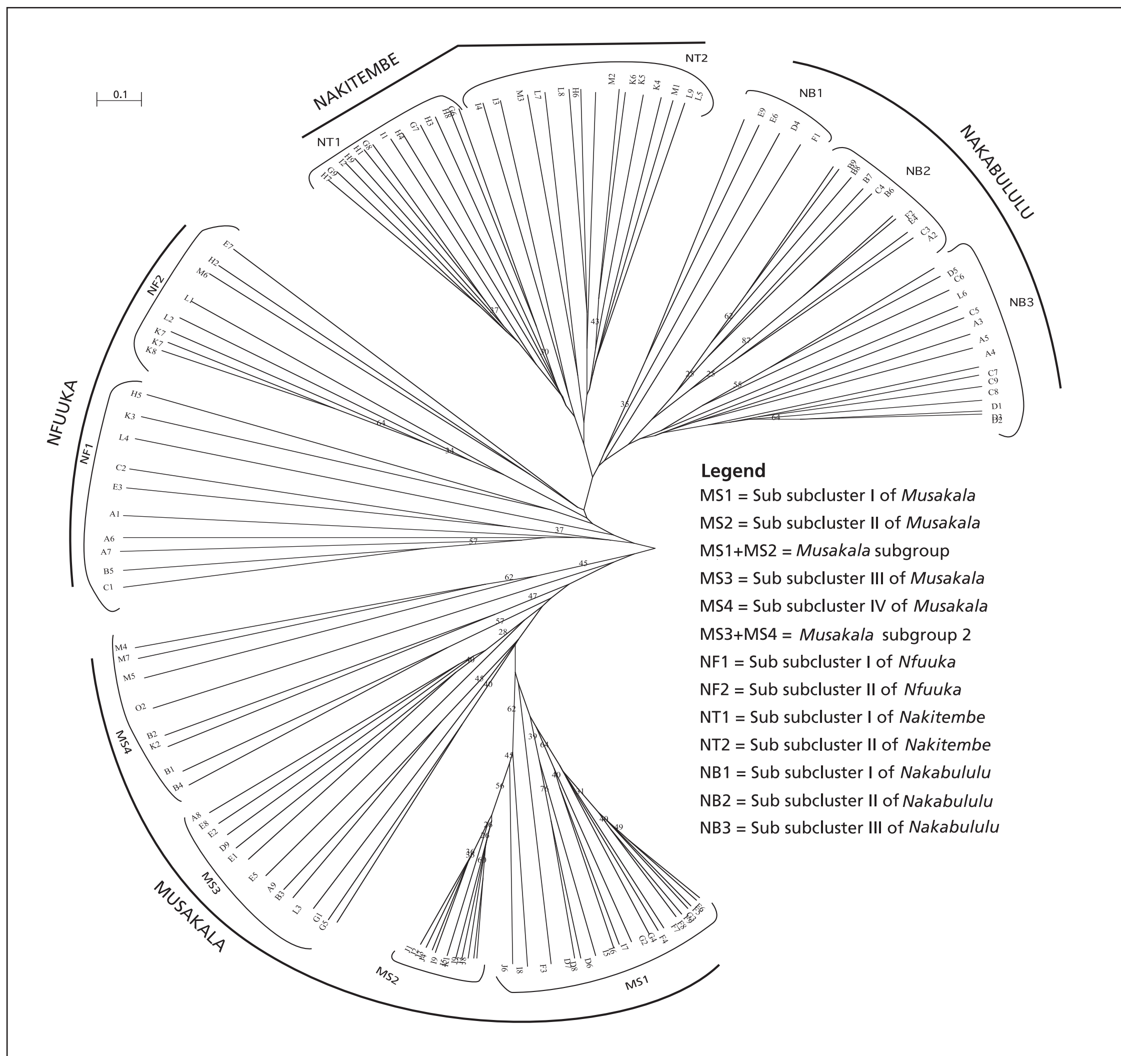

Figure 2. *Unrooted phenogram of 115 East African Highland banana accessions from Uganda using pooled AFLP data. Labels at branch points are bootstrap support values, and the bar on upper left hand corner of the phenogram is Nei's genetic distance scale. The codes refer to the accessions tested, which are presented in Table 1.*

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