Chapter 4

Genetic Diversity in Banana and Plantains Cultivars from Eastern DRC and Tanzania Using SSR and Morphological Markers, Their Phylogenetic Classification and Principal Components Analyses

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Abstract

Bananas and plantains are edible and vegetatively propagated parthenocarpic species of the genus *Musa.* They are used as staple food, dessert and cash crop by more than hundred millions of people throughout the world. However, the crop is threatened by several pests and diseases in central and eastern Africa. One way of partly solving this problem is to have diploids which have desirable traits currently lacking in the AAA-Lujugira-Mutika subgroup. The study assessed through 21 microsatellite markers pairs the cladistic closeness of the diploid AA-Mshale accessions with AAA-Lujugira-Mutika with the purpose of inclusion in breeding programmes. Results showed that the eight studied accessions of AA-Mshale were different from each other. AA-Mshale malembo was fairly well established to be among the ancestor of Lujugira-Mutika, suggesting the determinism of its pollen viability and the level of resistance to pests for including in breeding programmes. The use of two pairs of microsatellites per chromosomes linkage group established the existence of alleles' deletion, recombination or non-annealing. The closeness among AA-Mshale and AAA-subgroups (Ibota, Gros Michel and Green Red) so far established through other techniques was confirmed. The results recommend the use of microsatellite markers, covering 11 linkage groups for cultivar identification and diversity study.

Keywords: *Musa*, AA-Mshale malembo, AAA-EAHB, clade, SSR markers

1. Introduction

1.1. Background

Bananas and plantains are edible and vegetatively propagated parthenocarpic species of genus *Musa* belonging to the family *Musaceae* which according to Meng et al. [1] has wild seeded species native to South-East Asia. These seedless edible species are thought to have originated through intra- and interspecies crosses between *M. acuminata* Colla and *M. balbisiana* Colla, including some back crosses [2]. These species constitute a staple food, a key commercial crop and a major source of raw materials for both beverage and handicraft industries for hundred millions of people in the world. They include 20% of the population of the United Republic of Tanzania (URT), and its production promotes the country to be the second largest producer after Uganda in east Africa [3–5].

1.2. Problem statement and justification

The east African highland bananas (EAHBs) are currently threatened by several pests and diseases, which need diploid parents with farmers and other consumers' desirable traits for inclusion in the breeding programme [6]. The edible diploid landrace 'Mshale' (Mchare [7], AA genomic group) of URT was identified to be highly similar to *M. acuminata* spp. *malaccensis* cv. 'Pisang lilin'. Research using numerical taxonomy on AAA-EAHB genomic subgroup from Eastern DRC and Tanzania has shown certain level of relationship with 'Mshale malembo', suggesting that it is one of the ancestors [8, 9]. These observations were supported by Simmonds [7] and De Langhe et al. [10] but need to be confirmed at a molecular level. Such research has not yet been done and remains dearth for the inclusion of the Tanzania's landrace in breeding programme. Elsewhere, such research using the AFLP technique has been conducted by Ude et al. [11], on phylogenetic origin of AAA-Gros Michel and AAA-Yangambi km 5. The technique has shown that these cultivars have similar ancestors that have contributed to their development. In this respect, *M. acuminata* spp. *malaccensis* cv. 'Pisang lilin' was identified as a source of one of their genomes (A). This supported the use of landrace AA 'Paka' from Zanzibar in the improvement of 'Gros Michel' in Jamaïca [7, 10].

1.3. Hypothesis, technology justification and objective

AFLP technique shows a dominant mode of inheritance and hence constitutes its limiting factor for this study. On the other hand, research using SSR markers has confirmed these preceding findings [12]. Moreover, the fact that the genetic map has 11 linkage groups of Pisang lilin was also reported [13]. Therefore, the determination of identity and confirmation of the contribution of 'Mshale' in the AAA-EAHB using microsatellite markers determined from Pisang lilin could be a useful tool for the regeneration of subgroups escaping genetic erosion due to pests. This would constitute different scientific point of view from the current belief that AAA-EAHB comes from somaclonal variation [14]. The study aimed to establish the cladistic relationship of the banana landrace 'AA-Mshale' in AAA-EAHB which may constitute a way for reconstituting the EAHB through breeding.

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2. Materials and methods

2.1. Plant materials

Cigar (unfurled) leaf samples from 25 accessions of bananas and plantains (**Table 1**) were collected from the existing banana gene bank in the Horticulture Unit of Sokoine University of Agriculture (SUA). The 25 accessions consisted of eight edible diploids (AA), nine, four and two triploids (AAA, AAB, ABB), two tetraploids (AAAA) genomic groups which were determined through numerical morpho-taxonomic classification [15]. Apart from the diploids and triploids AAA-EAHB subgroup, the other subgroups and genomic group were added as

No	Name of cultivars	Genomic group	Subgroup, clone set
01	Unyoya	ABB	Pisang Awak
02	Bokoboko	ABB	Bluggoe
03	Mzuzu	AAB	French Plantain
04	Ngego I	AAB	French Plantain
05	Ngego Halisi	AAB	French Plantain
06	Kisukari	AAB	Silk/Kamaramasengi
07	FHIA 17	AAAA	FHIA
08	FHIA 23	AAAA	FHIA
09	Bukoba	AAA	EAHB-Musakala, cooking type
10	Embwailuma	AAA	EAHB-Nakitembe, cooking type
11	Mwanjunjila	AAA	EAHB-Nfuuka, cooking type
12	Muhowe	AAA	EAHB-Nfuuka, beer type
13	Kimalindi fupi	AAA	Dwarf Cavendish
14	Jamaïca	AAA	Gros Michel
15	Yangambi km 5	AAA	Ibotabota (or 'Ibota' in short)
16	Mzungu mwekundu	AAA	Red/Green-Red
17	Mshale malembo	AA	Mshale
18	Mshale makyughu	AA	Mshale
19	Nshonwa mshale	AA	Mshale
20	Ndyali	AA	Mshale
21	King banana	AA	Wild diploid
22	Huti	AA	Mshale
23	Ilalyi	AA	Mshale
24	Ijihu	AA	Mshale
25	Green bell	AA	Mshale

Table 1. Cultivars used in molecular characterization using SSR markers.

control to verify the accuracy of the ancestry. The SUA *Musa* sp. germplasm was an *in situ* field conservation located in the plateau zone of Morogoro Urban District of Tanzania [5].

2.2. DNA extraction

The DNA of the 25 accessions (**Table 1**) was isolated using DNeasy Plant Mini Kit (Qiagen, USA; www.qiagen.com) following the manufacturer's instructions, quantified in 2% agarose gel (in 0.5 TBE electrophoresis buffer) and stained in 5 μg/ml of ethidium bromide solution. The DNA quality was checked by ensuring that the 260/280-nm values ranged between 1.4 and 2.2 using spectrophotometer [12]. The PCR was performed using a Gene Amp PCR system 2700 thermocycler (Applied Biosystems). Each reaction was carried out in a total volume of 20 μl, containing 10 ng of genomic DNA, 1.2 mM $MgCl_2$, 10 mM dNTPs, 0.2 μM of each primer, 1.25 U of Taq polymerase and 10x Go Taq flex buffer (New England Biolabs, Inc.). Twenty-one SSR primer pairs (**Table 2**) distributed across the 11 linkage groups were used. This SSR primer selection was done among established linkage groups covering banana genome [13]. During

SSR	Motif	LG	Forward primer (F) / Reverse primer (R)	°C	bp
mMaCIR105	(CA)8,(CT)15	6	CATCCACTTGCTTTTCCA	52.0	264
			CTTCACGGCTTCCACA		
mMaCIR114	(AC)7,(CT)28	8	GCAAGCCAAAGGGAA	50.0	222
			ACCAACAAAGAATGGTGTAA		
mMaCIR115	(CA)2	11	CAAGAGACTACCACCGAAGA	53.0	114
			TGATTCTCACGACGTATGG		
mMaCIR117	(TC)20	7	GTTTGTGGAATAAGTGGGAA	53.0	214
			ATGAGGGAGTTAGTGGTGG		
mMaCIR119	(CA)9,(TA)6,(CA)5	10	TGAAAAGCAATCCAACCT	51.0	395
			ACCCTGAAATGTTTGTCTTT		
mMaCIR168	(CA)7	10	GCACCAAACCAGTCCTAC	54.5	243
			CGTCTCAGTTGCCGTG		
mMaCIR172	(CT)19	1	CAGCTAATGCCAAACCC	53.0	258
			CGACTTCGAGCGAGC		
mMaCIR174	(AG)13	2	GAACCCACCTCCCTCTT	54.2	167
			TGGGATTCCTGAGTGCT		
mMaCIR180	(CA)7	1	GCCTCAGCCTCATCATC	54.0	226
			CACCCACTCGACCCA		
mMaCIR189	(CT)3,(CT)16	2	GGGAGGGCAGAGGAA	53.0	259
			GCCGAACTTGGTAATGTG		
mMaCIR192	(TG)8	3	TGACCTAGCACAACGCA	53.5	133
			GCTTATGTTTCATCGCCTT		

SSR	Motif	LG	Forward primer (F)	°C	bp
			Reverse primer (R)		
mMaCIR210	(GA)3,(TG)12,(AG)5	7	GGAAGGTGGCATGAAAG	52.0	319
			TAACCTGATACCCATGTATTGA		
mMaCIR228	(CT)18,(AC)7	5	CAAGCATGTTAGTTTGGGA	52.0	197
			AAGGTGCATCCAAGGG		
mMaCIR241	(TC)20	3	GCTAAGCATCAAGTAGCCC	53.0	297
			ACGAACAAGCAATCAAAGTAG		
mMaCIR256	(CA)7	4	TTGCGGGAAACTGCT	53.0	280
			GTTGCACTGCCCACTT		
mMaCIR257	(CA)7	9	CTTTACCGAGTTGAGGG	50.0	234
			TCATATCAGAAGATAGCCAA		
mMaCIR273	(TC)22,(CT)6	9	TGGTTGAAGATTCCCAT	50.0	211
			GATCAAGAGGTGACAAACC		
mMaCIR274	(AC)11	5	TAGCTCTTTCAACACTCTCATC	53.0	150
			CTGGAGGCAGCGAAC		
mMaCIR280	(TC)7,(AC)7	4	GGGTCCCTGTTGGCT	54.0	221
			TTGCAGATTAGGGTGGG		
mMaCIR297	(TC)9,(AC)13,(CA)9	11	GAACTCGGATTGTTCCTTT	53.0	173
			AGGCTGATGGTAGCGAG		
mMaCIR301	(TG)11	6	CATGATGTTTGAGTTTGC	50.0	166
			CTGGAAAGCAACACCG		

Table 2. Primer sequences, SSR repeat motif, linkage groups (LG), theoretical annealing temperature (°C) and expected PCR product's size (bp).

amplifications, temperature cycling was conducted as follows: an initial denaturation step at 95°C for 5 min that was followed by 32 cycles of denaturation at 94°C for 1 min, annealing at each temperature as specified in **Table 2** per primer pair for 1 min, and extension (elongation) for 90 s at 72°C. A final extension was carried out at 72°C for 7 min. For gel electrophoresis, a 10-μl aliquot of each amplification reaction was separated at 100 V for 2 h, using 2% agarose gels (0.5× TBE buffer). Gel images were photographed under UV illumination to check for amplicon size and PCR specificity. Allele sizes were estimated against 2-Log DNA Ladder molecular size standards. All samples were run with three replications starting from DNA extraction to maintain the integrity of the sample.

2.3. Data analysis

Alleles (0, 1, 2, …) were scored from 21 SSR marker pairs in the 25 accessions and were used to build the phenetic and cladistic trees. The data were analyzed using Numerical Taxonomy and/or

Multivariate Analysis System package (NTSYSpc) version 2.1 (Exeter Software, Setauket, USA). The Manhattan method was used to assess similarity among the banana accessions. The genetic similarity matrices were then used to construct the dendrogram with unweighted pair group method with arithmetic mean (UPGMA) algorithms that employed the sequential, agglomerative, hierarchical and nested clustering procedure [16]. The cladistic kinship between accessions was determined based on neighbor joining coefficients using Dice dissimilarity coefficients (matrix using NTSYSpc 2.1. The scattered plot and accuracy of the trees were determined using principal component analysis (PCA) and cophenetic correlation method (from NTSYSpc 2.1). A two-way Mantel statistic test of 500 permutations was performed to get a cophenetic value.

3. Results and discussion

3.1. Results

3.1.1. Molecular/genetic relatedness among accessions

The coefficient of dissimilarity varied from 0.28 to 0.66, being <1 or 100% showing no duplication among accessions from the 21 loci covering 11 linkage groups used as shown in **Figure 1**. Hence, the eight accessions belonging to AA-Mshale group were found to be genetically different. The dendrogram (**Figure 1**) established two main clusters (A and B). In the first cluster (A), AAA-Lujugira-Mutika accessions ('Bukoba'/Musakala, 'Muhowe'/Beer (Mbidde) and 'Embwailuma'/Nakitembe) were clustered with the seven accessions of AA-Mshale ('Ndyali', 'Mshale makyughu', 'Ilalyi', 'Mshale malembo', 'Nshonwa mshale', 'Ijihu' and 'Huti'). They included the tie of AAAA-FHIA (17 and 23) accessions with 'Yangambi km 5' (AAA-Ibota), 'Mzungu mwekundu' (AAA-Green-red) and 'Green bell' (AAA-Cavendish). Whereas in the second cluster (B), six heterogenomic accessions named 'Kisukari' (AAB-Silk), 'Ngego I', 'Ngego Halisi' and 'Mzuzu' (AAB-French Plantain), 'Unyoya' and 'Bokoboko' (ABB) were tied to three homogenomic accessions (AAA) 'Jamaica' (Gros Michel), 'Kimalindi fupi' (Dwarf-Cavendish) and Mwanjunjila (EAHB having a yellow male bud). The accession 'King banana' (AA) was an outline.

The genetic variation causes were allelic deletion or non-annealing and heterozygosis. The mMaCIR168 primer showed allele deletion in cultivars 'Ndyali' and 'Mwanjunjila' (first one and third three after (left) Ladder, **Figure 2**), and mMaCIR189 showed heterozygosis in cultivars 'Mshale Makyughu', 'Ilalyi' and 'King banana' (first six, nine and second three after ladder) while both primers showed a homozygote allele in cultivar 'Mshale malembo' (the first number six after the ladder). Similarly, alleles' deletion (null alleles) was observed among 19 cultivars for primers mMaCIR117 and mMaCIR174. The alleles' sizes resemble those of Hippolyte et al. [13].

The observed mutation has negatively influenced the principal component analysis (PCA) that resulted in poor fit of the clustering analyses with a cophenetic coefficient of 0.72 from distance matrix and 0.67 from product-moment correlation matrix. Consequently, the variation has spread over the principal component (PC) so that the three first PCs cannot hold the maximum of the variation (**Figure 3**) and hence weakened the value of PIC (Polymorphism Information Content).

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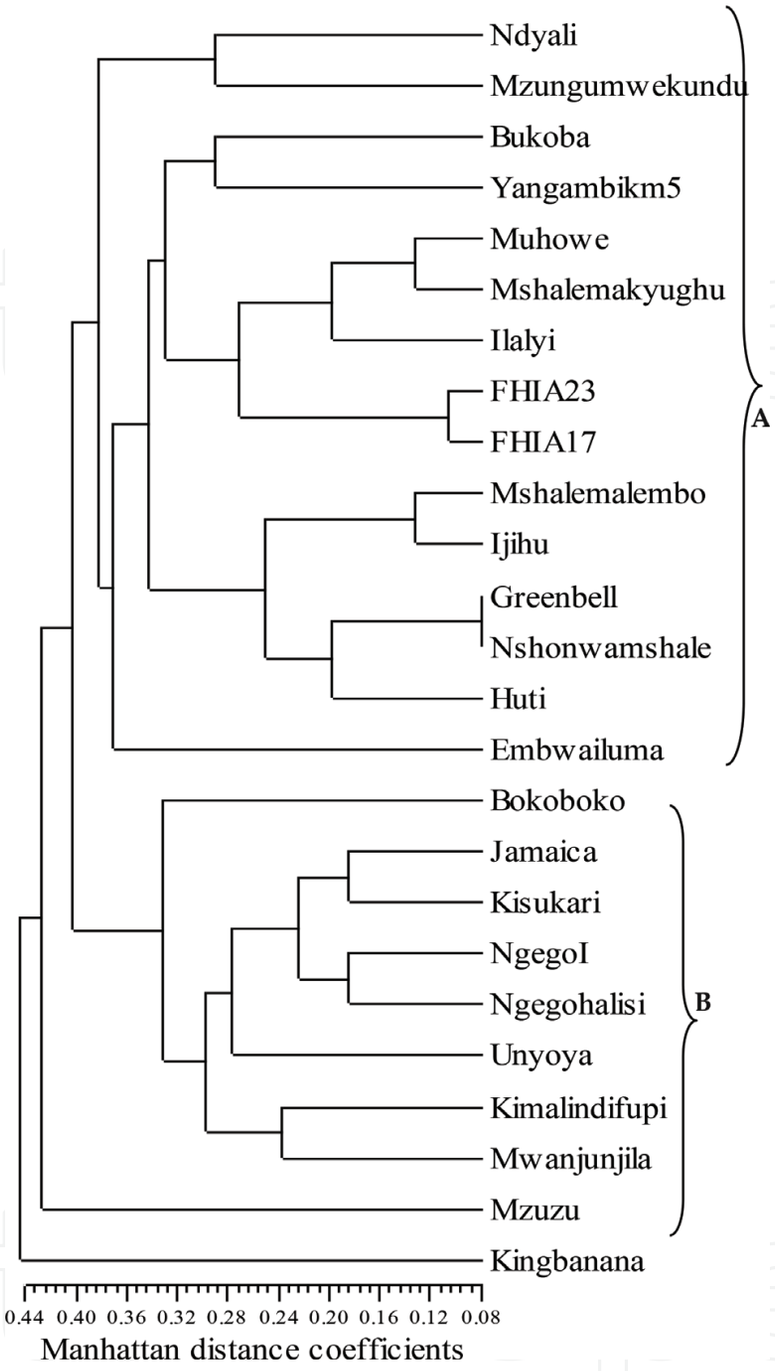

Figure 1. Phenogram from UPGMA clustering of the average Manhattan coefficients between the 25 *Musa* accessions using 21 microsatellite markers covering 11 linkage groups.

3.1.2. Cladistic relationship

The cladogram showed three clades which revealed mono-, para- and polyphyly (A, B and C, **Figure 4**). The eight AA-Mshale accessions were subdivided into two clades. The first clade (A) was a monophyletic group composed of eight accessions in which six belonged to AA-Mshale genomic group ('Ndyali', 'Mshale malembo', 'Ijihu', 'Nshonwa mshale', 'Huti' and 'King banana') and two of triploid ('Green bell' (AAA-Cavendish) and 'Mzungu mwekundu' (AAA-Green-red).

Figure 2. On gel image of alleles from mMaCIR117, mMaCIR168, mMaCIR174 and mMaCIR189 using 25 banana accessions (eight edibles diploids (AA-Mshale), nine AAA, two AAAA, four AAB and two ABB genomic groups) of (SUA) (Tanzania).

The second clade (B, **Figure 4**) that encompassed AAA-EAHB accessions was subdivided into two subclades (B1 and B2) and formed paraphyletic group with the first clade. The first subclade (B1) was made of three accessions, 'Mzuzu', 'Bukoba' and 'Yangambi km 5', that belonged to AAB-French Plantain, AAA-EAHB-Musakala and AAA-Ibota, respectively. Whereas, in the second subclade (B2), the AAA-EAHB accessions 'Muhowe' and 'Embwailuma' shared the ancestry with AA-Mshale (Mshale makyughu and Ilalyi) and AAAA-FHIA (17 and 23). The last clade (C) had 'Kimalindi fupi' (AAA-Cavendish), 'Mwanjunjila' (AAA-EAHB) and Jamaica (AAA-Gros Michel) sharing a common ancestry with AAB-Silk (Kisukari), AAB-French plantain (Ngego Halisi and Ngego I) and ABB (Bokoboko and Unyoya). The clade (C) established a polyphyly with the two first clade (A and B) that had AA genomic group accessions. Whereas, in reference to accession 'Jamaica', there was a paraphyly between the clades B and C.

3.2. Discussion

This clustering from dissimilarity using UPGMA fairly confirms the relationship established by numerical taxonomy between the AA-Mshale malembo and the AAA-Lujugira-Mutika group determined by several authors [2, 7–9]. Likewise, the observed alleles' differences among AA-Mshale accessions were in line with the morpho-taxonomic dissimilarity determined previously by the upcited authors. Moreover, the clone sets (Musakala, Nfuuka and Nakitembe) coined

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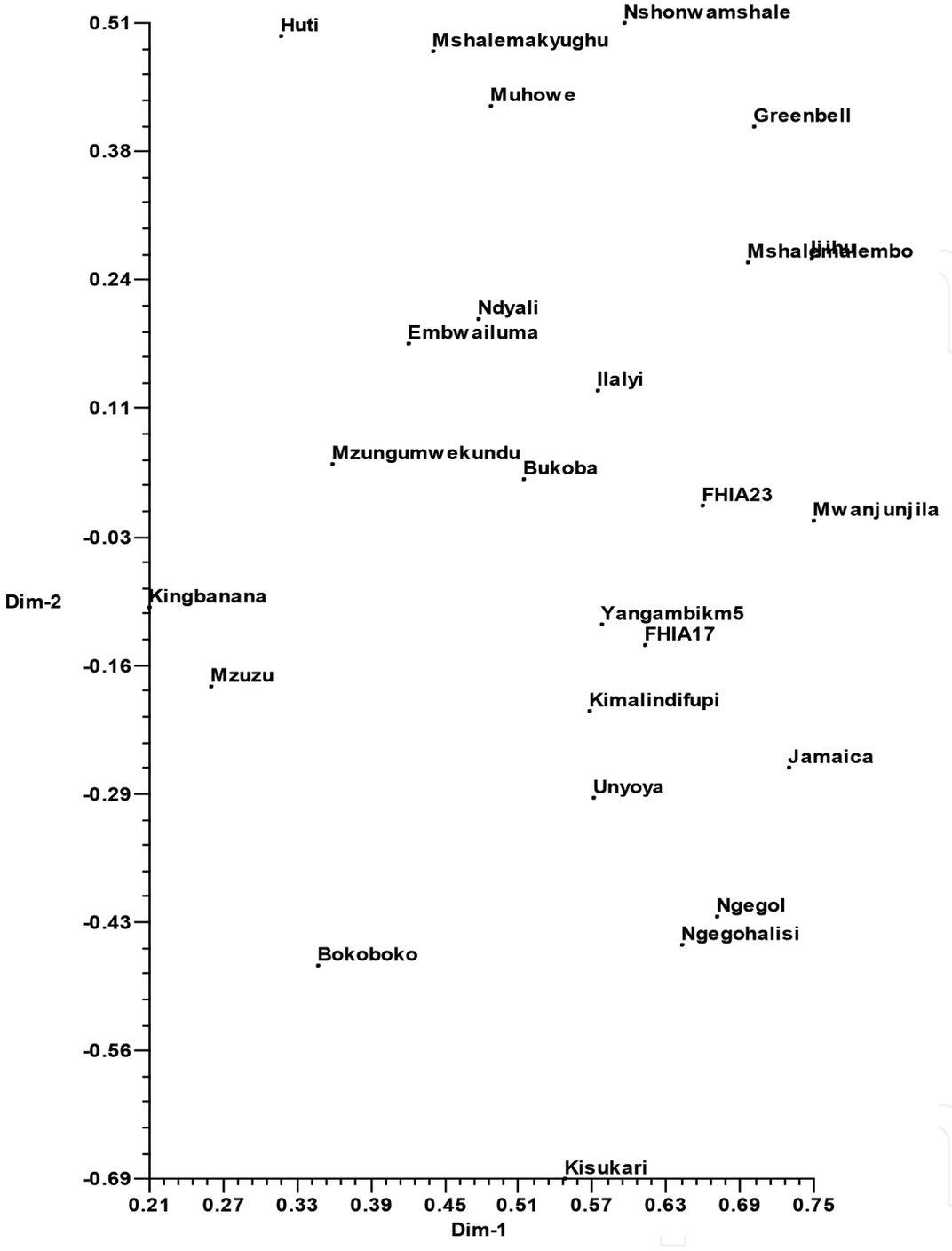

Figure 3. PCA showing the relative positions on the first (Dim-1) and second (Dim-2) PCs of the 25 banana accessions of the SUA's genebank using 21 microsatellite primers.

subjectively within the AAA-Lujugira-Mutika were linked with the different AA-Mshale accessions following their alleles' closeness [16]. Interestingly, the clustering of AAA-Cavendish, AAA-Gros-Michel, AAA-Ibota, AAB-Plantain and AAB-Silk subgroups as sympatric is similar to results of [11, 12, 17], while they used other techniques or primers partly covering the 11 linkage groups [13]. This once more established the usefulness and reliability of the alleles from the 11 linkage groups in diversity and cladistic study.

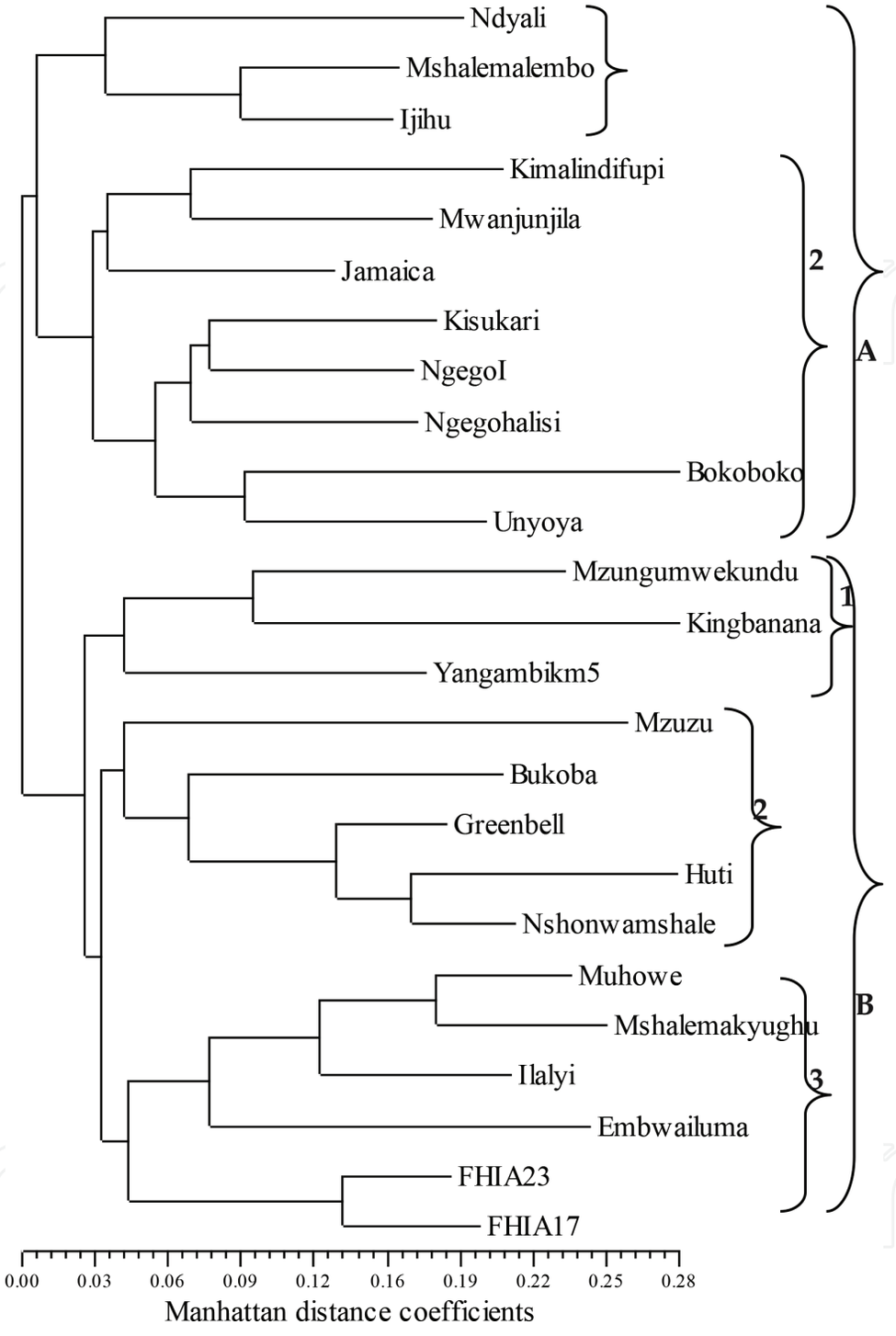

Figure 4. Cladogram from neighbor joining clustering of the Manhattan dissimilarity coefficients between the 25 *Musa* accessions from SUA genebank and 21 microsatellites.

The mono-, para- and polyphyletic relationships are in line with those revealed from numerical morpho-taxonomy [7–10]. The para- and polyphyletic relationship may be explained by the hypothesis of back-crosses developed [2]. The back-crosses theory explains the role of the observed alleles deletion and rearrangement (heterozygosis) in the evolution of AA-Mshale malembo in the AAA-EAHB. These relationships were also similar to results from other microsatellites covering 10

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linkage groups [17]. However, there is contrast with the statement of lack of convincing lineage between 'Mutika-Lujugira', 'Red', 'Ibota' and 'Plantain' subgroups, and the diploid *M. acuminata* accessions. This may be explained by the poor fit of the clustering analysis and the spread of principal components over the variables due to observed mutation.

4. Conclusion and suggestion

The eight accessions of AA-Mshale were determined at allele level to be different from each other. The contribution of accession AA-Mshale malembo in the ancestry of AAA-Lujugira-Mutika has been ascertained using simple sequence repeat tandem (SSR) markers. This suggests more studies on the parameters like pollen viability, germination and level of resistance to diseases and pests before inclusion in the breeding programme. The SSR markers constitute the best tool for cultivar phylogenetic identification, marker-assisted selection and diversity study.

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Conflict of interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this chapter.

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