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Molecular Characterization of Banana Cultivars (*Musa* Spp.) From Egypt Using AFLP

[1]Dina A. El-Khishin, [2]Ebtsam L. Belatus, [1]Amina Abd El-Hamid and [1]Khaled H. Radwan

[1]Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt.
[2]Horticulture Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

Abstract: A wide scope of morphological variation leading to cultivar confusion is a quite common problem in banana worldwide. Banana genetics is relatively unknown and is complicated by specific inter-hybridization, heterozygosity, and polyploidy. These factors make identification of closely related banana cultivars difficult, particularly when sterile. Amplified fragment length polymorphism (AFLP) analysis was employed to distinguish among eleven cultivars of *Musa* grown in Egypt. Ten primer combinations revealed unique molecular markers specific for each of the eleven cultivars, which can be further developed into specific probes for identification purposes. Results showed that such an assessment would be of great help in clarifying the nomenclature situation at the farmer's level. It would also allow for a standardization of cultivars at the national level by linking molecular genotypic studies with the current classification system of *Musa* cultivars based purely on morphological traits.
Finally, a greater effort should be directed at collecting and characterizing banana cultivars from all over Egypt in a national type project.

Key words: AFLP analysis, *Musa* spp. Genetic diversity, DNA fingerprinting

INTRODUCTION

Banana, together with and plantains, represents the forth most important crop in the developing countries. The worldwide production of bananas is 100 Metric Tons, which represents the food staple across most of the poorest parts of the world. In Africa the total banana consumption would comprise up to 400 kg per person per year.

All banana taxonomists seem to agree that no single scientific name can be given to all the edible bananas. *Musa* spp. originated mainly from intra-and interspecific hybridizations between two wild diploid species, *M. acuminate* Colla ('A' genome) and *M. balbisiana* Colla ('B' genome) [1]. Therefore, the cultivated varieties can present different genomic combinations: AA, AB, AAA, AAB, ABB, AAAA, AAAB, AABB and ABBB, diploids, triploids and tetraploids, depending on the basic number of chromosomes, two, three or four, respectively, being eleven, the basic number of chromosomes of the species. The main problem in banana's is that we lack a DNA profile library for *Musa* cultivars in Egypt, also the reassessment of morphological traits, and local names to specific cultivars needs to be re-evaluated in order to reflect the genetic uniformity of a cultivar.

DNA fingerprinting techniques would support a more reliable method for a better identification of both banana species and cultivars [2]. Taxonomic studies in *Musa* have been conducted using a wide array of techniques, such as morphological characters Simmonds [3] and Simmonds and Weatherup [4] isozymes Bhat *et al*., [5] cytogenetics Cheesman [6] and Osuji *et al*, [7] molecular cytogenetics (Osuji *et al*., [8] intergenic spacers Lanaud *et al*., [9] restriction length polymorphism (RFLP) Gawel and Jarret, [10] and Gawel *et al*., [11] random amplified polymorphic DNA markers (RAPD) Bhat *et al*., [12] inter simple sequence repeats (ISSR) Godwin *et al*., [13] microsatellites Grapin *et al*., [14] and finally genomics Heslop-Harrison and Schwarzacher, [15].

Amplification fragment length polymorphism (AFLP) is a DNA fingerprinting technique that was developed by Vos *et al.* [16]. It is based on selective PCR amplification of DNA restriction fragments. It can be used for DNA of any origin and complexity and is reproducible and reliable.

In a preliminary AFLP analysis of *Musa* breeding populations as discussed by Crouch *et al.* [17], AFLP was mentioned as potentially the most powerful tool in the molecular breeding of banana. As proper classification of *M. acuminata* is important in assisting in the selection of characters for banana breeding,

The objectives of the present study are (1) to examine the usefulness of AFLPs in differentiating

Corresponding Author: Dina A. El-Khishin, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt.

Table 1: The Banana Genotypes Studied.

No.	Species/cultivar	Location	Remarks
1	Red or green red	El-Kanater (Sahel area)	
2	Cultivar X1	El-Kanater (Sahel area)	unknown
3	Cultivar X2	El-Kanater (Sahel area)	like M. Ali
4	Cultivar X3	El-Kanater (Sahel area)	like Amble
5	Cultivar X4	El-Kanater (Almaaya area)	like Maghrabi
6	Maghrabi	El-Kanater (El-Islaheia area)	
7	Williams	El-Kanater (El-Islaheia area)	
8	Grand Nain	El-Kanater (El-Islaheia area)	
9	Williams	El-Kanater (station area)	
10	Poyo	Horticulture Research Institute	
11	Hindi	Horticulture Research Institute	

El-Kanater Agricultural Research Station

Table 2: Levels of polymorphism as revealed by AFLP analysis.

Combination no.	Primer Sequence	Total No. of Bands	Polymorphism %
1	E-AAC/M-CAA	137	96
2	E-AGC/M-CTT	120	94
3	E-AAG/M-CAC	81	88
4	E-ACC/M- CAT	88	94
5	E-AGG/M-CTT	60	92
6	E-AGC/M-CTG	45	95
7	E-ACA/M-CAG	60	88
8	E-ACT/M-CTC	70	91
9	E-ACG/M-CTG	89	92
10	E-AGC/M-CAC	110	92

cultivars of banana in Egypt, (2) to develop molecular markers for the most important banana cultivars being bred in Egypt and (3) to determine genetic relationships between the cultivars. A wide scope of morphological variation leading to cultivar confusion is a quite common problem worldwide, and in Egypt, therefore (4) the most important objective was to resolve the problem of cultivar confusion in *Musa*.

MATERIAL AND METHODS

Plant Material: Banana cultivars had been the subject of a systematic taxonomical study several decades ago [18]. The identified specimens were planted in a field collection at the El-Kanater Agricultural Research Station, in the Delta region. The collection was maintained over the years and still provides reliable reference material.

Young cigar leaf tissues were collected from plants cultivated in El-Kanater Experimental Research Station (ARC) and from Horticulture Research Institute (Table 1). Four samples of unknown origin were also collected and designated cultivar X1, X2, X3, and X4. Another seven cultivars namely, 'Green-Red'; 'Poyo'; 'M.Ali'; 'Maghrabi'; 'Williams' and 'Hindi' were also collected.

Leaf samples of different banana cultivars were used for AFLP analysis. Leaves were surface-sterilized according to the procedure described by Zhang *et al.*, [19]. They were imbedded in 95% ethanol for one minute and then they were swirled in 5% sodium hypochlorite solution (w/v) supplemented with Tween 20 for 5 min. Subsequently, leaves were thoroughly rinsed with autoclaved water followed by immersing in 95% ethanol for 30 seconds and were blotted to dry. Leaves were then wrapped in aluminum foil, frozen in liquid nitrogen, and stored at -80°C until further needed.

DNA Extraction: Plant genomic DNA was extracted from leaf samples using the CTAB method according to Reichardt and Rogers [20] for AFLP analysis.

AFLP Analysis: AFLP analysis was carried out according to Vos *et al.* [16]. Restriction digestion of the genomic DNA with *Eco*RI and *Mse*I were carried out overnight at 37°C. Heat inactivation followed, and then genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters overnight at 15°C to generate a DNA template for amplification. PCR was performed in two consecutive reactions. The template DNA generated was first preamplified using AFLP primers, each having one selective nucleotide. The PCR products of the preamplification reaction were then used as template for selective amplification using two AFLP primer combinations. Each primer contained three selective nucleotides Table (2). The final PCR products were run on a 6% denaturing polyacrylamide gel in 0.5X TBE buffer. Silver staining of the gel was carried out according to the manufacturer instructions (Promega Corp., Madison, WI) followed by overnight drying before being photographed.

Data Analysis: For AFLP analysis, only clear and unambiguous bands were visually scored as either present (1) or absent (0). Each band was interpreted as one allele. Bands with the same mobility were assumed to be homologous [21]. Each marker was treated as an independent unit character. The genetic similarities (GSs) and similarity matrices from AFLP data were calculated among species using Dice's coefficient [22]. Cluster analyses were based on a similarity matrix obtained with the un-weighted pair group method using arithmetic averages (UPGMA) Rohlf, [23] and relationships between species were visualized as a dendrogram. All data were scored in the form of a binary matrix. For each pair of species, the Dice's similarity index (GS) was calculated [22]. The calculations were performed with the SAS software for data analysis (SAS Institute Inc.).

RESULTS AND DISCUSSION

The aim of the present study was to study the genetic polymorphism among 11 banana cultivars, and to attempt to resolve the problem of classification of banana cultivars using AFLP technology.

Aflp Profile Analysis of Banana Cultivars: AFLP analysis using 10 primer combinations was performed to detect polymorphism among 11 banana cultivars (Figure 1). AFLP yielded a total of 860 selectively amplified bands ranging in size from 60 bp to 750 bp. A 91.6% of the total fragments were polymorphic. The number of amplified bands per primer combination ranged from 45 to 137 with an average of 86 bands per primer which indicates a high level of polymorphism among the cultivars studied. The maximum number of bands obtained was 137 using primer combination number (1) (E-AAC/M-CAA), whereas the least number of bands obtained was 45 using primer combination number (6) (E-AGC/M-CTG), Table (2), thus confirming the high multiplex ratio produced by AFLP markers. These estimates are in line with those of Powel *et al.,* [24], van Montagu, *et al.,* [25], Ferreira *et al*., [26], Noyer, *et al*., [27], Wong *et al*., [28] and Wong *et al*., [29] concluded that the multiplex ratio obtained from AFLP was higher than that for other techniques. The highest percentage of polymorphism (96%), was obtained using primer combination number (1) (E-AAC x M-CAA), whereas, the lowest percentage of polymorphism (88%) was revealed by two primer combinations namely (3&7) (E-AAG x M-CAC) & (E-ACA x M-CAG) respectively.
The AFLP profiles obtained can be used to distinguish between the different cultivars by their unique banding patterns (Figure1). These results compare favorably to those of Loh *et al.* [30] whose AFLP studies with 8 primer combinations yielded a total of 555 polymorphic and 58 monomorphic bands in 16 banana cultivars as well as too studies carried out by Wong *et al.* [29] in which 8 primer combination yielded of 453 (93%) polymorphic and 34 (7%) monomorphic from three taxa of wild banana (*Musa acuminate* Colla).

The study of Wan *et al.* [31] on 13 banana land races generated 65.2% frequency of polymorphism. The data obtained shows AFLP to be a good method of choice for molecular studies in banana cultivars, by virtue of its ability to pick up more polymorphisms per primer pair as well as it being a reliable and easily repeatable technique.

Cultivar Identification: AFLP analysis of ten primer combinations on 11 banana cultivars was used to identify unique molecular markers specific for each cultivar. Unique markers are identified as bands that specifically identify a cultivar from the others by their presence (positive) or absence (negative). All primer combinations used revealed unique markers giving a total of 58 markers. Primer combination number (5) gave the highest unique number (10 unique markers), while primer combinations number (3, 10) gave the least number of unique markers (2 unique markers).
The highest number of unique markers was observed with cultivar (2) which gave a total of 13 markers, while the rest of the cultivars gave a total of unique markers ranging from 2-6 (Table 3). These results are in accordance with those of Loh *et al.* [30]; Wong *et al.* [28, 29] and Noyer *et al.* [27].

Table 3: Unique positive and/or negative AFLP markers, markers size and total number of markers characterizing each of the eleven banana cultivars.

Primer Combination		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	Total Unique markers	Grand total
Cultivars													
Red	(+)				700			270					
					300			240				6	6
					230			100					
	(-)												
X1	(+)	700	500			240	280	300					
		320		540		170	255	180					
						130	200					12	13
	(-)									320		1	
X2	(+)					200				400		2	2
	(-)												
X3	(+)	200	275			300						3	6
	(-)	195								330			
										210		3	
X4	(+)						290						
							250						
							230		100			4	6
	(-)					95				110		2	
Maghrabi	(+)					190							
						100						2	2
	(-)												
Williams	(+)					280							
						60	200	260				4	5
	(-)	400										1	
Grand Nain	(+)					670							
						640						2	3
	(-)										100	1	
Williams	(+)			500									
				470	380		190					4	6
	(-)		350							150		2	
Poyo	(+)		310			650						2	3
	(-)									70		1	
Hindi	(+)				410			300					
								220				3	6
	(-)								320	80	400	3	

(+) Positive Unique Markers (-) Negative Unique Markers

Thus, AFLP has proven to be useful in distinguishing between banana cultivars. Further development of these unique markers into genetic probes would aid in selection and identification of different cultivars.

Genetic Diversity Between Cultivars: Banana classification has gone through a lot of debates throughout time, an earlier classification by Simmonds, [32] and Robinson, [33] (Figure 2) shows that the Genus *Musa* is originated mainly from intra-and interspecific hybridizations between two wild diploid species, *M. acuminate* Colla ('A' genome) and *M. balbisiana* Colla ('B' genome) [1]. The International Network for the Improvement of Banana and Plantain (INIBAP) organized a survey of banana diversity in the Middle East in collaboration with the Food and Agriculture Organization (FAO) in 2002, covering Jordon, Egypt and Oman [34]. This is considered the newest classification available for edible bananas in Egypt (Figure 3). In the current classification, all of the Cavendish varieties belong to the AAA group, the Plantain is a special AAB subgroup, while the 'Sapientum' falls into the groups AAB, ABB and even

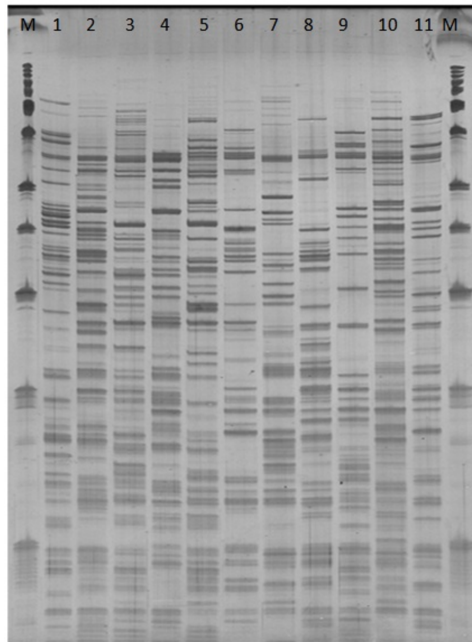

Fig. 1: AFLP profiles of the eleven banana cultivars, 1,2,3,4,5,6,7,8,9,10,11 respectively**.** Primer combination (4.4), (M) molecular weight standard (100 bp ladder).

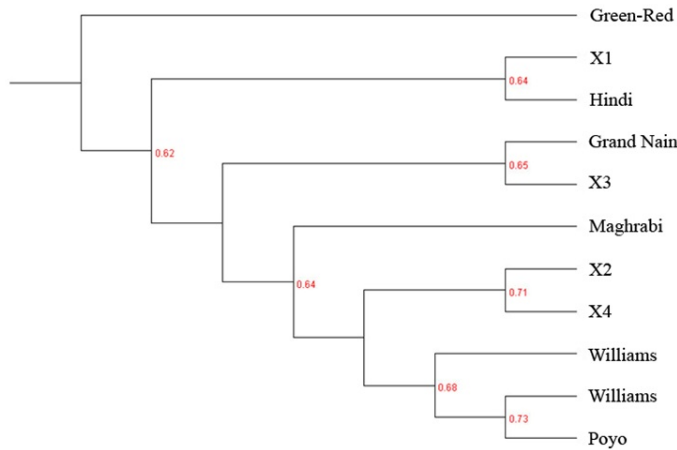

Fig. 2: Dendrogram constructed with UPGMA cluster analysis of AFLP data showing the genetic relationships among the eleven *Musa* cultivars.

some AAA. The classification process went in two steps: assessment of the genomic group (AAA, AAB, etc.) by using the Shepherd-Simmonds list of critical morphological characteristics Simmonds and Shepherd, [1], followed by the tentative cultivar identification.

The AFLP data was used to calculate Dice's genetic similarities between the 11 cultivars using the Diversity Database Fingerprinting Software. A maximum similarity of 72.6 % was observed between 'Poyo' & 'Williams' cultivars which belong to the same subgroup of Giant Cavendish (semi-dwarf range). The 'Red and 'Green-red' cultivar separated itself in a single cluster which agrees with its origin belonging to the Red & Red-green sub-group (Figure 4 and Table 4). All the cultivated bananas used fell into two major clusters one having 'Hindi' and' X1' in one cluster, while the others were grouped in the other cluster which was further subdivided into five groups of which the cultivars 'Maghrabi' & 'Williams' formed separate ones by themselves (Figure. 4 and Table 4).

If we were to apply either of the classification methods used by Simmonds, [32] and Robinson, [33], (Figure 2) or by De Langhe, [34] (Figure 3) in this study, we will find that results do not show segregation of cultivars based on their overall hypothetical genetic homologies. We find that 'Hindi' (Dwarf Cavendish AAA) grouped with unknown X1, 'Grandnain' (Giant cavendish AAA) grouped with another unknown X3

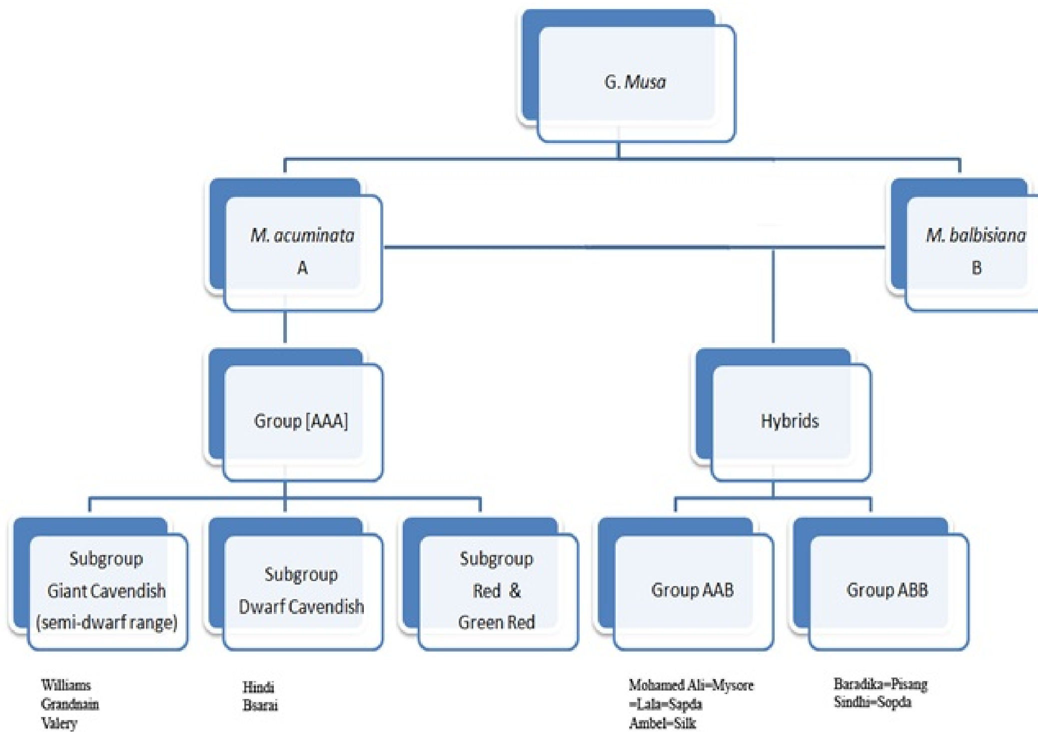

Fig. 3: Banana classification according to (Simmonds, 1966 and Robinson, 1996).

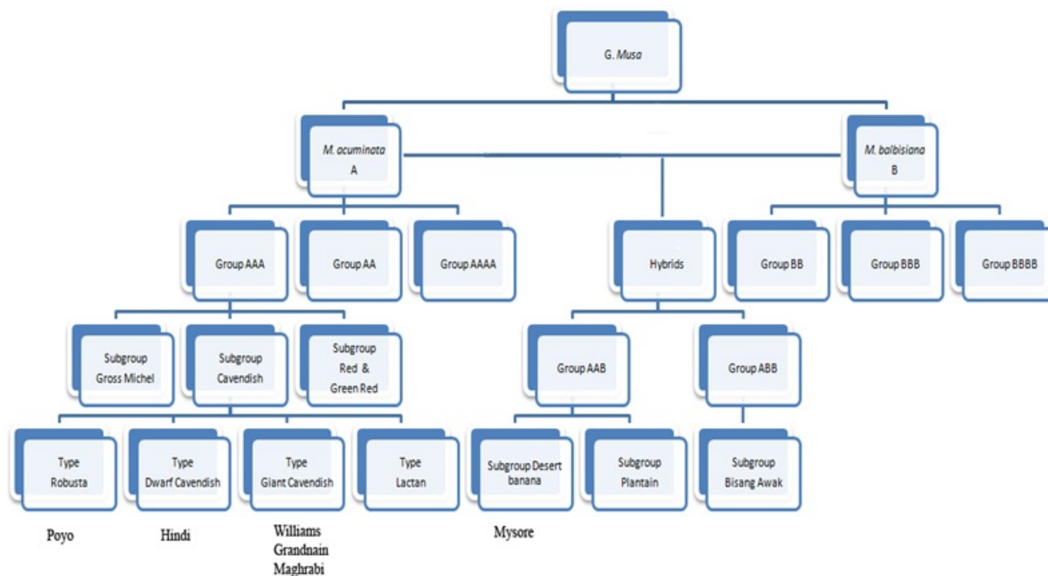

Fig. 4: Banana classification according to (De Langhe 2002).

(like Amble), 'Maghrabi' (Giant cavendish AAA) grouped with unknown X2 (like M.Ali) and X4 (like Maghrabi), and finally 'Williams' rightfully grouped with itself and with 'poyo' both belonging to the same (Giant cavendish AAA) group. The unknowns used in this study were of confused identity when sampled, we here were trying to help in clarifying the situation at the farmer's level.

In conclusion, this study has demonstrated that AFLPs can detect polymorphism among *Musa* cultivars with utmost precision and are capable of generating fingerprints for the discrimination and characterization of these cultivars, which can be applicable for other plant or animal species. The supposed difference between these cultivars is the subject of a rather worldwide confusion. The regular appearance of

Table 4: Genetic Similarity matrix calculated according to Dice's coefficient based on AFLP data.

	Red	X4	X2	Poyo	X3	Maghrabi	Williams	Grand Nain	Williams	Hindi	X1
Red	100										
X4	64.8	100									
X2	63.5	70.9	100								
Poyo	63.5	72.1	68.5	100							
X3	61.4	65.2	63	65.9	100						
Maghrabi	60.6	65	63.3	67.2	60.4	100					
Williams	59.6	67.8	65.5	71.7	61.5	61.9	100				
Grand Nain	59.6	65.6	60	70.3	65.5	65.3	65.7	100			
Williams	58.3	66.7	63.9	72.6	61.2	63.8	64.2	63.5	100		
Hindi	57.2	65.4	61.5	67.9	58.5	61.4	60.4	59.9	60.1	100	
X1	54.9	65.8	59.7	69.5	57.1	57.4	60.4	64.2	56.6	63.9	100

mutants in size makes the problem worse. Such assessment would be of great help in clarifying the situation at the farmer's level. It would also allow for a standardization of Cavendish cultivars at the national level.

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