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Evaluation of Genetic Diversity in Omani Banana Cultivars (*Musa* cvs.) using AFLP Markers

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Abstract: The aim of the present study was to investigate the genetic diversity among eighteen banana cultivars collected from Al-Batinah, Al-Dhakhliya and Dhofar regions of the Sultanate of Oman using AFLP markers. Eleven AFLP primer combinations were used to develop banana DNA fingerprints. Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis yielded three distinct taxa. Banana cultivars, Bahri, Omani, Maisori Fardh, Sokari and Zanzibar from Al-Dhakhliya region grouped in cluster 1, whereas cultivars from Dhofar, Dwarf spotted Cavendish, Somali, Abubaker Philipino, Maisori Fardh, Milk Banana, Plantain Kenya and Sawara Red grouped in cluster 2 and Williams, Somali, Malindi, Red Banana, Maisori Fardh and Nagal cultivars from Al-Batinah region grouped in cluster 3. Multivariate analysis (NTSYS 2.2) of genetic data yielded a total of 1397 alleles, of which 1322 (94.68%) appeared to be polymorphic. The primer combination E-ACT/M-CAC produced 98.15% polymorphic alleles, whereas E-ACA/M-CTG primers showed 160 alleles, the highest number as compared to all other primers used in this study. AFLP based fingerprinting clearly indicated high genetic diversity among banana cultivars grown in different regions of the Sultanate of Oman.

Key words: AFLP, Omani banana, *Musa* sp., genetic diversity, polymorphism

INTRODUCTION

The edible bananas (*Musa* sp.), which originated in South East Asia and the Western Pacific area and were then introduced into other continents (Simmonds, 1966) are amongst the important food crops in the subtropics and tropics. Westward, banana likely followed the major trade routes that transported other fruits and it is known to have arrived in East Africa around 500 AD. Bananas were not carried to Europe until the 10th century and Portuguese traders obtained it from West Africa, not Southeast Asia, during the age of discovery. Plants were taken from West Africa to the Canary Islands and South America in the 16th century and spread throughout the Caribbean with settlement of the area in the 16th-17th centuries. Bananas are now grown pantropically in more countries than any other fruit crop in the world Bananas and plantains belong to the Musaceae, known simply as the banana family. There

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are 25-80 species in the genus *Musa*, depending on the taxonomist. *Musa* is important not only for fruit production, but the genus has provided man with food, clothing, tools and shelter prior to recorded history. All banana and plantain cultivars are believed to have originated from hybridization between two wild species, *Musa acuminata* Colla and *Musa balbisiana* Colla (Bhat *et al*., 1992; Loh *et al*., 2000; Simmonds, 1966, 1995). Hybrids of *M. acuminata* and *M. balbisiana* are sometimes given the names *Musa X paradisiaca* L., *Musa X sapientum* L., or perhaps most accurately, *M. acuminata X M. balbisiana* Colla. Taxonomically, banana cultivars and hybrids are classified based on ploidy analysis and a set of 15 morphological descriptors into genomic groups, differing for genome constitution and ploidy (Simmonds and Shepherd, 1955; Simmonds and Weatherup, 1990). The main genomic groups are AA, AAA, AAB and ABB, although AB, AAAB AABB and ABBB are also possible (Stover and Simmonds, 1987). Highly related clones or cultivars resulting from mutations in a single genotype are allocated to so-called subgroups, characterized by specific morphological and fruit quality attributes (Simmonds, 1973). In many countries (e.g., Ecuador, Costa Rica, Colombia), the production of bananas for export is mainly based on the Cavendish subgroup (group AAA), while a great number of cultivars and landraces (groups AAA, AAB and ABB) are cultivated for local consumption in Africa, Asia and Latin America (Jenny *et al*., 1997, 1999).

The taxonomy of the cultivated bananas has long been a contentious issue and because it relies heavily on morphology, the literature is plagued with contradictions. Clone Klue Tiparot, originally regarded as tetraploid ABBB has been reclassified as triploid, ABB (Jenny *et al*., 1997; Horry *et al*., 1998) and banana clones Monthan Saba and Bluggoe based on molecular data belongs to ABB were previously classified as BBB on the basis of morphological characters (Pillay *et al*., 2000).

Plant morphology is often considerably affected by the environment and for their consistent classification of hybrids, their evaluation in different environment is necessary. The difficulties associated with the use of whole-plant or floral morphology has led researchers to develop other techniques for the correct identification of *Musa* species and cultivars. To overcome these limitations, DNA fingerprinting techniques as a reliable alternative have been used to study the genetic diversity and taxonomy of the cultivated bananas. These include isozyme analysis (Bhat *et al*., 1992); restriction fragment length polymorphism (RFLP) (Gawel *et al*., 1992; Faure *et al*., 1993; Carreel *et al*., 2002); random amplified polymorphic DNA (RAPD) (Bhat and Jarret, 1995; Pillay *et al*., 2000, 2001); amplified fragment length polymorphism (AFLP) (Loh *et al*., 2000; Ude *et al*., 2002a, b) and microsatellites or simple sequence repeats (SSRs) (Lagoda *et al*., 1998a, b; Crouch *et al*., 1998a; Kaemmer *et al*., 1997; Grapin *et al*., 1998). Of all these techniques, microsatellites have proved to be the best marker for banana typing (Grapin *et al*., 1998), as they are highly polymorphic, show a co-dominant mode of inheritance, are reproducible and easy to interpret. In AFLP analysis of *Musa* breeding population, AFLP was found as potentially most powerful tool in the molecular breeding of banana and AFLP markers were found tightly linked to loci influencing parthenocarpy (Crouch *et al*., 1998b). AFLP analysis is clearly a powerful technique in terms of its ability to identify a large number of polymorphic bands without any prior knowledge of the organism. Unfortunately, the information content of these banding patterns is restricted, as they must initially be treated as dominant markers. However, when AFLP analysis is applied to large populations, circumstantial allelic relationships may be sufficient for practical purposes. Software has been developed to distinguish homozygotes and heterozygotes on the basis of band intensity. Yet, such an approach may be frequently confounded by the presence of bands of intermediate intensity.

AFLP assays are also technically demanding and expensive in that they require a number of DNA manipulations and a complex visualization procedure. In addition, they require relatively large amounts of reasonably high quality DNA. The use of poor quality DNA may lead to incomplete digestion, which can result in artificial polymorphisms. RAPD analysis has been used to differentiate *Musa* genome groups (Howell *et al*., 1994), more closely related *Musa* germplasm and hybrids in banana and plantain breeding populations. RAPD assays have proven to be powerful and efficient means of assisting introgression and backcross breeding. However, RAPD analysis has several disadvantages including the dominant nature of the marker system and reproducibility problems, which may limit their application in Marker Assisted Selection (MAS). This has led to a focus on the development and utilization of primers for *Musa* microsatellites (Jarret *et al*., 1994; Kaemmer *et al*., 1997), which have been considered optimum markers in other systems due to their abundance, polymorphism and reliability. Microsatellite markers, otherwise known as Simple Sequence Repeat Length Polymorphisms (SSRLP) are generated by highly specific PCR amplification and, therefore, should not suffer from the reproducibility problems experienced with RAPD analysis. All marker systems have different advantages and disadvantages in specific applications. Thus, it is important for molecular breeding programs to develop capacity in several assays in order that the most suitable system can be chosen and rapidly applied for any particular application. In addition, different DNA marker assays detect different types of genetic variation (Bhat *et al*., 1997).

Many pests and diseases have significantly affected *Musa* cultivation. More virulent fungal strains of *Fusarium oxysporum* Schlecht f. sp. *cubense* (E.F. Smith) Snyd and Hans (Panama disease or wilt) and *Mycosphaerella fijiensis* Morelet (black Sigatoka) have spread dramatically during the past 20 years. As a consequence of these threats to *Musa* cultivation, there has been renewed interest in *Musa* breeding programs. Although some programs have addressed improvement of export bananas, generally the goal has been to improve cultivars for local consumption in the tropical world (Ortiz and Vuylsteke, 1996). *Musa* breeders face many problems intrinsic to this crop that slow the genetic improvement of bananas and plantains, viz. low fertility due to triploidy, slow propagation, long time span from one generation to the next (almost two years from seed to seed) and a large area requirement for field testing. Some of these obstacles have been overcome through conventional methods by screening for seed-fertility and through ploidy manipulations and interspecific hybridization (Rowe and Rosales, 1996; Vuylsteke *et al*., 1997). Likewise, routine biotechniques such as *in vitro* germination of seeds and rapid micropropagation for station trials have allowed more rapid breeding progress in bananas and plantains. More recently, successful cross breeding programs are adopting emerging biotechniques such as genetic engineering and molecular marker-assisted breeding to enhance the effectiveness of their operations (Crouch *et al*., 1998b).

Bananas cultivated in the Sultanate of Oman are thought to have originated as spontaneous variants from cultivars introduced over the centuries from East Africa and India and little is known about their genetic diversity. Despite the advancements in DNA fingerprinting technology that have been used in the analysis of genetic diversity of *Musa* sp. (Tenkouano *et al*., 1999; Pillay *et al*., 2001; Ude *et al*., 2002a), existing classification of banana cultivars in Oman are primarily based on morphological characters (Al-Hosni, 2009; De Langhe, 2002), which is subject to environmental influences. The present study was conducted to investigate the AFLP marker based genetic diversity and genetic relationship among banana cultivars grown in different regions of the Sultanate of Oman.

MATERIALS AND METHODS

Sample Collection

Leaf sample from eighteen Omani banana cultivars and hybrids, Maisori Fardh, Malindi, Nagal, Red Banana, Somaili and Williams from Al-Batinah region and Bahri, Maisori Fardh, Omani, Sokari and Zanzibar from Al-Dhakhliya region and Abubaker Philipino, Dwarf Spotted Cavendish, Maisori Fardh, Milk Banana, Plantain Kenya, Sawara Red and Somali from Dhofar region in the Sultanate of Oman were collected during 2007-2009 for present study (Table 1). For each cultivar, leaf samples were collected from randomly selected 3-4 replicate plants, giving a total of 61 replicate samples for the 18 banana cultivars studied (Table 1). The present study was conducted in Plant Biotechnology Laboratory, College of Agricultural and Marine Sciences in Sultan Qaboos University.

Extraction of DNA

Total genomic DNA was extracted from mature leaves bulked from 3 samples per replicate of each cultivar. One gram of leaf sample was pulverized in liquid N_2 and extracted in 3 mL of modified CTAB buffer (Doyle and Doyle, 1990). The extracted genomic DNA (400 ng) was digested with 12 U of *Eco*RI and 8U of *Mse*I at 37°C for 3 h to generate restriction fragments followed by ligation to *Eco*RI and *Mse*I adaptors (Vos *et al*., 1995). Pre-selective amplification of digested/ligated DNA was performed in 20 µL reaction volume puRETaq RTG PCR beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) containing 4 µL digested/ligated DNA, 1 µL AFLP pre-selective primer pair and 15 µL AFLP CORE mixture (Applied Biosystems, Foster City, CA). The pre-selective amplification was performed in a thermocycler programmed as: 1 cycle at 72°C for 30 sec, 30 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec with a final cycle at 72°C for 2 min. Ten microliter of pre-selective amplified product was electrophoresed on 1.5% agarose to determine the size of the amplicons.

Selective Amplification

The selective amplification of the target DNA sequences was carried out with 11 primer combinations (Table 2) as reported previously (Loh *et al*., 2000; Pillay *et al*., 2003;

Table 1: Banana cultivars and their genomic group from different location in Oman used for AFLP analysis

Banana variety	Cultivar code	Reported genotype*	Location
Abubaker	AP	ABB	Dhofar
Bahri	DA		Dhakhliya
Dwarf Spotted Cavendish	DS	AAA	Dhofar
Maisori Fardh	MF	AAB	Dhofar
Maisori Fardh	DB	AAB	Dakhliya
Maisori Fardh	BJ	AAB	Batinah
Malindi	BG		Batinah
Milk Banana	MB	AAB	Dhofar
Nagal	BK	ABB	Batinah
Omani	DC		Dakhliya
Plantain Kenya	PK	AAB	Dhofar
Red Banana	BH	AAA	Batinah
Sawara Red	SR	AAA	Dhofar
Sokari	DE		Dakhliya
Somali	S	AAA	Dhofar
Somali	BI		Batinah
Williams	BF	AAA	Batinah
Zanzibar	DD	AAB	Dakhliya

*De Langhe (2002)

Table 2: AFLP bands obtained form the 18 banana cultivars using 11 primer combinations

Primer combination				
*Eco*RI	*Mse*I	Total No. of bands	Polymorphic bands	Polymorphism (%)
AAC	CAA	111	106	95.50
ACC	CAT	138	131	94.93
AAC	CAG	112	106	94.64
ACC	CTT	141	136	96.45
ACG	CTA	105	97	92.38
ACA	CAG	141	134	95.04
ACT	CTC	141	134	95.04
ACT	CAT	115	107	93.04
ACA	CAA	125	118	94.40
ACA	CTG	160	147	91.88
ACT	CAC	108	106	98.15
Total		1397	1322	94.68

Wong *et al*., 2002) to generate AFLP fingerprints. The selective PCR amplification was carried out in 20 µL reaction volume using puRETaq RTG PCR beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) that contained 3 µL pre-selective amplified product, 1 µL FAM labeled*Eco*RI primer, 1 µL *Mse*I primer (unlabeled), 15 µL AFLP CORE mixture (Applied Biosystems, Foster City, CA). PCR was done for 13 cycles at 95°C for 30 sec, 72°C for 60 sec with final cycle at 72°C for 60 sec. AFLP products were then resolved by loading onto an ABI-3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using 25 µL loading buffer master mix and 0.5 µL selective PCR amplified product. All samples were run in quadruplet to maintain the integrity of the data analysis.

Data Analysis

Raw data obtained from the genetic analyzer was analyzed using GeneMapper 4.0 program (Applied Biosystems, Foster City, CA) and banding matching was performed at 1% optimization parameter and 1% band tolerance. The amplified fragments (band) generated using 11 primer combinations with the 18 cultivars of banana and plantain were scored manually for their presence (denoted as 1) and absence (denoted as 0). The number of alleles and discriminatory power of each primer to distinguish between varieties were also assessed. The data was analyzed using NTSYS 2.2 program (Rohlf, 1998). The genetic similarity (GS) values between pairs of samples were estimated according to Jaccard's similarity coefficient (Gs_{ij} = a/a+b+c), where, Gsij = measure of genetic similarity between individuals, i and j; a = number of polymorphic bands that are shared by individuals i and j; b = number of bands present in i and absent in j and c = the number of bands present in j and absent in i. The genetic relationship among and within the Omani banana cultivars were determined by generating distance matrix and subsequently grouped to construct unrooted consensus tree using UPGMA (Sneath and Sokal, 1973) and Hierarchical SAHN methods with NTSYS 2.2 program (Rohlf, 1998).

RESULTS

AFLP Analysis of Omani Banana Cultivars

Eighteen banana leaf samples in the replicates of 3-4 were collected from Northern (Al-Batinah), Southern (Dhofar) and interior (Al-Dhakhliya) regions of the Sultanate of Oman (Table 1). Amplification of digested/ligated products obtained from pre-selective

amplification using primers complimentary to *Eco*RI and *Mse*I adaptor sequences, yielded DNA smear of expected sizes ranging from 100-1100 bp. AFLP profiles of 18 banana cultivars were generated by selective amplification using 11 primer combinations of *Eco*RI and *Mse*I primers, which identified 1322 polymorphic bands out of total 1397 bands (94.68% polymorphism) (Table 2). The 11-primer combinations showed polymorphism ranging 91.88 to 98.15% with E-ACT/M-CAC producing highest polymorphic bands (98.15%) and E-ACA/M-CTG lowest 91.88% (Table 2). The size of DNA fragments obtained from AFLP profiles of 18 banana cultivars ranged from 50-600 bp with some unidentified unique bands.

Genetic Diversity Analysis

Genetic diversity among 18 banana cultivars were analyzed by unweighted pair-wise method of UPGMA and Jaccard's similarity coefficient of NTSYS 2.2. program. Six banana cultivars, viz., Maisori Fardh, Malindi, Nagal, Red Banana, Somali and Williams clustered in 3 clusters based on Jaccard's similarity coefficient by unweighted pair-wise method of UPGMA (Fig. 1). Cluster 1 included Williams and Somali cultivars, cluster 2 Malindi and Red Banana and cluster 3 Maisori Fardh and Nagal (Fig. 1). All cultivars showed diversity among and within the replicates. Pair wise genetic similarity estimates (GSE) were recorded in Williams 0.771 to 1.000; Somali 0.700 to 1.000; Malindi 0.655 to 1.000; Red Banana

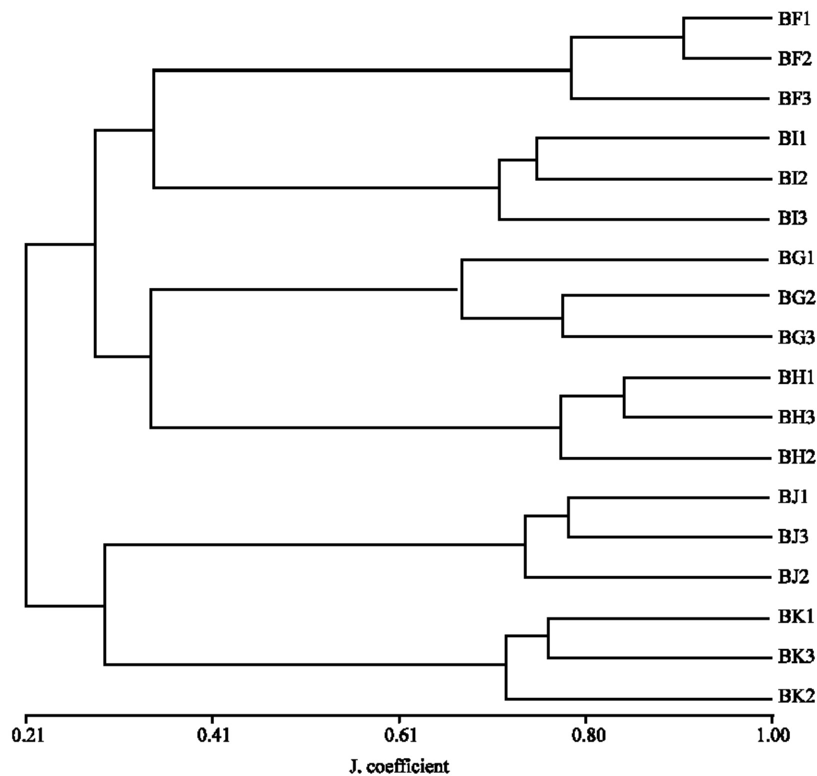

Fig. 1: Phylogenetic tree generated with Jaccard's similarity coefficient method of NTSYS-pc using AFLP data from 18 samples representing 6 *Musa* cultivars from Al-Batinah region

0.778 to 1.000; Maisori Fardh 0.692 to 1.000 and Nagal 0.708 to 1.000. Similarly 5 banana cultivars from Al-Dhakhliya formed 3 clusters having Bahri and Omani, Maisori Fardh and Sokari and Zanzibar in cluster 1, 2 and 3, respectively (Fig. 2). Pair wise GSE was recorded low among all banana cultivars from Al-Dhakhliya. GSE between Bahri cultivars was 0.411 to 1.000; Omani 0.429 to 1.000, Maisori Fardh 0.504 to 1.000, Sokari 0.465 to 1.000 and lowest GSE in Zanzibar ranged 0.303 to 1.000. Banana cultivars from Dhofar formed 4 clusters based on Jaccard's similarity coefficient including Dwarf Spotted Cavendish and Somali in cluster 1, Abubaker Philipino in cluster 2, Maisori Fardh, Milk Banana and Plantain Kenya in cluster 3 and Sawara Red in cluster 4 (Fig. 3). Pair wise GSE between Dwarf Spotted Cavendish ranged from 0.600 to 1.000, Somali cultivars 0.591 to 1.000, Abubaker Philipino 0.800 to 1.000, Milk Banana 0.625 to 1.000, Plantain Kenya 0.676 to 1.000 and in Sawara Red 0.452 to 1.000.

AFLP data obtained from 61 leaf samples representing banana cultivars from 3 regions, Al-Batinah, Al-Dhakhliya and Dhofar were analyzed by Jaccard's similarity coefficient using UPGMA cluster analysis method to determine genetic diversity in banana cultivars between regions. The cluster analysis created 3 distinct taxa representing 3 regions (Fig. 4). Banana cultivars from the same region clustered together. Although diversity was evident among different samples from the same cultivar but replicates of the same sample for each cultivar showed similar AFLP fingerprint and grouped into their respective cultivars.

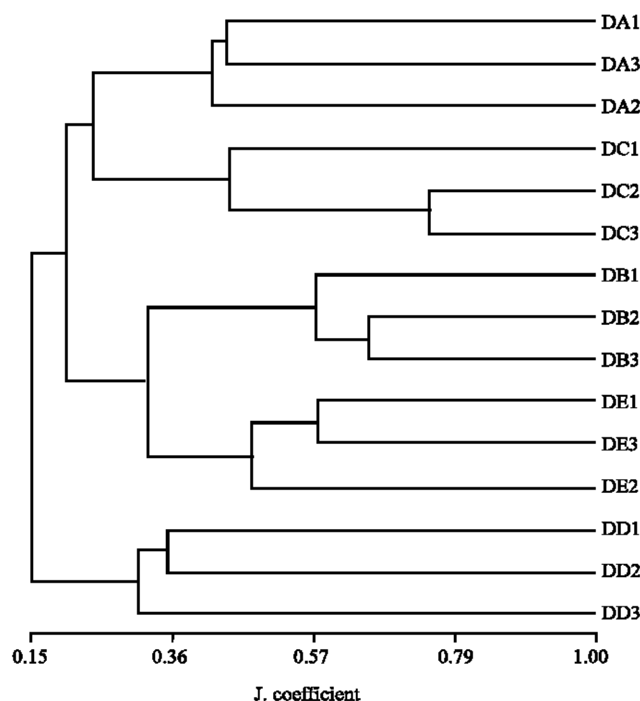

Fig. 2: Phylogenetic tree generated with Jaccard's similarity coefficient method of NTSYS-pc using AFLP data from 15 samples representing 5 *Musa* cultivars from Al-Dhakhiliya region

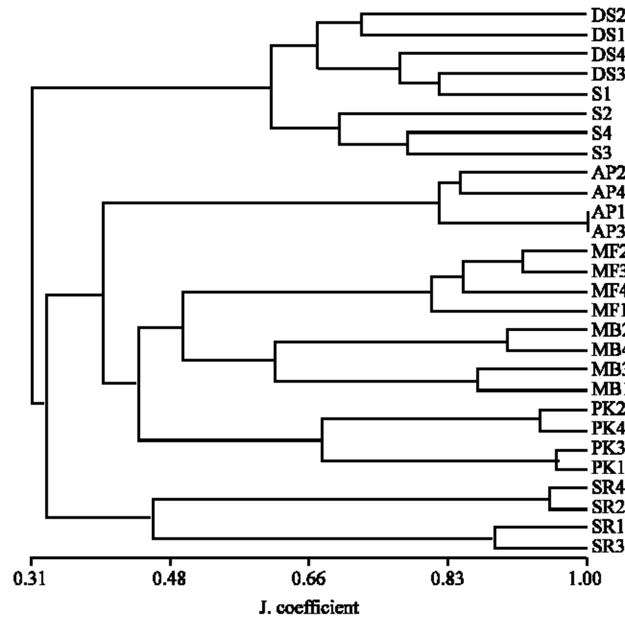

Fig. 3: Phylogenetic tree generated with Jaccard's similarity coefficient method of NTSYS-pc using AFLP data from 28 samples representing 7 *Musa* cultivars from Dhofar region

DISCUSSION

Omani farmers, regardless of genetic and morphological diversity in banana have named banana since ancient time in their own way in different regions of Oman, which led to spread of new names of banana cultivars known worldwide. The present study aimed to correct the ambiguity in naming banana in Oman using AFLP based DNA fingerprinting technique. AFLP profiles of 18 banana cultivars from different regions of Oman with 11 primer combinations specific to *Eco*RI and *Mse*I sites yielded 1397 DNA bands with 94.68% polymorphic. In our results, AFLP based DNA fingerprints of Omani banana provided genetic evidence that clonally propagated bananas neither shared genetic similarity between three regions nor have a common origin. All 18 banana samples used in present study showed different AFLP profiles suggesting that they may be genetically either unrelated or distantly related. Cultivars grouped genetically according to their geographical location irrespective of their common names such as Maisori Fardh and Somali are present in different regions. Based on Jaccards similarity coefficient of AFLP data, Somali and Maisori Fardh cultivars groups with their region and are distantly related to the same cultivar from other region (Fig. 4). We have found AFLP amplified fragments in the range of 50-600 bp. The variation noted in the fragment size was attributed to the variation in the selective sequence of the *Eco*RI and *Mse*I primers (Van Treuren, 2001).

The literature regarding molecular markers in the characterization of natural banana cultivars is ample, which has demonstrated the importance of studies on genetic diversity in banana breeding programs. Silva *et al.* (2002) have investigated AFLP based genetic diversity in *Musa acuminata*, *M. balbisiana* and other natural banana hybrids suggesting the presence of new relationship within *M. acuminata* complex different than those of morphological data. Restriction Fragment Length Polymorphism (RFLP) profiles of

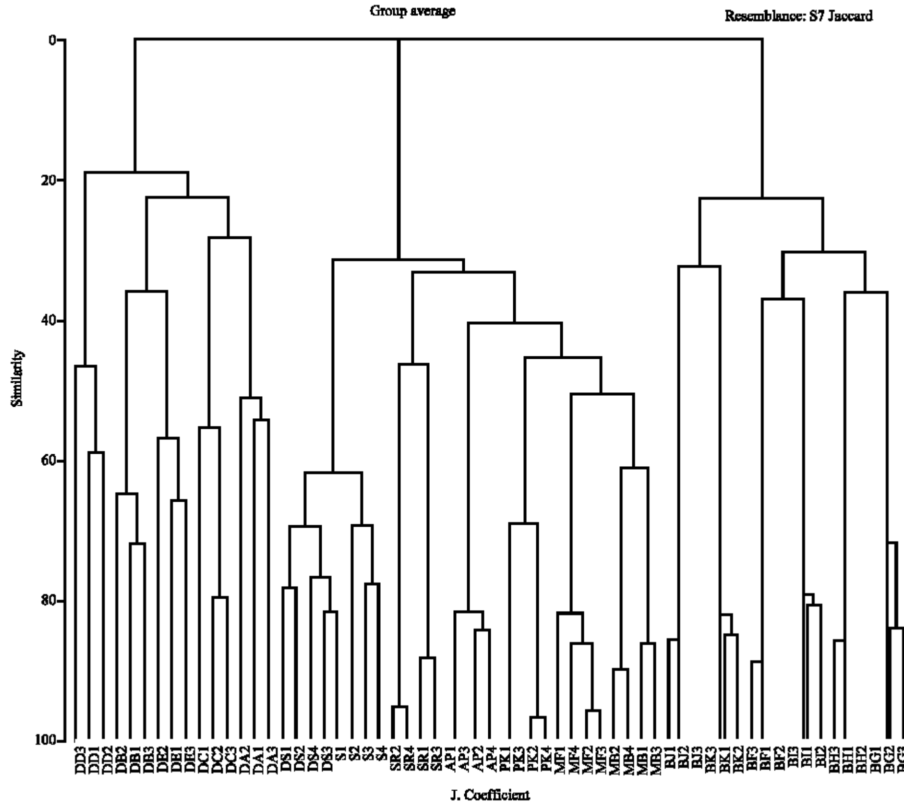

Fig. 4: Phylogenetic tree generated with Jaccard's similarity coefficient method of NTSYS-pc using AFLP data from 61 samples representing 18 *Musa* cultivars from Al-Batinah, Al-Dhakhliya and Dhofar regions

mitochondrial and chloroplast DNA were useful in elucidating the center of domestication of parthenocarpic banana varieties and complimenting the morphological data (Ferreira *et al*., 2004).

Opara *et al*. (2010) have investigated genetic diversity in 7 banana cultivars from Dhofar region of Oman using AFLP with 12 primer combinations and found 92.50% polymorphism. They further classified banana cultivars by phylogenetic, hierarchical clustering and principal component analysis, which showed significant differences between clusters found with molecular markers and those clusters created by previous studies (De Langhe, 2002) using morphological analysis. Pollefeys *et al*. (2004) suggested that morphologically *Musa* is diverse with well-defined characters giving accurate indicators of genome constitution and ploidy, however, phenotyping for many physiological characters such as biotic or abiotic stress tolerance is difficult because of the plant size and long life cycle. *Musa acuminata* Colla is highly variable and present several unresolved nomenclature problems. UPGMA cluster analysis of *M. acuminata* RFLP profiles yielded three distinct taxa, *M. acuminata* subsp *truncata*, subsp *malaccensis* and subsp *microcarpa* from Malaysia (Wong *et al*., 2001). Genetic diversity of 30 plantains in Cameroun by SSR and AFLP markers confirmed narrow genetic base supporting the hypothesis that cultivars may have arisen from vegetative multiplication of a single seed (Noyer *et al*., 2005). Whereas, Wan *et al*. (2005)

have concluded that UPGMA cluster analysis of 13 banana landraces AFLP data indicate that although genetic diversity of Myanmar banana landraces was different from that of international standard cultivars, the average diversity levels between them were not distinctly different.

The AFLP technique used in the present study was found to be a useful tool in determining the genetic diversity among banana cultivars grown in different regions of Oman and realizing the ambiguity in naming banana cultivars in Oman. Our findings are similar to previous reports that have successfully used AFLP markers to acquire genetic diversity in bananas (Ude *et al*., 2002a, b; Loh *et al*., 2000; Wong *et al*., 2002; Wang *et al*., 2007; Opara *et al*., 2010). Since no historical records exist about the actual origin of bananas now cultivated in Oman, AFLP marker based technique should now be employed to determine the relationships between Omani banana cultivars and bananas found elsewhere along the Indian Ocean (Southern and East Africa and India) and other parts of the world.

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