The African Journal of Plant Science and Biotechnology ©2008 Global Science Books

Diversity Assessment among Mango (*Mangifera indica* L.) Cultivars in Egypt using ISSR and Three-Primer Based RAPD Fingerprints

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ABSTRACT

Mangoes are an important fruit crop in Egypt and all over the world. Statistics provided by the Egyptian Ministry of Agriculture and Land Reclamation indicate that a total of 151,000 Fadden (equiv. 63419.310.464 ha) are planted with mango trees, with a total production of 0.596 million t in Egypt alone. The average yield per Fadden is usually about 5.41 t. For improving the yield and yield attributes, varieties are often produced and evaluated under different conditions. Utilization of molecular marker analysis provided new insights to breeders for molecular assisted selection (MAS). Depending on the marker system used, the genetic similarity analyses varied dramatically. In this report, genomic variation within twelve mango cultivars, widely used in fresh market mango production in Egypt, were investigated using two different molecular marker systems; RAPD (random amplified polymorphic DNA) and ISSR (inter-simple sequence repeat). A new strategy was used to increase RAPD potential in genetic diversity by using three different primer combinations per reaction. Different dendrograms constructed for the RAPD and ISSR results individually and collectively revealed that similarity and clustering is very dependant on the marker system used.

Keywords: comparative analysis, genetic diversity, marker assisted selection, molecular breeding

INTRODUCTION

The mango (*Mangifera indica* L.) is considered one of the oldest cultivated trees in the world. It is now cultivated commercially throughout the tropics and in many subtropical areas where it has been considered to be the "king of fruits" (Adato *et al*. 1995). On a world wide basis, mangoes comprise one of the largest fruit crops grown and consumed by human, and currently ranked fifth in total production among major fruit crops, after citrus (all types), *Musa* (banana and plantains), grapes, and apples (FAO production year book 2002).

Nowadays, plant genetic resources are one of the most valuable assets available to mankind. Therefore, protection and conservation of these resources for future generations are very valuable (Arumuganathan and Earle 1991; Anad 1997). This is an essential component for effective and efficient management of plant genetic resources as well as their utilization in the characterization of germplasm (Bally *et al*. 1996). Such characterization is essential not only for identification of various species but also to determine genetic relatedness among them (Belaj *et al*. 2001). The information generated could be used successfully in breeding programs wherever possible.

Thus, utilization of molecular markers to detect some economically important inherited traits in mango cultivars is very important and highly required (Omayma 2003). In this regard, RAPD (random amplified polymorphic DNA) assay is one of the widely used and an easy PCR-based technique for producing molecular markers. It was developed in 1990 and it based on the amplification by the polymerase chain reaction (PCR) of random DNA segments, using single primers of arbitrary nucleotide sequence (Williams *et al*. 1990). The potential of the original RAPD assay to generate polymorphic DNA markers with a given set of primers was further increased by combining two primers in a single PCR (Klein-Lankhorst *et al*. 1991). RAPD assay was adapted in mango to direct the amplification of a genome-specific "fingerprint" of DNA fragments (López-Valenzuela *et al*. 1997; Lowe *et al*. 2000; Kumar *et al*. 2001). This technique was extensively used with other economically horticultural fruit crops such as almond (*Prunus dulcis*) (Bartolozzi *et al*. 1998), olive (*Olea europaea L*.) (Besnard *et al*. 2001), plums (*Prunus domestica*) (Boonprakob *et al*. 2001), low bush blueberry (*Vaccinium corymbosum*) (Burgher *et al*. 2002), citrus species (Cabrita *et al*. 2001; Awad 2003), coconut palm (*Cocos nucifera L*.) (Carde *et al*. 2003), pecan (*Carya illinoinensis*) (Conner *et al*. 2001), apricot (*Prunus armeniaca* L.) (Hormaza 2001), paw paw (*Asimina triloba* L.) (Huang *et al*. 1997), Indian cashew (*Anacardium occidentale L*.) (Dhanaraj *et al*. 2002), apple (*Malus domestica*) (Conner *et al*. 1997) and *Actinidia* sp. (Huang 2002).

On the other hand, Inter simple sequence repeat (ISSR) technique is also a PCR-based method which involves amplification of a DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction (Meyer *et al*. 1993; Gupta *et al*. 1994; Wu *et al*. 1994; Ziêtkiewicz *et al*. 1994). It was also used extensively for producing molecular markers in horticultural plants such as pear (*Pyrus communis*) (Corvo *et al*. 2001), gooseberry (*Ribes grossularia* subgenus *Grossularia*) (Lanham and Brennan 1999) and *Ribes nigrum* L. (Lanham *et al*. 2000).

In this investigation, we aimed to provide a comparative assessment by using ISSR and three-primer RAPD analysis to study genetic diversity among mango cultivars. Thus, we hypothesized that if one primer used in RAPD analysis was not sufficient, we could expect more bands to be detected

Received: 25 July, 2008. Accepted: 11 September, 2008.

Original Research Paper

Table 1 Characteristics associated for each mango cultivars used in this study.

Cultivar	Origin	Tree size	Malformation	Alternate bearing	Cold	Time of ripening	Type of embryo	Skin color
Alphonse (Alfons)	India	Medium	-	Irregular	Moderate	August to first of October	Mono	Yellow with red spots
Langra	India	Big	Tolerant	Irregular	Tolerant	Mid August to mid September	Mono	Green
Hidi Khassa	Selected seedy clone in Egypt	Medium	Moderate	Regular	Moderate	Late August to September	Poly	Green
Fajri Kalan	India	Big	Tolerant	Regular	Tolerant	First of October	Mono	Green with yellow
Sukkary	Selected seedy clone in Egypt	Big	Moderate	Moderate	Tolerant	July to August	Poly	Yellow
Zebda	Selected seedy clone in Egypt	Big	Tolerant	Regular	Tolerant	Late September to October	Poly	Dark green
Ewais (Oweisi)	Selected seedy clone in Egypt	Big	Moderate	Regular	Tolerant	September	Poly	Yellow
Sidik	Selected seedy clone in Egypt	Big to medium	Tolerant	Irregular	Tolerant	August	Poly	Green with red spots
Hindi sannara	India	Medium	Moderate	Regular	Sensitive	Late July to August	Poly	Greenish yellow
Sabra*	South Africa	Small	-	-	-	Mid June to July	poly	Yellow with red cheeks
Peach*	South Africa	Small	-	-	-	August to September	poly	Orange-yellow color with red cheeks
13/1*	Egypt	Small	-	-	-		poly	Green

* Used as dwarf rootstocks

by using three primers. At the same time, if three RAPD primers were included in the same PCR reactions, new amplicons would be produced. For this purpose, we analyzed each of those techniques individually with twelve different mango cultivars from different genetic and geographical origins in Egypt to compare their robustness and reliability in breeding programs.

MATERIALS AND METHODS

Plant material and cultivation

Twelve of the most important mango cultivars in Egypt (Alphonse, Langra, Hindi Khassa, Fajri Kalan, Sukkary, Zebda, Ewais, Sidik, Hindi Sannara, Sabra, peach and 13/1 rootstock, were used in the study. To confirm the originality of trees for the above mentioned cultivars, trees were selected and labeled during the bearing season (**Table 1**). Most of the cultivar samples were taken from experimental trees grown at the orchard of the Horticulture Research Institute in El-Kanater Kalubia governorate, Egypt, 30° 5′ 71″ S and 31° 22′ 72″ W, 22 m above sea level. In addition, some samples were taken from a private farm near Cairo, Egypt.

Statistical analysis

All obtained data in the experiment was subjected to statistical analysis of variance according to Snedecor and Cochran (1967) and the comparison of cultivar means was done using LSD test at $P < 0.05$.

DNA extraction

DNA samples were extracted from young, fresh leaves (0.1 g) by the CTAB (cethyltrimethylammonium bromide) method followed by an RNase-A treatment (Sigma, St. Louis, MO; R-4875) for 30 min at 37°C in each case according to Mansour *et al*. (2005). The quality and quantity of extracted DNA was measured (2 µl) by a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Delaware, and USA). DNA samples were adjusted to a concentration of 50 ng/µl with ddH_2O and subjected to PCR amplification.

RAPD PCR reactions

Amplification reactions were performed according to (Williams *et al*. 1990) in volumes of 25 µl. Briefly the reaction mixture containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 µM of each dATP, dCTP, dGTP and TTP (Pharmacia), 0.2 µl primer, 25 ng of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Promega). To increase the potential of PCR reaction, various combinations of three decamer oligonucleotides had been used in the single-primer PCR as suggested by (Klein-Lankhorst *et al*. 1991). The amplification was performed in a Perkin Elmer 2400 Thermal Cycler programmed for 5 minutes at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 34°C, 2

Table 2 Sequence of the RAPD and ISSR primers applied.

RAPD	Sequences (5′- 3′)
P1	GTA GAC CCG
P2	GGA CCC TTAC
P3	GTC GCC GTC A
P4	GGT CCC TGA C
P5	TGG ACC GGT G
P6	AGG GGT CTT G
P7	TTC CCC CGC T
P8	TTC CCC CCA G
P9	ACT TCG CCA C
P10	CAA TCG CCG T
P11	AGG GAA CGA G
P12	TGC GCC CTT C
P13	TTC GCA CGG G
P14	GTG AGG CGT C
P15	CAA ACG TCG G
P16	CTG CTG GGA C
P17	GTG ACG TAG G
P18	CCA CAG CAG T
P19	TGA GCG GAC A
P20	GTG AGG CGT C
ISSR	
814	$(CT)_8TG$ (#814)
844A	$(CT)_8AC$ (#844A)
844B	$(CT)_8G$ (#844B)
17898A	$(CA)_6AC$(#17898A)
17898B	$(CA)_6GT$ (#17898B)
17899A	$(CA)_6AG$ (#17899A)
17899B	$(CA)_6GG$ (#17899B)
HB8	$(GA)_6GG$ (#HB8)
HB9	$(GT)_6GG$ (#HB9)
HB10	$(GA)_6CC$ (#HB8)
HB11	$(GT)_6CC$ (#HB11)
HB12	$(CAC)_3GC$(#HB12)
HB13	$(GAG)_3GC$ (#HB13)
HB14	$(CTC)_3GC$ (#HB14)
HB15	$(GTG)_2GC$ (#HB14)

min at 72°C, using the fastest available transitions between each temperature (ramp time), followed by one cycle of 72°C for 20 min; and 4°C thereafter. The annealing temperature varied according to the melting temperature (™) for the lowest primer in the combination (**Table 2**). The core program increased from 40 to 45 cycles, if amplification was weak, to get a slight increase in the amount of PCR products.

ISSR PCR reactions

A set of 15 anchored micro satellite primers was procured from (Metabion, Germany) (**Table 2**). PCR amplification was performed according to (Dangi *et al*. 2004). Briefly, 20 ng of DNA was added with 10 mM Tris-HCI pH 7.5, 50 mM KCI, 1.5 mM $MgCl_2$, 0.5 mM spermidine, 2% formamide, 0.1 mM dNTPs, 0.3 uM primer and 0.8 U of *Taq* DNA polymerase (Promega Taq DNA polymerase, USA) in a 25 µl reaction using Perkin Elmer 2400 thermocycler. All the chemicals required for the reaction mixture were obtained from (Sigma-Aldrich, USA). After initial denaturation at 94°C for 5 min, each cycle consisted of 30 sec denaturation at 94°C, 45 sec of annealing at 50°C, 2 min extension at 72°C along with 5 min extension at 72°C at the end of 40 cycles. The annealing temperature varied according to the melting temperature of each primer (**Table 2**). Moreover, the core program increased from 40 to 45 cycles, if amplification was weak, to get a slight increase in the amount of PCR products.

Gel electrophoresis

Amplified fragments, 10 µl, were separated by agarose (1.6%) gel electrophoresis, stained with ethidium bromide (0.5 ng/µl) at 80 V in 1X TBE buffer and photographed on a UV transilluminator (Pharmacia) by Canon S5 digital camera with UV filter adaptor. A negative control which contained all the necessary PCR components except template DNA was included in the PCR runs.

Fragments analysis

Amplification products were scored independently as 1 and 0 for presence and absence of bands, respectively, and the obtained binary data were used for the analyses. Only sharp PCR fragments were scored (not "ghost"). Fragments at low intensities were only scored as present when they were reproducible in repeated experiments using GelAnalyzer 3 (Egygene) software. Unequivocally reproducible bands were scored and entered into a binary character matrix (1 for presence and 0 for absence). The genetic similarity among accessions was determined by Nei's genetic distance (Nei 1987) modified to accommodate dominant (e.g., RAPD-like) markers. A dendrogram was constructed based on the matrix of distance using Unweighted Pair Group Method with Arithmetic averages (UPGMA). All the calculations were performed by using the NTSYS-pc 2.02 software package (Numerical Taxonomy System, Exeter Software) (Rohlf 2000). The statistical stability of the clusters was estimated by a bootstrap analysis with 1000 replications using Winboot software (Yap and Nelson 1996).

RESULTS

Assessment of genetic diversity using triple-primer RAPD assay

In this investigation, RAPD amplification reactions were performed using various combinations of three different decamer oligonucleotides that had been previously tested in the single-primer PCR (**Table 2**). An example of such experiment is shown in (**Fig. 1B**). In all cases, the combination of three primers (in a 1: 1: 1 ratio) resulted in the appearance of new bands that were not produced when each primer was used separately (**Figs. 1A, 1B**). The total number of bands resulting from triple-primer RAPD is not the simple sum of resulting bands number from each single RAPD primer following amplification. The number and sizes of the amplified products varied depending upon the sequences of random primers and DNA samples used. A considerable amount of polymorphism was detected for all the used combination. The sizes of the amplified fragments were ranged from 100 bp to 2 Kbp. A total of 340 bands were scored, in average 37 ± 2 band per primer/gel, 10 ± 2 polymorphic, 24 ± 2 unique bands and 34 ± 2 polymorphic (with unique). It also reveals (90.70%) polymorphism. While some bands were monomorphic shared by all cultivars, there were specific bands specific for each one (**Fig. 1**). Genetic similarity was calculated from the Nei's similarity index value for all the 12 mango cultivars considering RAPD scoring results, then used to construct a dendrogram using Unweighted Pair Group Method with Arithmetic averages (UPGMA) (**Fig. 1C**).

Using ISSR markers to assess diversity in mango cultivars

The ISSR technique uses microsatellites, usually 16-25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter- SSR sequences of different sizes (Ziêtkiewicz *et al*. 1994). In this investigation, a set of 50 ISSR primers was used for initial screening of 12 mango cultivars of which 25 gave amplification (**Fig. 2A**). However, only 15 ISSR primers detected intraspecific variation in mango cultivars generating clear reproducible patterns and revealing on average 20 bands per gel/primer in the range of 100 bp to 2 kbp (**Table 2**). Among these bands, 5 were polymorphic bands and 15 were unique bands revealing 90.70% polymorphism (**Table 3**). Based on ISSR gels patterns, the similarity index values were used to construct a dendrogram using UPGMA (**Fig. 2B**). The resulted dendrogram shows different clusters showing variation occurring in the frequencies of SSR motifs and thus the possibility of different cladistic origin. Both Fajri and 13/1 were closely grouped in one cluster showing possible ancestry SSR motifs. The rest of the cultivar was grouped individually (**Fig. 2B**).

DISCUSSION

Molecular genetic applications in mango are quite limited (Galan Sauco 1993; Davis 2000). However, those applications have the potential to resolve many serious production problems in production of mango cultivars and to improve breeding methodologies (Omayma 2003). For instance, the breeding timetable would be freed from the constraints of juvenile period and the additional years to tree evaluation (Litz 1997). In addition to morphological and chemical analysis, utilization of molecular analysis in this study provided new insight for breeders to molecular in molecular-assisted selection (MAS).

The successful application of a mapping marker system depends on its ubiquity and widespread and even distribution within the genomes for detection of polymorphisms between different varieties and individuals (Ye *et al*. 2005). RAPD analysis takes advantage of the fact that a single short (10-bp) oligonucleotide primer may hybridize with genomic sequences at many sites, and some of the sites (oriented correctly) may be close enough to each other for a PCR amplification to take place (Williams *et al*. 1993). However, the potential of the original RAPD assay to generate polymorphic DNA markers with a given set of primers was further increased by combining two primers in a single PCR (Klein-Lankhorst *et al*. 1991). There may be regions in which a single RAPD primer will fail to produce a band, but when more primers are used together, the distance between their binding sites and their orientation are both correct for production of a band by PCR. Thus, the possibility for production of new bands (revealing new genomic loci) was highly enhanced with more selective combinations of RAPD primers. The above logic was demonstrated in this study and revealed by proper selection by mixing three RAPD primers in the same PCR reactions. Genetic diversity parameters (average number of alleles per polymorphic locus, percent polymorphism, average heterozygosity and marker index) were calculated for single-primer RAPD, three-

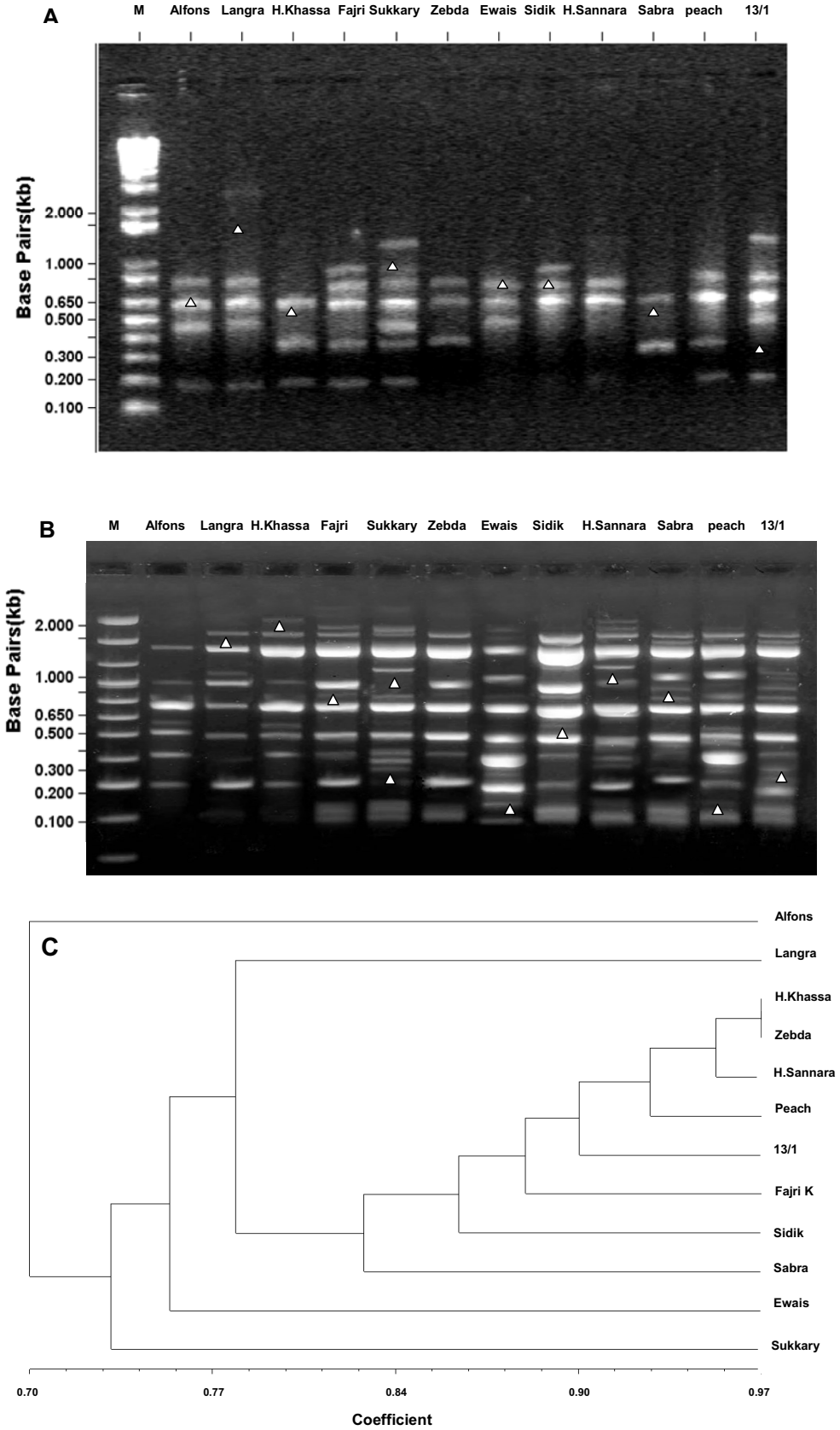

Fig. 1 RAPD markers associated with polymorphisms among mangos cultivars. (**A**) RAPD amplification with single primer P4. (**B**) RAPD amplification with triple-primers P4, P5 and P6. (**C**) Dendrogram based on algorithm of unweighted pair group method with arithmetic averages using RAPD results between in different mango cultivars in Egypt.

Table 3 Comparison of DNA marker systems in mango (*Mangifera indica* L.) cultivars.

Marker system	№ of Primers	Gel polymorphism			Average № of bands/primer	Polymorphism (%)	Mean of band frequency
		Polymorphic (without unique)	Unique bands	Polymorphic (with unique)			
Single-primer RAPD	20	8 ± 2	17 ± 2	24 ± 2	24 ± 2	87.34	0.125
Three-primer RAPD	20	10 ± 2	24 ± 2	34 ± 2	37 ± 2	91.892	0.250
ISSR	15	5 ± 2	15 ± 2	20 ± 2	20 ± 2	90.70	0.150
Total ≈	35	23	56	78	81		0.525

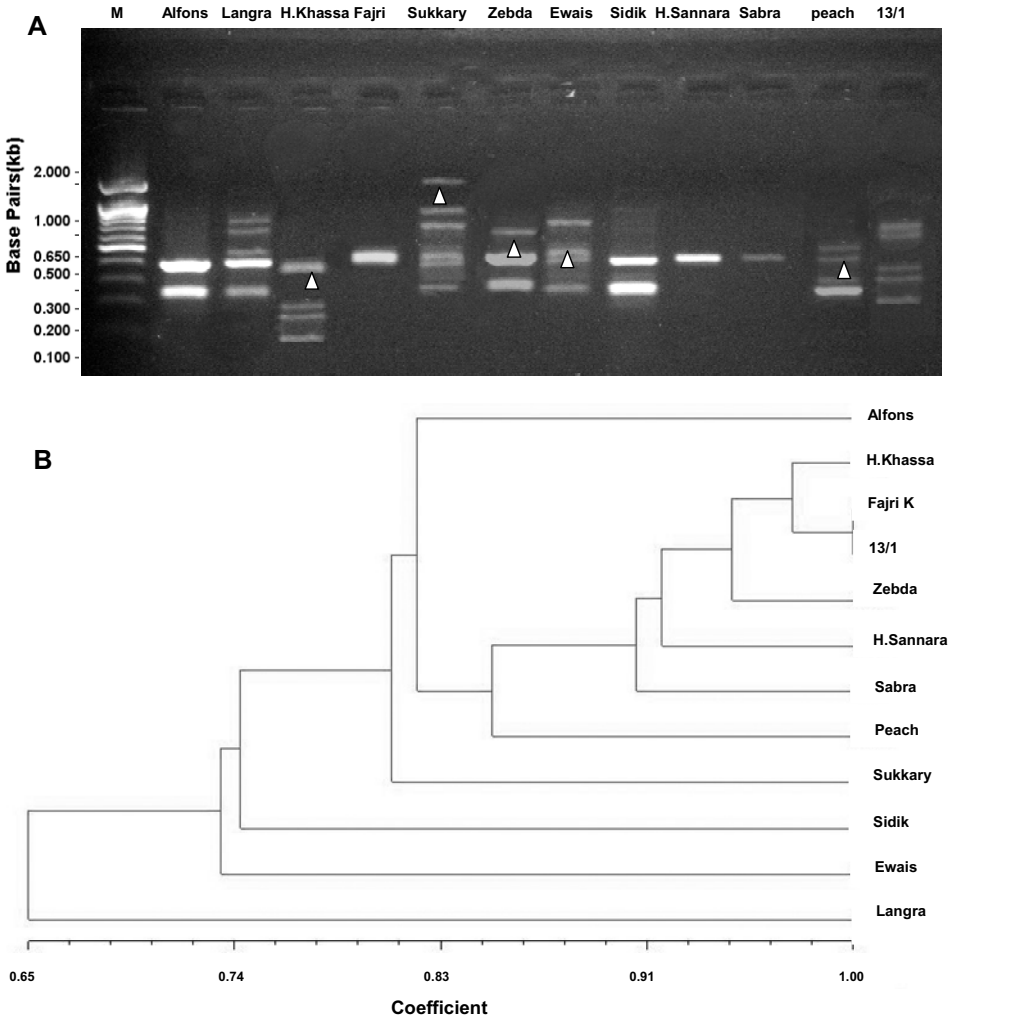

Fig. 2 ISSR markers detecting polymorphisms between mangos cultivars. (**A**) PCR amplification with HB11 primer. (**B**) Dendrogram based on algorithm of unweighted pair group method with arithmetic averages using ISSR results between in different mangos cultivars in Egypt.

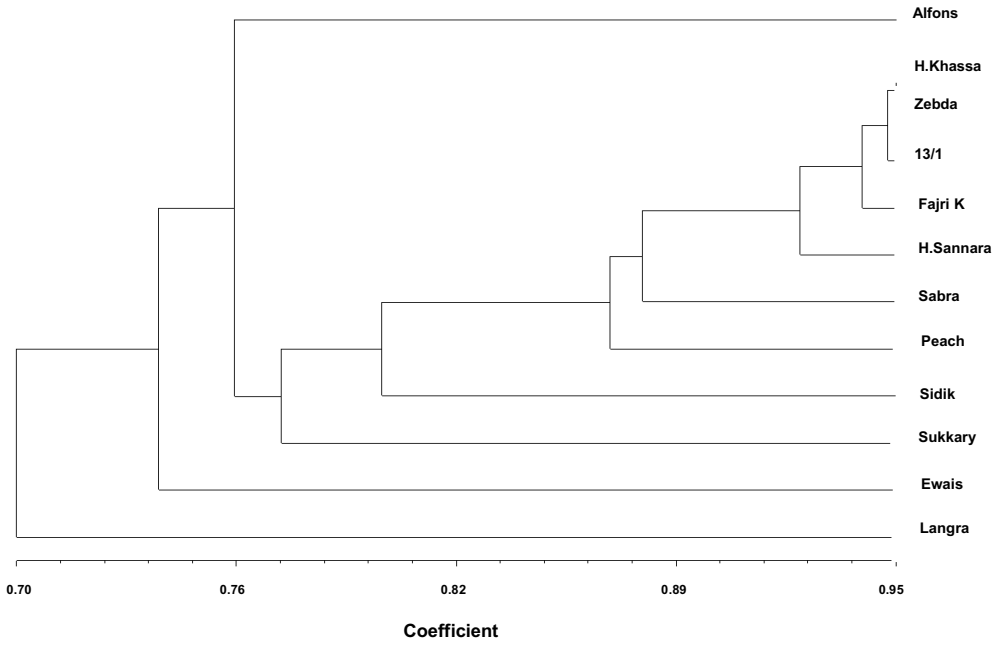

Fig. 3 Collective polymorphisms analysis between mango cultivars using both ISSR and three-primer-based RAPD.

Primer RAPD and ISSR approaches in all the cultivars. The results revealed high and clear reproducible fragment patterns for three-primers RAPD above single primer RAPD and ISSR in the range of 100 bp to 2 kb (**Fig. 3**). The UPGMA analysis showed that most of the different fresh-market mango cultivars from different geographical regions were distributed in different groups. These observations of genetic diversity analysis might provide further facilities to predict field performance. In addition, three-primer RAPD analysis could provide useful genetic markers associated

with certain characters which should be further analyzed and sequenced in the further applications in mangoes. These applications include, commercial variety protection, assessment of seed purity, marker assisted plant breeding and the verification of labeling and identity of plants in production and marketing.

ACKNOWLEDGEMENTS

The project was granted by the by Bibliotheca Alexandrina Center for Special Studies and Programs, Grant No. 050176 and National Research Center Mango project. The authors would like to thank Dr. Sanna Ebaid Horticulture Research institute for providing us by samples of Sabra and peach rootstocks.

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