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Molecular discrimination and genetic relationships between some cultivars of *Cucurbita pepo* ssp. *pepo* using random amplification of polymorphic DNA (RAPD) analysis

Safaa Abdel Aleem Radwan

Department of Botany, Faculty of Science, University of Tanta, Tanta, Egypt.

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***Cucurbita pepo* ssp. *pepo*; zucchini group is a widely grown and economically important group belonging to genus *Cucurbita*, and being one of the easiest groups to cultivate in temperate climate with overwhelming production. Since, RAPD analysis provides a fast and reliable method for molecular characterization and investigation of the intraspecific genetic relationships; it had been used in this study to discriminate and clarify the genetic diversity between seven cultivars of *C. pepo* ssp. *pepo* originated worldwide. Seven out of 20 decamer arbitrary primers showed polymorphism in the RAPD profile. The polymorphism was investigated by 87 consistent amplification products. Some of those fragments were uniquely amplified in single cultivar. Thus, they can be used as molecular markers for cultivar identification. The results of Jaccard similarity and the phenogram ascertained the wide genetic base of the Egyptian landrace El-Escandrani with the lowest loading component of 0.400. Therefore, it could be recommended as a reservoir of alleles useful for breeding programs in parental crosses. The multivariate analysis using the principle component analysis separated all the cultivars on the first component indicating the high correlation between them. The strongest correlation was confirmed between the two MHTSQ hybrids with Mansoura cultivar from Italy, with a loading component of 0.85.**

Key words: Genetic diversity, *Cucurbita pepo*, cluster analysis, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Cucurbita pepo is a worldwide cultivated fruit and economically important species of the genus *Cucurbita* L. It belongs to the family Cucurbitaceae, one plant group with the most species supply human with edible products and useful fibers (Bisognin, 2002). In the genus Cucurbita, five domesticated species were characterized, including *Cucurbita argyrosperma*, *Cucurbita ficifolia*, *Cucurbita maxima*, *Cucurbita moschata* and *C. pepo*, in addition to ten wild species. (Robinson and Decker-Walters, 1997). Most species originated in Mexico with only the species *C. maxima*, being native to South America.

C. pepo is the most diverse of these species and has traditionally comprised two subspecies; pepo and ovifera (Decker, 1988; Sanjur et al., 2002; Nesom, 2011). The former appears to be originated in Mexico while the second originated in the eastern half of United States. Each subspecies encompass several horticultural cultivar groups. *C. pepo* subspecies pepo L. includes pumkin, vegetable marrow, cocozelle and zucchini. *C. pepo* subspecies ovifera (syn. texana) includes acorn, scallop, croockneck and straightneck (Ferriol et al., 2003; Paris, 1986; Paris et al., 2003).

On the basis of archaeological records, *C. pepo* appears to be one of the first domesticated species. The oldest have been declared in the eastern of the United States (4000BC.) and in Mexico (8750BC).

*Corresponding author. E-mail: safaa_radwan@hotmail.com. Tel: 002-040-3344352. Fax: 002-040-3350804.

The records also reveal that the species may have been domesticated having Cucurbita fraterna and C. texana as possible progenitors. The species was introduced to Europe only about 500 years ago (Whitaker, 1947). Zucchini group is today the most widely grown and economically important group of *C. pepo* ssp. pepo. It is one of the easiest fruits to cultivate in temperate climate. It has a reputation for overwhelming production (Paris, 2010).

In a culinary context, the zucchini is treated as a vegetable, which means it is usually cooked and presented as a savory dish or accompaniment. However, botanically, the zucchini is an immature fruit, being the swollen ovary of the zucchini flower. Zucchini is considered on the list of the very low calories vegetables that contains no saturated fats and cholesterol. It is a moderate source of folates which is important in cell division and in DNA synthesis. It is a very good source of potassium, a vital intra-cellular electrolyte. Fresh fruits are rich in vitamin A, flavonoid, polyphenolic antioxidants such as carotenes, Lutein and zeaxanthin. These compounds help scavenge harmful reactive oxygen species ROS. It is also a good reservoir of antioxidant vitamin C, vitamin B-complex and mineral like iron, manganese, phosphorus and zinc (Achi et al., 2005).

Characterization based on morphological and horticultural traits have some disadvantages as being influenced by environmental factors (Dey, 1997; Sammour et al., 2007). It is tedious, unreliable and time-consuming. Instead, molecular characterization have proven many advantages, being able to distinguish polymorphisms which not produce phenotypic variation, highly polymorphic, easy and fast to detect and not possessing pleiotropic effects. Moreover, they can facilitate rapid screening of large numbers of genotypes for polymorphic loci (Williams et al., 1990).

Random amplified polymorphic DNA (RAPD) has established as a good genetic marker system to assay and evaluate the genetic diversity among species, among populations and furthermore among individuals in the same population (Ding et al., 2009; Mathew et al., 2010). It is an informative marker that screen large in the genome; either the expressed or the non-expressed and even the regulatory sequences (Rakhee et al., 2004; Elena et al., 2010).

Most studies proved RAPD marker reliability in estimating interspecific genetic relationships. The advantages of RAPD over other DNA-Based methods include a lack of the requirement for sequence information of the species, ease and speed of the assay, little amount of DNA required, no use of radioactivity and the ability to provide markers in genomic regions with repetitive DNA sequences (Dos-Santos et al., 1994; Hallden et al.1994; Thormann et al. 1994).

There are quite few studies concerning the molecular analysis of Cucurbita species. The RAPD markers were used to analyze the genetic diversity among *C. moschata* Lanraces from Korea, Southern Africa and other geographical origins (Youn and Chung, 1998; Baranek et al., 2000).

Ferriol et al. (2003) studied the genetic diversity among nineteen spanish accessions of C. maxima using two different molecular markers; sequence related ampli-fied polymorphism (SRAP and RAPD. More recently Ferriol et al. (2004a, b) employed the SRAP and RFLP molecular marker for analyzing the diversity among a large number of *C. Moschata* and *C. maxima* Landraces., Most of the studies that discuss the genetic variability within *C. pepo* using different molecular markers (RFLP, AFLP, RAPD, ISSRs) have been focused on the assess-ment of the genetic and evolutionary relationships between the wild and domesticated types, between the two subspecies or among the cultivars groups with only few representatives of landraces (Ferriol et al., 2003; Paris et al., 2003).

Therefore, the objectives of this study is to; First, discriminate and clarify the genetic relationships among the available seven *C. pepo* ssp. pepo cultivars using RAPD analysis as a basic requirement of further crop improvement. Second: to compare the natural variation present in a collection of the Egyptian landrace and the other commercial cultivars and hybrids to test the efficacy of RAPD for *C. pepo* cultivar identification.

MATERIALS AND METHODS

Plant material

Six cultivars and one Landrace belonging to *C. pepo* subspecies pepo (zucchini group) were donated from the Vegetables and Horticultural Research Center, The Agricultural Ministry). The represented Zucchini cultivars were Al-Escandrani landrace, D64-27, Amira, Mansoura, MHTSQ-05, MHTSQ-06 and Amjjed. The cultivars of this group were originated in different countries (Table 4).

Plant germination

Seeds were germinated at 25 to 30°C in the soil. Young fresh leaves were collected and stored in a -80°C deep freezer until use.

DNA extraction

DNA was extracted and purified from all samples using Qiagen DNeasy™ Plant Minikit following the protocol of the manufacturer (Qiagen Inc, Valencia, CA).

RAPD analysis

RAPD was performed as described by Williams et al. (1990) with minor modifications. Briefly, polymerase chain reaction (PCR) amplification was performed in 25 µL reaction mix containing 20.40 ng genomic DNA, 0.5 unit Taq polymerase (Sigma), 0.2 mM each of dATP, dCTP, dGTP, dTTP, 10 Pico mole random primer 5 µL amplification buffer. 1.5 µL of $MgCl_2$ and 9.75 µL of distilled H_2O. The reaction was assembled on ice, overlaid with a drop of mineral oil. Amplification was performed for 45 cycles using Biometera Uno

Table 1a. Name and sequences of the selected twenty random primers used in RAPD-PCR analysis. The highlighted primers are the primers resulted in polymorphism.

Primer name	Sequence (5'→3')
A-01	CAGGCCCTTC
A-02	TGCCGAGCTG
A-03	AGTCAGCCAC
A-04	AATCGGGCTG
B-01	GTTTCGCTCC
B-02	TGATCCCTGG
B-03	CATCCCCCTG
B-04	GGACTGGAGT
B-10	CTGCTGGGAC
B-14	TCCGCTCTGG
B-19	ACCCCGGAAG
G-01	CTACGGAGGA
G-02	GGCACTGAGG
G-03	GAGCCCTCCA
G-04	AGCGTGTCTG
J-11	ACTCCTGCGA
Z-01	TCTGTGCCAC
Z-02	CCTACGGGGA
Z-03	CAGCACCGCA
Z-04	AGGCTGTGCT

Table 1b. Name and sequences of the primers resulted in polymorphism using RAPD-PCR analysis.

Primer Name	Sequence (5'→3')
A-04	AATCGGGCTG
B-01	GTTTCGCTCC
B-02	TGATCCCTGG
B-04	GGACTGGAGT
G-02	GGCACTGAGG
Z-02	CCTACGGGGA
Z-03	CAGCACCGCA

thermal cycler, as follows: One cycle at 95°C for 3 min and then 44 cycles at 92°C for 2 min, 37°C for 1 min and 72°C for 2 min. Reaction was finally incubated at 72°C for 10 min and further incubated on 4°C . Seven primers were selected for RAPD analysis based on their ability to amplify *C. pepo* genome and produce reproducible amplification patterns (Table 1).

The amplification products were separated by electrophoresis on 1.5% agarose in 50X TAE buffer (Tris-acetate EDTA buffer: 242 g Tris-base, 57.1 ml glacial acetic acid and 100 ml EDTA (0.5 M pH 8.0) stained with 0.2 µg/ml ethidium bromide and photographed under UV light. Sample was loaded by using 10 µl PCR-product and 2 µl loading buffer. 100 bp DNA ladder (Fermentas) was used.

Data analysis

The band identification was based on the mobility of DNA fragments by numerous side-by-side comparisons of DNA extracts. The genetic diversity among the accessions was evaluated by Jaccard similarity index, multivariate analysis (cluster and principal component analysis (PCA) analyses). The analyses were performed using the frequencies of scored bands calculated for the accessions. The dendrogram was constructed through the average linkage-joining rule, using the software package “SYSTAT for Windows”, Version 7.0 copyright (C) 1997, SPSS INC. The RAPD data were analyzed using POPGENE version 1.31 Microsoft Window-based Freeware for Population Genetic Analysis.

RESULTS

Twenty (20) primers were used to elucidate the genetic diversity between seven *C. pepo* cultivars by amplifying the extracted DNA using RAPD-PCR analysis. The sequences of these primers are listed in Table 1a and b.

The RAPD profile of the amplified products is shown in Figure 1. The number of bands and the degree of polymorphism revealed by each primer are given in Table 2.

Totally, 87 consistent and differential amplification products were generated with seven (out of 20) decamer arbitrary primers. For each genotype, fragments were amplified per primer between 8 to 18 with an average of 13 bands/primer (Table 2). In general, the levels of polymorphism were varied with different primers among the different squash cultivars. The percentage of polymorphism produced by each primer differed from one primer to the other. Out of the total bands, 69 bands were polymorphic (Figure 1). The maximum value of polymorphism was 100% produced by the primers OPA-04 and OPZ-03. The minimum value of polymorphism was 27% produced by the primer OPB-02 with an average polymorphism of 70.9% across all the genotypes.

The different cultivars could be distinguished either by fragments combination or by specific cultivar fragments. Twenty five (25) bands were unique amplification products and could be used as molecular marker to distinguish each cultivar. The RAPD profile is shown in Figure 1a, b, c and d. The bands generated by the primer OPB-04 with the sequence length of 391 and 351 bp were unique for D64-27 cultivar originated in Italy; while, the bands generated by the primer OPZ-02 with the sequence length of 716 and 360 bp were unique for Al-Escandrani landrace from Egypt. There was only one case, where a specific band, present in all other cultivars, was absent in a single cultivar (D64-27) (the band with sequence length of 140 bp and generated by the primer OPB-04).

The level of genetic diversity among RAPD fragments was calculated with Jaccard coefficient of similarity (Table 3). The overall mean similarity index calculated by Jaccard similarity index (JSI) for pair wise combination of the amplified fragments generated by the seven arbitrary primers on the genomic DNA of the seven cultivars of *C. pepo* ranged from 0.352 to 0.816 with an average of

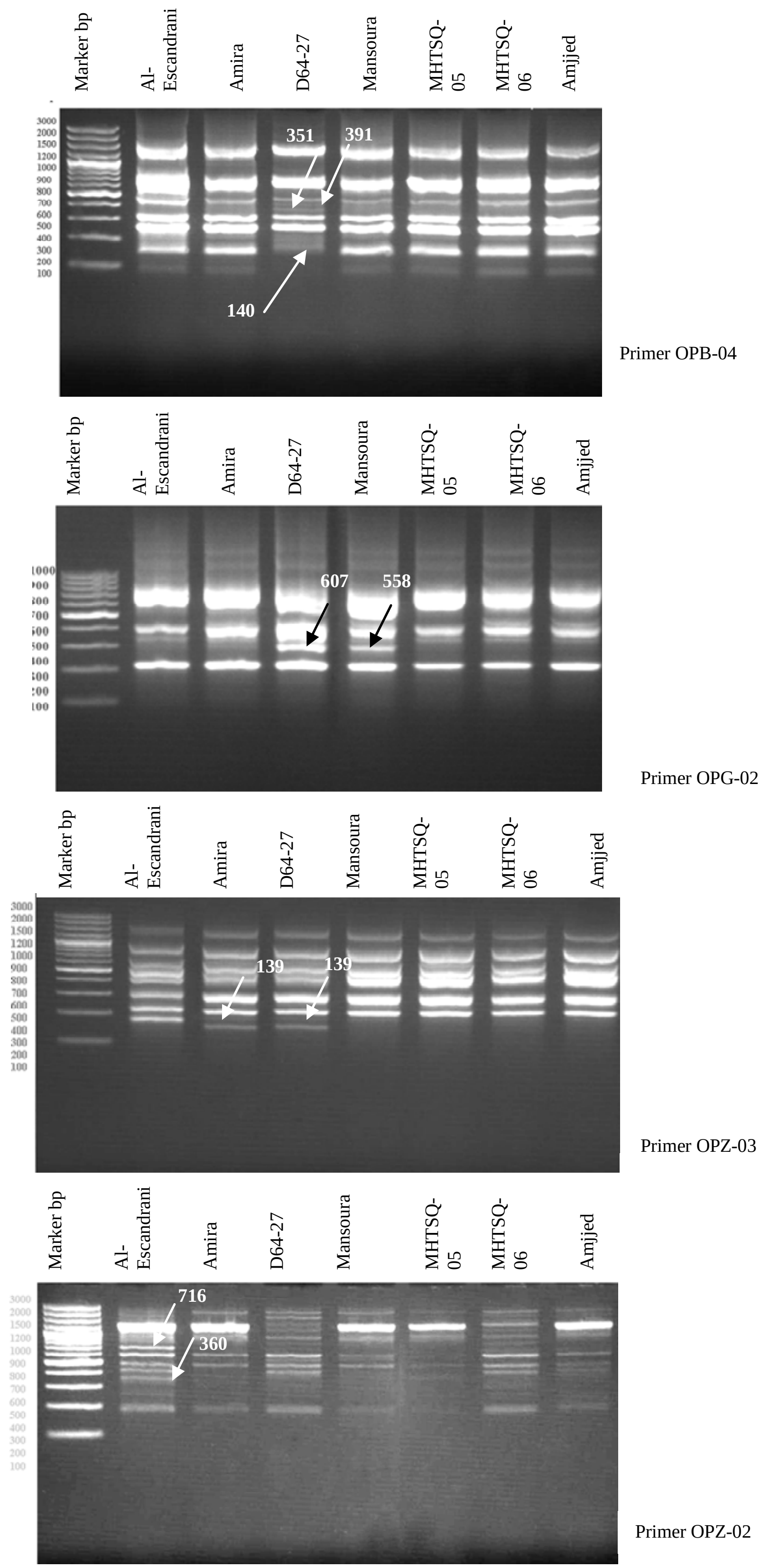

Figure 1. RAPD profiles illustrating bands amplification in the seven cultivars of *C. pepo* using four primers B04, G02, Z03 and Z02. DNA ladder Mix (100-3000 bp). Markers bands are indicated by arrow with molecular size.

Table 2. RAPD polymorphism among seven squash cultivars.

Unique product	Polymorphism (%)	Number of polymorphic bands	Total number of bands	Primer
6	100	13	13	OPA-04
-	38	3	8	OPB-01
1	27	3	11	OPB-02
2	64	7	11	OPB-04
6	88	14	16	OPZ-02
7	100	18	18	OPZ-03
3	79	11	14	OPG-02

Table 3. Jaccard Binary similarity coefficients between cultivars of *C. pepo*.

	Acc.1	Acc.2	Acc.3	Acc.4	Acc.5	Acc.6	Acc.7
Acc.1	1						
Acc.2	0.597	1					
Acc.3	0.486	0.678	1				
Acc.4	0.524	0.655	0.548	1			
Acc.5	0.469	0.618	0.469	0.792	1		
Acc.6	0.5	0.567	0.5	0.75	0.816	1	
Acc.7	0.371	0.444	0.352	0.6	0.654	0.717	1

0.682. The cultivars of *C. pepo* were divided into three groups: (1) the cultivar MHTSQ-06 and the cultivar MHTSQ-05 with the highest index of similarity that equal 0.816. Both cultivars were H1 hybrid result from cross between Al-Escandrani landrace as one parent and an American inbred line as the other parent; (2) the cultivar Amjjed originated in USA with the cultivar MHTSQ-06, the cultivar MHTSQ-06 with the cultivar Mansoura originated in Italy and the cultivar MHTSQ-05 with the cultivar Mansoura with a high mean indices of similarity equal 0.717, 0.750 and 0.792, respectively; (3) The lowest Jaccard similarity index was recorded between the cultivar Amjjed from USA with both the landrace Al-Escandrani from Egypt and the cultivar D64-27 from France with the mean similarity index of 0.371 and 0. 352, respectively.

Genetic diversity among the seven cultivars of *C. pepo* was investigated by cluster analysis using Euclidean distance matrix on average linkage. The phenogram constructed using each cultivar as an operational taxonomic unit (OUT) and including all the DNA fragments generated by the seven primers (Figure 2). The cluster analysis showed two main groups. G1 group contained the cultivar Al-Escandrani (the Egyptian Landrace) and G2 group contained the rest cultivars. G2 group is subdivided into two subgroups (G2a and G2b).

G2a included the two cultivars Amira and D64-27. Both cultivars originated in France. G2b separated the rest cultivars to two clusters. The first cluster included the cultivar Amjjed from USA solitary. The second cluster gathered two H1 hybrids (Al-Escandrani landrace x USA inbred line) with a third cultivar named as Mansoura and originated in Italy.

The principal component analysis (PCA) is one of the multivariate approaches to grouping based on the similarity coefficients of variance – covariance value of the component traits of the entities. The matrix of eigenvectors and values of the principle components PCs resulting from the interaction of the RAPD data (Table 4), indicated that all the DNA fragments generated by the seven primers on the DNA of the seven cultivars of *C. pepo* influencing 71.47% of the variability accumulated up to the first two components of PCA. The first component explained 51.24% of the total variation. The second component showed 20.23%. All the studied cultivars were separated on the first principal component.

The factorial plot showed all the cultivars aggregated in one direction. In parallel with the cluster tree, the cultivar MHTSQ-06, the cultivar MHTSQ-05 and the cultivar Mansoura have the same loading component of about 0.85, though they aggregated near to each other on the plot. Also the cultivar Amira and the cultivar D64-27 were aggregated together with approximately the same loading component of about 0.7.

DISCUSSION

The knowledge of genetic diversity of a crop is essential for the parental selection, which maximizes the genetic

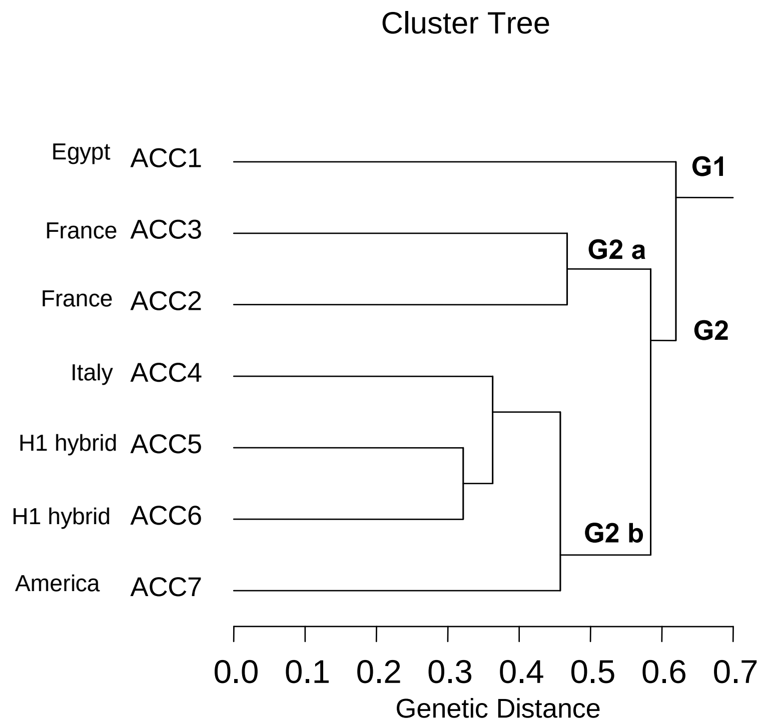

Figure 2. Dendrogram showing the genetic relationships among the seven cultivars of *C. pepo* based on genetic distance.

Table 4. Name, Origin, matrix of eigenvectors and values of the principle components for RAPD data of *C. pepo* cultivars.

Acc. Number	Name	Origin	Principal component 1	Principal component 2
1	Al-Escandrani	Egypt	0.4	-0.469
2	Amira	France	0.714	-0.506
3	D64-27	France	0.704	-0.649
4	Mansoura	Italy	0.886	0.008
5	MHTSQ-05	H1 hybrid	0.892	0.204
6	MHTSQ-06	H1 hybrid	0.862	0.29
7	Amjjed	USA	0.632	0.627
	Variance explained by components		3.587	1.417
	Percent of total variance explained		51.242	20.237
	Accumulated eigenvectors		51.242	71.48

improvement. Though, identification and utilization of the diverse germplasm is a core issue for plant breeding. The accurate description of different genotypes and the patterns of the genetic diversity help determining future breeding strategies and facilitate introgression of diverse germplasm into the current commercial squash genetic base. Furthermore, the estimated genetic diversity of these cultivars increases the use and interest of data already existing in the gene banks.

Our present study investigates a high intraspecific genetic diversity among the studied *C. pepo* cultivars. The variation assessed by 87 RAPD polymorphic bands generated by seven primers in the represented germplasm. The DNA fragments generated by RAPD primers were different in number, intensity and position indicating high genetic variation between the studied cultivars. The average polymorphism was 70.9% among the *C. pepo* cultivars which confirmed the high genetic variation between them. The primers OPB-01 and OPB-02 were characterized with the lowest number of polymorphic bands (three bands); whereas, the primers OPA-04 and OPZ-03 were characterized by the highest number of generated polymorphic bands (13 and 18 bands, respectively). The variation between reproducible bands generated by each primer depends on primer, sequence and the extent of variation in specific genotype (Chan and Sun, 1997; Shiran et al., 2007; Shukla et al., 2006).

With RAPD technique, some fragments were uniquely amplified in single cultivar such as the bands with the sequence length of 391 and 351 bp in the D64-27 cultivar from France and the bands with the sequence length of 716 and 360 bp in the Egyptian landrace El- Escandrani. In fact, these fragments are of great interest in optimal management of germplasm collections, as they facilitate the identification of cultivars and duplicates and verify possible pollen or seed contamination during conservation activities (Ferriol et al., 2004 a,b).

The output of Jaccard binary similarity coefficient and cluster analysis based on all DNA fragments generated by the seven primers showed the strongest homogeneity between the MHTSQ-05 cultivar and MHTSQ-06 cultivar with mean similarity index of 0.816. Both hybrids resulted from cross between El-Escandrani landrace x American inbred lines. A strong homogeneity was also found between Mansoura cultivar from Italy with either the MHTSQ-05 and MHTSQ-06 cultivars with mean similarity indices of 0.792 and 0.750, respectively. This homogeneity might be attributed to that these cultivars originating from one progenitor; Amjjed cultivar from USA.

The cluster analysis based on the comparison in the similarity matrix between the RAPD fragments generated by the seven primers resulted in more reliable data. From the dendrogram, we can predict that there was a strong relation between the genetic diversity and the geographical origins; whereas, the cultivars from different geographical locations were often unique and tend to be clustered in one part of the dendrogram. This result clearly suggests that the variation analyzed is determined not only by genetic factors but also by environmental differences. This agreed with Naghavi and Jahansouz (2005).

The constructed phenogram exhibited that all the genetic distance among the examined cultivars was ~59%. The Egyptian landrace El-Escandrani clustered independently from all the other studied cultivars. This finding indicated its unique banding pattern over the rest cultivars. The results ensured the wide genetic base of landraces, as they are characterized by a specific adaptation to the environmental conditions of the area of cultivation. Munazza et al. (2009) reported that the assessment of genetic diversity within and between landraces should have priority for variety improvement. Therefore, our results recommended the Egyptian zucchini landrace that have a level of diversity higher than that of the commercial cultivars and hybrids. So, it can be considered as a reservoir of alleles useful for breeding; because divergent genotypes may have a good breeding value (Gwanama et al., 2000).

The dendrogram also separated the cultivars originated from Europe and USA together with the hybrids that have one parent from USA; all in one major cluster. The Amira cultivar and the D64-27 cultivar were separated in distinct group. Both originated in France. This finding ascertained the fact that the distribution of the cultivars here is dependent on the geographical location. This agreed with Gwanama et al. (2000), who studied the genetic relationships among accessions of C. moschata in Zambia and Malawi. He observed that the accessions were grouped according to criteria of the geographic origin and the degree of breeding. The two hybrids MHTSQ-05 and MHTSQ-06 resulted from cross between El-Escandrani landrace x American inbred lines were separated in a second distinct group at a genetic distance of 30%. In general, a lower genetic variability is found within commercial hybrids compared with commercial cultivars and landraces, which is consistent with the narrower origin of hybrids and genetic erosion due to intensive breeding (Formisano et al., 2010). Moreover, both MHTSQ-05 and MHTSQ-06 cultivars were located together with Mansoura cultivar originated in Italy in one cluster. All these cultivars had Amjjed cultivar from USA as a possible ancestor. This finding ascertains the fact that Central and North America are the primary centers of diversity. It may also indicate the great diversification that took place in this species after the first *C. pepo* fruits and seeds arrived in Europe from America (Ferriol et al., 2003).

It is notably on the phenogram that Mansoura cultivar from Italy was located between the two cultivars originated in France on one side and the two MHTSQ hybrids on the other side. This result may be due to the fact that, since the 16th century, various squash cultivars migrate from different parts of South America where the primary center of diversity to reach Europe (Ferriol et al., 2004 a,b). It also may be due to the geographical location of Italy nearby France. Therefore, spreading new types selected in the two continents both ways through migration, hybridization and introgression. Meanwhile, *C. pepo* species is an out crossing and open-pollinated species. This character generates a great diversity of phenotypes; most of them being intermediate forms (Ferriol et al., 2003). It also suggested that the Italian *C. pepo* cultivars did not originate from squashes coming from a single American origin.

On the dendrogram, the Amjjed cultivar originated in USA was located far from the Egyptian landrace El-Escandrani; with a low similarity index of 0.371. This finding may due to the different geographical origins. However, both can be considered a vital genetic resource as parent for further breeding programs. Actually, the maximum variability for selection in segregating populations may be achieved by utilizing genotypes from different cluster as parents for crosses (Gwanama et al., 2000). The multivariate methods of analysis such as PCA used in the present study provided an effective way of evaluating germplasm material in order to identify materials that could be further evaluated or utilized.

In our results, the principal component analysis for the studied cultivars based on all DNA fragments generated by seven primers shows that the first two components accounted for 71.47 of the total variance of all cultivars. The separation of almost all cultivars was on the first component. This result indicates a high degree of correlation among the studied cultivars. The Egyptian landrace El-Escandrani had the lowest loading component of 0.400 indicating its low correlation with the other cultivars as it is selected for the Egyptian growing conditions. The PCA also confirmed the strong correlation between the two MHTSQ hybrids with Mansoura cultivar from Italy. This result was clarified by the similar high loading component of about 0.85.

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