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Full Length Research Paper

AFLP aids the selection of explicit (*Cucurbita pepo)* crosses resulting in hopeful vigor hybrids.

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In this study, morphological characterization and the Amplified Fragment Length Polymorphism technique (AFLP) were used to estimate the genetic diversity among fifteen pure inbred lines (S1, S2, --- and S15) of *C. pepo*. Out of the 28 primers screened, eleven EcoR1 and Mse1primer combinations were selected and amplified 622 loci of which 520 (85.3%) were polymorphic. Genetic distance values were calculated and they ranged from 0.11 to 0.56 indicating high polymorphism among studied accessions that were classified into two main clusters using the unweighted pair group's method with arithmetic mean (UPGMA). Phenotypic studies indicated that S15 x S13 and S15 x S14 crosses were favorable as they were the most distant parents. Based on AFLP data, S1 was found to be genetically the most distant accession. Thus, S1 x S7 (PDV 0.52) and S1 x S5 (PDV 0.56) crosses were performed and their progeny was evaluated in the field mainly for productivity. S1 x S5 and S1 x S7 plants had higher yields (1.27 and 1.62Kg per plant) than their respective parents and than the commercial hybrid, Amjad (1.12kg per plant). In contrast, yields of S15 x S13 and S15 x S14 plants (0.91 and 0.89kg per plant), chosen according to morphological classification, were much lower than those resulting from AFLP crosses. AFLP data seems to be more accurate than the morphological one and should be deployed in determining parents for crosses. However the new hybrids should be tested for other important characteristics such as disease and pest resistance before they can be considered in any breeding programs.

Key words: AFLP, *C. pepo*, vigor hybrid, Genetic distance, plant breeding.

INTRODUCTION

The Cucurbitaceae constitute one of the largest families in the plant kingdom. They include a huge number of edible plant species, comprising about 80 genera with over 800 species. One of the most important genera represented in the archeological record of the New World is Cucurbita L. which includes 5 domesticated species and 22 wild species (Al-Qas Yousuf, 2011).

C. pepo, a vegetable cultivated worldwide, is of American origin. It is one of the most important species and is extremely variable in fruit characteristics (Ahmed et al., 2003; Al-Qas Yousuf and Jubouri, 2011). Fruits are used as vegetables for human consumption or as livestock fodder, while the seeds are processed to extract oil. *C. pepo* is naturally cross-pollinated but self-compatible. Inbreeding does not induce significant loss of vigor (Katzir et al., 2004).Traditional cultivars offered by seed companies are true inbred lines; they offer an easy start for the development of F1 hybrids, which yield 50–100% more than open-pollinated cultivars. F1 seed is obtained either by hand pollination, or by planting both parents in the field side by side. FAO statistics for 2002 estimate world production of pumpkins, squashes and gourds at 17.7 million t from 1.4 million ha. China is by far the most important producer (4 million t), followed by India (3.5 million t), Ukraine (0.9 million t) and the United States (750,000 t). In Iraq, *C. pepo* is an important crop with a yield of over 116100 t from 9100ha (Iraqi agricultural statistics, 2008).

Based on allozyme variation, *C. pepo* which was domesticated at least 5000 years ago consists of three subspecies: C. pepo subsp. fraterna (Bailey) Andres, *C. pepo* subsp. texana (Scheele) Filov, and *C. pepo* subsp.

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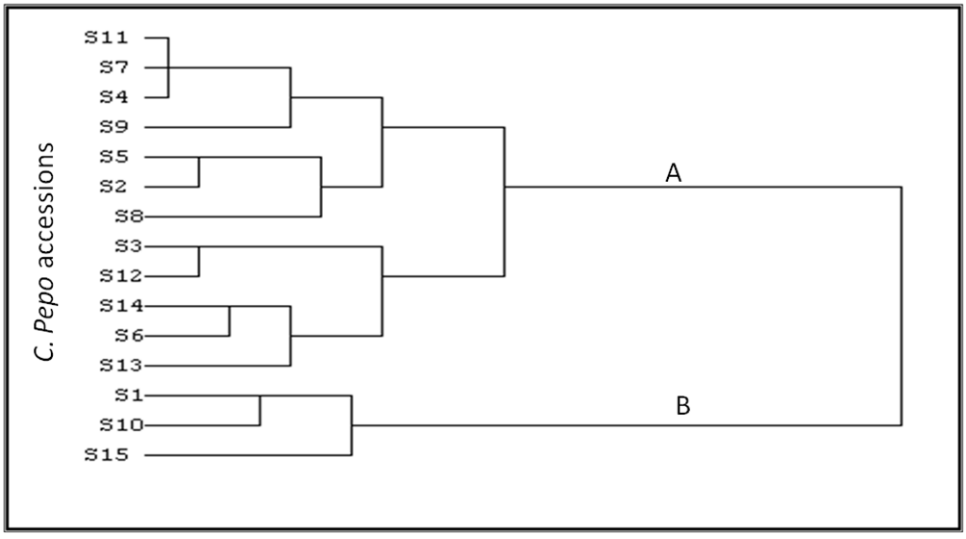
C. pepo accession code	Plant height Cm	Number of fruitful branches	Number of leaves per plants	Number of female flowers	Fruit length Cm	Fruit size Cm	Fruit weight g	Number of fruit per plant	Yield Kg
S1	120.0	1.03	70.6	17.4	14.3	4.16	163.3	9.50	1.03
S2	89.7	1.13	59.0	16.6	11.1	4.30	151.0	8.96	1.34
S3	59.9	1.00	50.7	12.6	12.8	4.60	168.3	6.40	1.08
S4	65.0	1.13	57.1	20.1	16.3	3.76	142.3	11.0	1.55
S5	89.2	1.20	58.0	9.06	16.1	3.86	154.0	5.33	0.80
S6	50.2	1.16	57.3	13.5	15.0	3.86	147.6	8.36	1.23
S7	57.8	1.33	57.6	16.7	16.3	3.46	145.3	7.73	1.11
S8	75.8	1.00	53.5	10.1	12.2	4.16	129.3	6.76	0.87
S9	67.3	1.60	67.6	14.6	13.4	4.06	139.6	6.16	0.87
S10	132.4	1.00	65.9	19.9	13.7	3.93	150.0	10.5	1.62
S11	57.0	1.40	57.6	17.6	15.6	4.10	151.0	9.26	1.39
S12	70.4	1.00	45.0	12.6	15.5	4.00	170.3	6.00	1.02
S13	42.0	1.23	45.9	7.53	10.5	4.53	165.5	4.60	0.76
S14	45.7	1.00	43.0	11.3	13.2	4.03	144.6	7.13	1.04
S15	128.0	1.53	87.7	23.6	12.1	4.40	144.6	9.16	0.87
L.S.D	17.9	0.39	14.7	3.48	1.68	0.78	37.6	2.24	0.32

Table 1. Morphological characteristics evaluated in 15 Iraqi accessions of *C. pepo.*

Figure 1. Cluster analysis of 15 *C. Pepo* accessions using morphological data.



pepo. These three subspecies contain wild and cultivated, inedible, small-fruited sorts (gourds) and eight groups of edible, large-fruited cultivars, pumpkins and squash (Yuan et al., 2008). In order to assess the genetic diversity, several morphological and molecular markers are used. Molecular markers are generally superior to morphological, pedigree, heterosis and biochemical data (Yee et al., 1999; Rademaker et al., 2000; Ferriol et al., 2004b).

One of these markers is the Amplified Fragment Length Polymorphism (AFLP), a high resolution technique that has been proved to be comparable with DNA pairing studies and RFLPs when genetic relatedness or intra-pathovar diversity is examined, and has shown more discriminatory power than random amplified polymorphic DNA (RAPD) or PCR using repetitive element primers (rep-PCR) (Andre et al., 1996; Bradeen et al., 2001). Unlike STS (Sequence-Tagged-Site

Table2. Sequences of oligonucleotide adapters and primers used in the pre-amplification step and the eleven selective AFLP primer combinations.

NAME	Reaction	sequence
EcoRI adapter		5'- AATTGGTACGCAGTCTAC
	Ligation	3'-CCATGCGTCAGATGCTC
MseI adapter		5'-TACTCAGGACTCAT
		3'-GAGTCCTGAGTAGCAG
EcoRI (E)	Pre-amplification	GACTGCGTACCAATTC
MseI (M)		GATGAGTCCTGAGTAA
1- E+ACT/M+CAT		GACTGCGTACCAATTCACT
		GATGAGTCCTGAGTAACAT
2- E+ACA/M+CAA		GACTGCGTACCAATTCACA
		GATGAGTCCTGAGTAACAA
3- E+ACA/M+CTG		GACTGCGTACCAATTCACA
		GATGAGTCCTGAGTAACTG
4- E+ACG/M+CTG		GACTGCGTACCAATTCACG
		GATGAGTCCTGAGTAACTG
5- E+ACT/M+CAA		GACTGCGTACCAATTCACT
		GATGAGTCCTGAGTAACAA
6- E+ACA/M+CAT	Selective amplification	GACTGCGTACCAATTCACA
		GATGAGTCCTGAGTAACAT
7- E+ACG/M+CAG		GACTGCGTACCAATTCACG
		GATGAGTCCTGAGTAACAG
8- E+ACA/M+CAG		GACTGCGTACCAATTCACA
		GATGAGTCCTGAGTAACAG
9- E+ACG/M+CAT		GACTGCGTACCAATTCACG
		GATGAGTCCTGAGTAACAT
10- E+ACT/M+CAG		GACTGCGTACCAATTCACT
		GATGAGTCCTGAGTAACAG
11- E+ACA/M+CTC		GACTGCGTACCAATTCACA
		GATGAGTCCTGAGTAACTC

Site) PCR and SSR, AFLP markers do not require a priori knowledge of DNA sequences and can detect all types of polymorphism including single base changes, insertions and deletions (Agarwal et al., 2008). On the other hand, SSR detects only changes in number of repeats (Barr et al., 2001).

In contrast to other molecular markers, AFLP can amplify in a single assay around 150 loci compared to 1 in SSR technique. Lately, various DNA-sequence polymorphisms have been employed to examine genetic relationships among C. pepo accessions with ever-increasing precision. These have been reviewed by Lebeda et al. (2007). The markers employed have included RAPDs, AFLPs, ISSRs, and SRAPs. A study of a wide-based collection of C. pepo using AFLPs, ISSRs has helped clarify a number of genetic relationships within the species, most notably differentiating the subspecies of C. pepo (Paris et al., 2003; Ferriol et al., 2003).

Additionally, ISSR technique was used for assessing relationships in the genus Cucurbita L. The results showed a dichotomy between accessions of C. pepo subsp. pepo and those of C. pepo subsp. texana and C. pepo subsp. fraterna, with cultivar-groups tending to form sub-clusters within their respective subspecies (Katzir et al., 2000; Ferriol et al., 2004a). Genetic distances among subspecies and cultivar-groups have not been rigorously defined, however, nor have results obtained from different marker systems been compared.

Therefore, the objective of the current study was to assess the genetic relationship within the selected 15 local accessions of C. pepo through the use of AFLP markers to identify suitable parents for potential crosses that might result in vigorous hybrids. The knowledge of

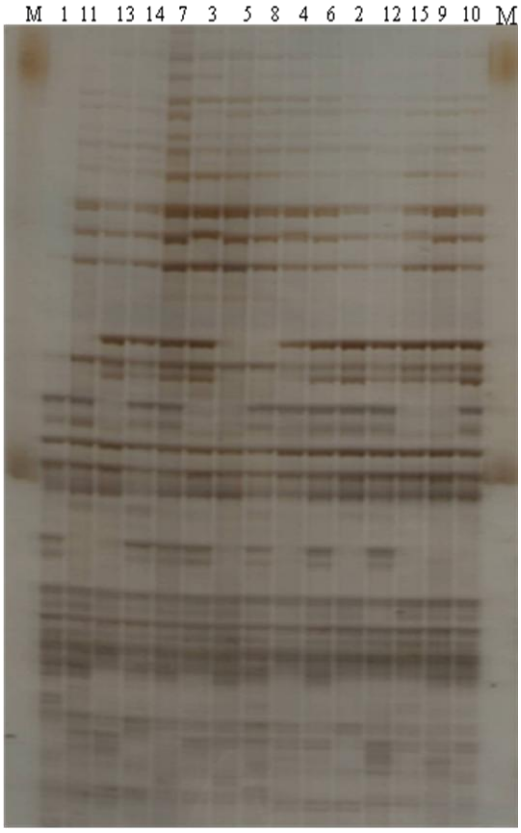


Figure 2. A portion of silver stained AFLP gel generated from 15 accessions of *C. pepo* using *Eco*RI+ACA/*Mse*I+CAA primer combination. M: marker.

the diversity of the studied accessions will facilitate their use in intended breeding programs and help in the selection of the most genetically distant parents for crosses.

MATERIALS AND METHODS

Plant Materials

A collection comprising 15 local accessions of Cucurbita pepo with good fruit characteristics was chosen for the current study.

Plant Cultivation and Pollination

Seeds of the selected accessions of *C. pepo* (S1, S2, S3, S4, ---- and S15) obtained from the Ministry of Agriculture were planted in rows 2.25 m apart (two rows each is 4m long for every accession), with plants spaced 0.8 m apart in rows. Male and female flowers were covered in paper bags one day before blossoms to ensure self pollination. Hand pollination was carried out, bags were left until three days later, fruits were left until ripened, and seeds were collected and stored at 4°C. When desired crosses were needed, a similar approach was followed, but pollens were obtained from the desired accession and used to pollinate the target one.

DNA Extraction

DNA was isolated from fresh young leaves using the Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). DNA concentration was determined by the GenQuant RNA/DNA Calculator (Amersham Biosciences Europe, Germany) according to manufacturer's protocol. A minimum of five seedlings per accession were sampled in bulk.

AFLP Analysis

AFLP analysis was performed based on the protocol described by Vos et al. (1995). Genomic DNA (40-50 ng) was restricted with EcoRI and MseI (0.25 U each) in a restriction buffer (10 mM Tris-HCL pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate) in a final volume of 5 µl. EcoRI and MseI adapters were subsequently ligated to the digested DNA fragments in a final volume of 10 µl.

Pre-amplification reaction was performed using E and M primers (EcoRI and MseI) with no selective nucleotide

at the 3`end to increase the number of amplified fragments. Table 2 presents the oligonucleotide adapters and primers sequences used in this study. The pre-amplification was carried out in 8.5 µl volume containing 250 ng of DNA using the following cycling parameters: 20 cycles of 30 sec at 94°C and 40 sec at 56°C and 50 sec at 72°C. The pre-amplified DNA was diluted 10 times by adding double distilled H2O2 using 2.5 µl as a template for the consequent selective amplification in which EcoRI and MseI primers with three selective nucleotides were employed.

DNA was amplified as follows: 12 cycles with annealing temperatures from 68°C to 59.6°C (decreasing by 0.7°C in each cycle), then 23 cycles at 59°C annealing temperature. In each cycle, the denaturation and elongation steps were the same: 94°C for 30 sec and 72°C for 1 min, respectively. To assure reproducibility, AFLP analysis was repeated twice starting from restriction. PCR products were mixed with 4 µl loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue) and denatured for 3 min at 95°C. AFLP fragments were separated on 6% polyacrylamide gel with 7 M urea, and 1x TBE buffer (12.1 g Tris, 5.11 g boric acid, 0.37 g EDTA).

Gels were run at constant power (1500 V, 80 W, 100 mA, 3 h and 20 min), and visualized with silver staining (Silver Sequence kit, Promega, Cat. Q4132, USA) following the manufacturer's instructions. Glass plates were treated as described in the instructions replacing Sigma-Cote solution with Repeal-Silane (Pharmacia Biotech, Sweden).

Band Scoring and Data Analysis

The number of polymorphic and monomorphic bands was determined for each primer pair, Reproducible and clear DNA fragments between 50 and 500bp were scored as present (1) or absent (0) and entered into a data matrix. The unweighted pair group method with arithmetic averages (UPGMA) and percent disagreement value of the statistical program were used to construct the matrices and the phylogenetic trees (Nei and Lei 1979; Statsoft, 2003).

Hybrid Production

Based on AFLP data, genetically distant accessions were hand crossed. The obtained seeds were cultivated as described above and resulting plants were evaluated for several fruit characteristics.

RESULTS AND DISCUSSION

In 2009, 15 promising local accessions of *Cucurbita pepo* were chosen and cultivated in the field of Baghdad University. The genetic material of these accessions was increased by self breeding and large amounts of seeds were obtained. Morphological and molecular studies using AFLP technique were conducted on these accessions to determine the genetic relatedness and select the most genetically distant ones for possible crosses hoping for vigorous hybrids.

Several morphological characteristics including plant height, fruit size, fruit weight and yield, etc (Table 1) were taken to evaluate the chosen accessions. All studied accessions exhibited a high variability for all the morphological characteristics. Based on the morphological data, accessions were distributed into two main clusters (Figure 1) in which cluster A was further divided into two sub-clusters (Figure 1). The lowest percentage of morphological similarity which represents the highest genetic dissimilarity was observed between accessions S13 and S15 and the highest was between S11, 7 and S4.

Based on these results, S15 x S13 and S15 x S14 crosses were favorable and thus were carried out. It is well documented that morphological features are traditionally used to assess genetic variation in *C. pepo*, but many cases are controlled by quantitative factors and / or affected by environmental conditions (Ferriol et al., 2004b). Accordingly, to evaluate the precision of the morphological classification, a molecular study was conducted. Several molecular marker types are currently available and feasible. The choice of a specific molecular marker technique depends mainly on its simplicity, credibility and reproducibility (Rademaker et al., 2000; Jones et al., 1997).

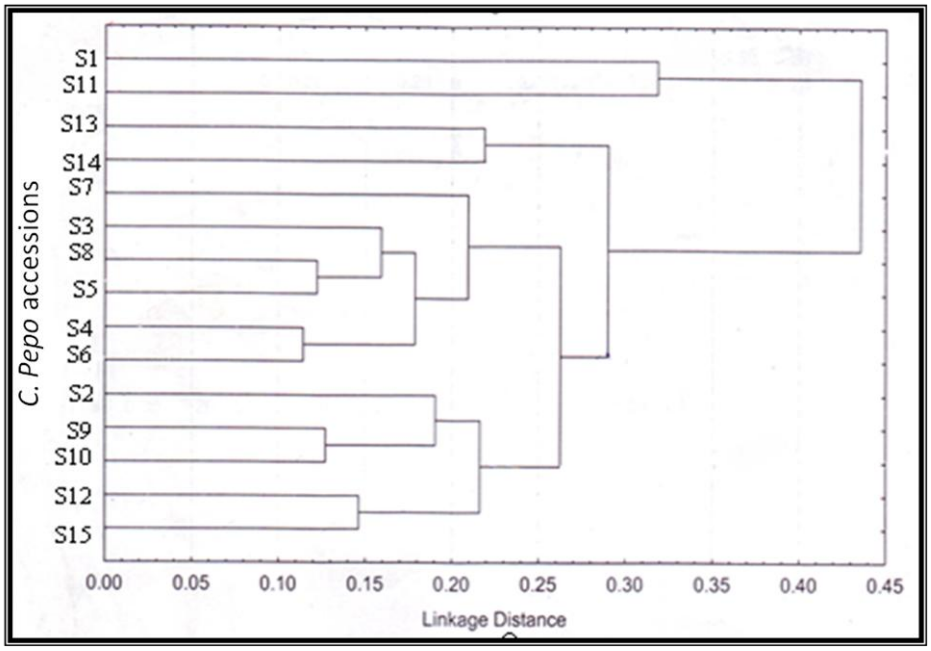
After more than two decades, AFLP for fingerprinting (Vos et al., 1995) is still a reliable technique that has proven its value in mapping and phylogenetic studies with a wide range of cereals (Medina et al., 1999; Gobert et al., 2002). It can detect all types of polymorphism and performs better than a series of other molecular marker techniques (Agarwal et al., 2008; Miyashita et al., 1999). Consequently, AFLP analysis was chosen to assess the genetic diversity within *C. pepo* accessions.

Out of the twenty-eight primers screened, eleven were chosen and each of the selected primer combinations yielded 32 to 71 amplification products. Table 2 shows sequences of the eleven selective AFLP primer combinations used in this study. The primer combination (E-ACT x M-CAG) gave the highest number of scorable bands (71) compared with the pair (E-ACA x M-CAG) which yielded the lowest one at 32 bands (Table 2). Overall, primers chosen produced a total of 622 easily scored bands of which 520 (85%) were polymorphic.

Figure 2 illustrates a portion of silver stained AFLP gel generated from 15 accessions of *C. pepo* using EcoRI+ACA/MseI+CAA primer combination (Figure 2).

The un-weighted pair group method with arithmetic averages (UPGMA) and percent disagreement values (PDV) of the statistical program were used to estimate the degree of genetic relatedness among all studied ac-

Figure 3. AFLP cluster analysis of 15 *C. Pepo* accessions using 11 primer combinations.



C. pepo accession code	Plant height Cm	Number of fruitful branches	Number of leaves per plants	Number of female flowers	Fruit length Cm	Fruit size Cm	Fruit weight g	Number of fruit per plant	Yield Kg
S1	120.0	1.03	70.6	17.4	14.3	4.16	163.3	9.50	1.03
S5	89.2	1.20	58.0	9.06	16.1	3.86	154.0	5.33	0.80
S3	59.9	1.00	50.7	12.6	12.8	4.60	168.3	6.40	1.08
S7	57.8	1.33	57.6	16.7	16.3	3.46	145.3	7.73	1.11
S13	42.0	1.23	45.9	7.53	10.5	4.53	165.5	4.60	0.76
S14	45.7	1.00	43.0	11.3	13.2	4.03	144.6	7.13	1.04
S15	128.0	1.53	87.7	23.6	12.1	4.40	144.6	9.16	0.87
C.C. (Amjad)	53.3	1.60	47.0	19.1	12.6	3.46	113.5	9.90	1.12
S1 X S7	49.3	1.00	43.0	14.6	13.9	4.06	157.2	9.40	1.62
S1 X S5	72.6	1.50	52.0	19.9	13.4	3.73	150.0	6.60	1.27
S15 X S13	82.1	1.00	50.1	7.6	10.2	3.10	111.0	6.26	0.91
S15 X S14	70.4	1.00	48.0	9.5	11.0	4.20	120.3	6.13	0.89
S1 X S11	71.0	1.00	48	6.51	9.4	3.13	108.1	5.60	0.77
N.C.									
L.S.D	13.4	0.63	9.07	2.48	0.53	0.41	40.20	2.02	0.385

Table 3. Morphological characteristics evaluated in selected *C. pepo* accessions and their crosses.
C.C.: Commercial control, N.C.: Negative Control (parents with low genetic distance 0.32)

cessions based on common amplified fragments. The matrix data (not shown) were put in a dendogram that shows a close representation of the values obtained in the PDV matrices. AFLP data separated the 15 pure accessions of *C. pepo* in two distinct clades, A and B where the latest was sub-clustered in three groups rather than two in the morphological one.

Figure 3 clearly shows that S1 is genetically the most dis-

tant accession from all others particularly from S7 and S5. Thus, S1 x S7 (PDV 0.52) and S1 x S5 (PDV 0.56) crosses were performed. Seeds from all crosses based on morphological and molecular data were obtained and cultivated, and their plants were compared mainly for productivity with their parents and with the commercial hybrid, Amjad (Peto seed, USA, Table 3). S1 x S5 and S1 x S7 plants had higher yields (1.27 and 1.62Kg per plant) than their respective parents (Table 3) and also had higher yield than the commercial hybrid Amjad (1.12Kg per plant).

In contrast, S15 x S13 and S15 x S14 plants which were selected according to morphological classification had lower yields (0.91 and 0.89 Kg per plant) than AFLP based crosses and Amjad (Table 3). In conclusion the AFLP technique used in the current study demonstrates great utility for estimating the genetic diversity among fifteen pure lines of summer squash over morphological characterization. Consequently, the use of molecular markers would shorten the time and efforts needed for selecting genetically different or similar parents useful for certain crosses and thus would cut the cost of producing new hybrids. Obtained hybrids S1 X S7 and S1 X S5 are still to be tested for other important characteristics such as disease and pest resistance before they can be considered in any breeding programs.

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