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Genetic Fingerprint of Some *Cucurbita pepo* (Summer squash) Genotypes Using Molecular and Biochemical Techniques

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Abstract

This study was conducted to fingerprint of seven squash genotypes using RAPD –PCR and SDS electrophoresis techniques . RAPD profile generated high level of polymorphism. OPA-03 and OPC-19 primers showed high value for number of main, amplified and unique bands and success in giving a distinct fingerprint for all genotypes while the primer OPT-08 gave fingerprint for one genotype .The highest value for primer efficiency and discriminatory value were produced by the primers OPD-13 and OPN-06.

Phylogenetic tree of squash grouped the seven genotypes in to two major groups . First small main group include one genotype while the other large main group include six genotypes which can divided in to two subgroups. The first small subgroup include one genotype while the other large subgroup include five genotypes.

Highest genetic distance was 0.84 observed between genotypes Delicata(5) and Straight neck (7). While the lowest genetic distance 0.12 observed between Elite(2) and Ford hook (3) genotypes.

SDS electrophoresis pattern of total seed protein showed low level of polymorphism due to producing four monomorphic bands from six main bands . Protein profile gave fingerprint to one genotype . RAPD-PCR technique showed high polymorphism 63.6 compared with low polymorphism 33.3which produced by SDS electrophoresis technique. Thus RAPD markers provides an excellent tool for future studies of squash genotypes fingerprinting using both OPA-03 and OPC-19 primers .

Key words: *Cucurbita pepo*, Random Amplified Polymorphic DNA (RAPD),Cluster analysis , , fingerprint , protein profile .

Introduction

Cucurbita pepo (Summer squash) belongs to *Cucurbita* genus which is a member of the economically important Cucurbitaceae family which contains five domesticated species all native to the American origin [1] and [2] .

C. pepo supply carbohydrates , soluble fibers and pro vitamin A from the flesh and protein vegetable oil and vitamin E from the edible seeds and increasing of its consumption because of important in healthy diet [3].

This crop is highly resistance to drought their seeds contain (23-35%) of protein and (25-55%) of oil..Squash has attracted the attention of many growers and plant breeders within the past 50 years [4].

It is a highly polymorphic vegetable species, both in vegetative and reproductive characteristics, with a wide range of genetic variation occurring within it [5] and [6] . Morphological features are traditionally used to assess genetic variation in *Cucurbita* species, however many cases are controlled by quantitative factors and/or affected by environmental modification [7].

Among the different types of molecular markers available, Random Amplified Polymorphic DNA (RAPD) are useful for the assessment of genetic diversity because of their simplicity, fast and easy to perform and comparatively cheaper than other markers and require no prior knowledge of DNA sequences [8].

The potential applications of RAPD fingerprinting in molecular biology include : determination of taxonomic identities, detection of inter specific gene flow assessment of kinship relationships ,analysis of mixed genome samples and production of specific probes and gene mutation [9]. RAPD markers are extensively used to analyze genetic diversity in *Cucurbita* [10].

RAPDs markers were used effectively to construct a partial map of 75% of *Cucurbit*a genome in include *B* gene for fruit which turn yellow befor anthesis ,the *M* gene for silver mottling leaves and a locus controlling the intensity of rind colour on mature fruit (qualitative trait loci) , on other hand quantitative traits loci associated with fruit shape and the depth of the indentations between leaf primary veins were identified [3].

Biochemical markers for analysis of total seed storage proteins are widely used [11],Its powerful tool in differentiation of hybrids and wild varieties [12] cultivars fingerprinting [13], Phylogenetic diversity relationships of varieties [14] and varieties identification [15]. Seed protein is not sensitive to environmental fluctuations; its banding pattern is very stable which advocated for cultivars identification purpose in crop. It has been widely suggested that such banding patterns could be important supplemental method for

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cultivars identification, particularly when there are legal disputes over the identity of a cultivar or when cultivars are to be patented[16]

The electrophoresis of seed storage protein is a method to investigate genetic variation and to classify plant varieties The technique of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is commonly used for separation of seed storage proteins [17]

For an effective breeding program, information concerning the extent and nature of genetic diversity within and between species is essential for any crop improvement ,it is particularly useful for organizing germplasm characterizing individual accessions and genotypes identification of cultivars as well as a general guide in the selection of the parents for hybridization to maximize the expression of heterosis [11] and [18].

Materials and Methods:

1-DNA extraction and PCR amplification : Seven of squash genotypes with diverse fruit shape and colour were included in this study: **1.**Waltham, **2.**Elite, **3.**Fordhook,**4.** D46-27, **5.**Delicata, **6.** Zucchini, **7.**Straight neck**.** Fresh young leaves at age of two weeks were used to for DNA extraction using The Genomic DNA Mini Kit (Geneaid Biotech. Ltd; Taiwan Company).

PCR reaction mixture was prepared as follows: 5µl template DNA and 2 µl of primer (10 pmole/µl), were added to each AccuPower® TLA PCR PreMix tube. Sterilized deionized distilled water was added to AccuPower® TLA PCR PreMix tubes to the final volume of 20 µl. The tubes were mixed with vortex to dissolve the lyophilized blue pellet, and briefly spine down . Six oligonucleotide primers were examined for fingerprinting genotypes as listed in Table 1) .

Amplification were performed in thermocycler programmed according to annealing temperatures as follows:1. one cycle of 5 min at 94C°, for 40cycle of each 1 min at 94C°, 2 min at 40C° and 2 min at 72C°, with a final extension for one cycle of 5 min at 72C°. The amplified DNA product were separated by electrophoresis in 1.2 % agarose gels stained with ethidium bromide (5 µl of ethidium bromide solution (10 mg/ml) for 3 hr at 70V and then visualized under UV light and photographs.

Presence of a product was identified as (1) and absence was identified as (0). Data were scored for all genotypes, their amplification product and primers. The data then entered into NTSYS-PC (Numerical Taxonomy and multivariate Analysis System), Version 1.8 (Applied Biostatistics) program [19]. A Dendrogram was constructed based on genetic

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distance : genetic distance(GD)=1- genetic similarity (GS) using the Unweighted Pair-Group Method with Arithmetical Average (UPGMA).

Table 1: Primers and their sequence

Primer	Sequence (5'→ 3')	Primer	Sequence (5'→ 3')
OPA-03	AGTCAGCCAC	OPN-06	GAGACGCACA
OPC-19	GTTGCCAGCC	OPT-08	AACGGCGACA
OPD-13	GGGGTGACGA	OPW-04	CAGAAGCGGA

2- Total seed protein extraction and electrophoresis :

Total seed proteins were extracted separately from 0.02 g air-dried seed meals in 1000 µL extraction buffer (0.0625 M Tris HCl pH 6.8, 2% SDS, 10% sucrose and 0.002% bromophenol blue, 5% b-mercaptoethanol) for 24 hours at –4°C. After that time, the extracts were centrifuged for 10 minutes at 1000xg [20].

Lammeli method was applied for preparation of SDS-PAGE (sodium dodecyl sulfate-polyacryl amide gel) [21]. Gel at concentration of 12.5 percent was prepared as in Table 2.

Table 2: Gel solution composition for slab gels at concentration of 12.5%

Solutions	Separation gel 12.5%	Stacking gel 4.5%
Sol. A	7.5 ml	0.9 ml
Sol. B	4.5 ml	-
Sol. C	-	1.5 ml
Sol. D	0.07 ml	0.018 ml
Temed	0.01 ml	0.01 ml
Water	1 ml	3.6 ml

A current of 1.5 mA per well with a voltage of 80 V was applied until the tracking dye crossed the stacking gel. Later the current was increased to 2 mA per well and voltage up to 120 V. The electrophoresis was stopped when the tracking dye reached the bottom of the resolving gel.

Staining using coomaasie brilliant blue solution continue for about overnight while destaining continued till bands clearly visualized in white fluorescent light.

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Results and discussion

1-Molecular analysis :

The results in table 3 showed that both primers OPA-03 and OPC-19 produced higher value for main bands ,unique bands and amplified bands and this clearly reflect on their ability to gave distinctive DNA fingerprint for the seven genotypes. Higher number of amplified bands giving an excellent chance for detecting DNA polymorphisms among individuals [22]. The presence of unique bands indicate that each cultivar had one or more novel sequences which was not found in other cultivar. They are more useful for cultivar identification to differentiate specific cultivar from others [23].

Primer OPN-06 showed higher value for polymorphism ,primer efficiency and discriminatory value .These three criteria are concerned directly with primer ability to produce polymorphic bands which were 7 bands compared with the rest primers. amplification pattern and genotypes DNA fingerprint were illustrated in figure (1).

Table3: Summarized results of all data obtained by six RAPD primers including fragment size range (bp), no. of main bands, no. of amplified bands, no. of monomorphic bands, no. of polymorphic bands, no. of unique bands, polymorphism, primer efficiency , discrimination value and no.of identified genotypes

No.	Primer	Fragment size range (bp)	No. of main bands	No. of amplified bands	No. of monomorphic bands	No. of polymorphic bands	No. of unique bands	Polymorphism (%)	Primer efficiency	Discriminatory value (%)	No.of identified genotypes
1	OPA-03	390-2126	26	32	0	4	22	15.3	0.12	15.3	7
2	OPC-19	486-3364	31	37	0	5	26	16.12	0.13	19.2	7
3	OPD-13	257-833	12	28	0	6	6	50	0.21	32	2
4	OPN-06	228-914	11	26	0	7	4	63.6	0.26	26.9	5
5	OPT-08	318-716	6	25	3	1	2	16.6	0.04	3.8	1
6	OPW-04	276-645	7	18	0	3	4	42.8	0.16	11.5	3
		Total no. of bands	93	166	3	26	64	-		-	

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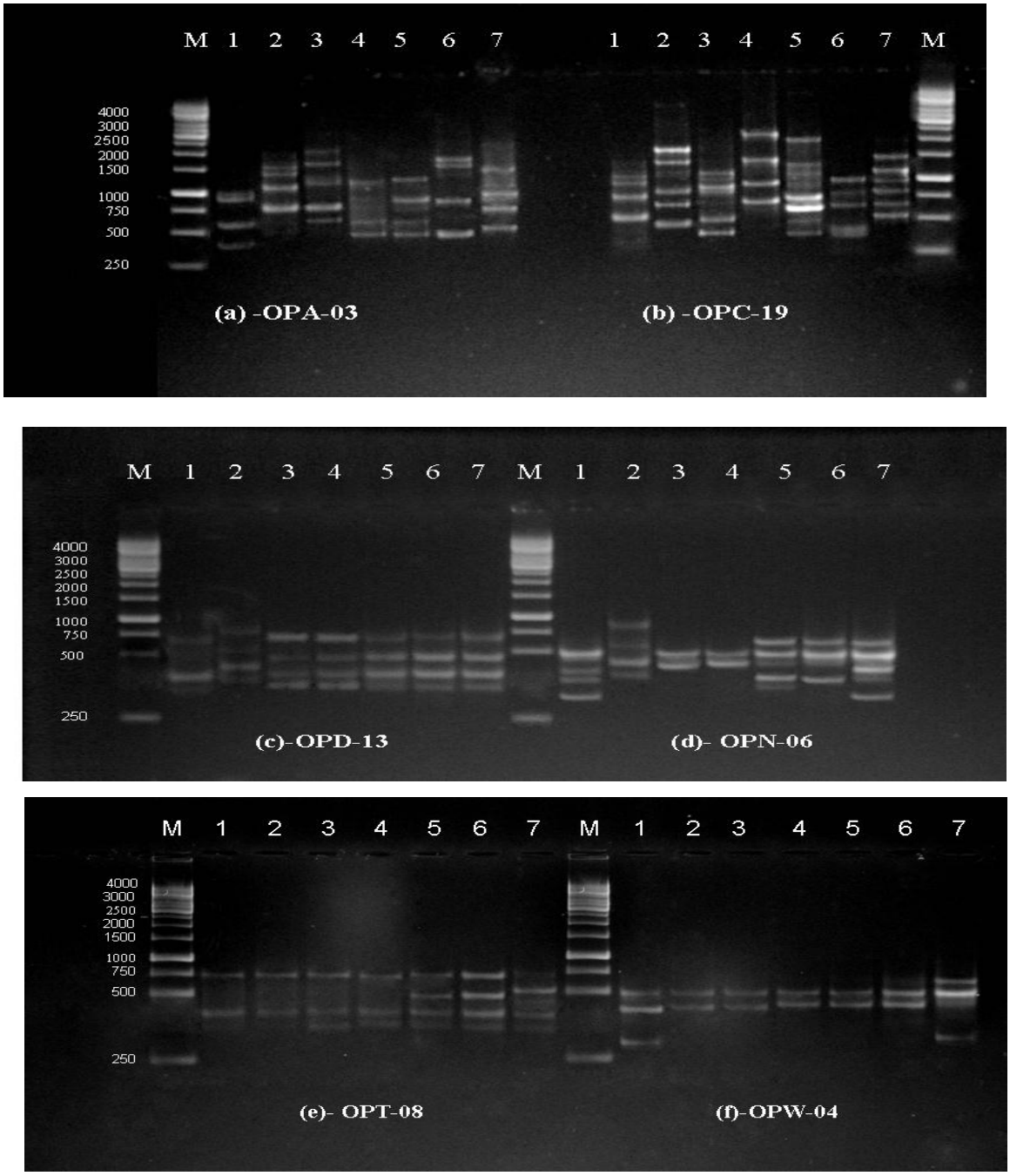

Fig. 1:RAPD fingerprints of seven squash genotypes generated by the six primers : (a) OPA-03 ,(b)OPC-19, (c) OPD-13,(d) OPN-06, (e) OPT-08 ,(f) OPW-04

Figure (2) indicates that Phylogenetic tree of squash grouped the seven genotypes in to two major groups . first small main group include one genotype while the other large main group include six genotypes which further can divided in to two subgroups. The first small subgroup include one genotype while the other large subgroup include five genotypes . The

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phyllogram can be used in the formulation of breeding plans. for example, crosses between closely related genotypes are less likely to produce heterosis [24]

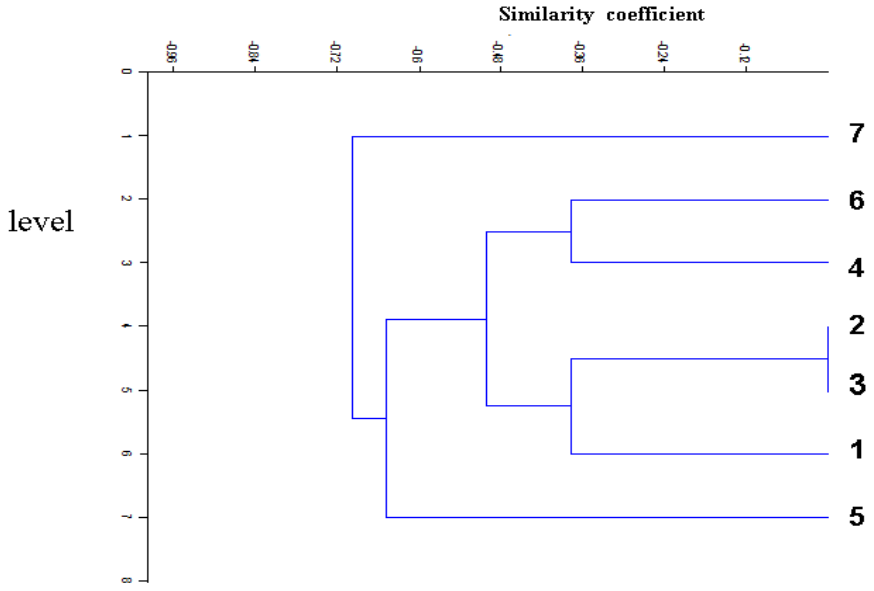

Figure 2: UPGMA dendrogram illustrating the trees of genetic relationship between seven squash genotypes using RAPD primers

The data in table (4) indicate that the highest genetic distance was 0.84 observed between genotypes Delicata(5) and straight neck (7). While the lowest genetic distance observed between Elite(2) and Fordhook (3) genotypes. High genetic distance reflect the low similarity value between genotypes which directly concern with geographic origin of genotypes [25]. The genetic distance value provides a useful estimation of relationship between a specific pair and a small number of genotypes [24]

Table 4 : The genetic distance values between seven squash genotypes studied using RAPD analysis

	1	2	3	4	5	6	7
1	0.000						
2	0.3679	0.000					
3	0.3779	**0.1244**	0.000				
4	0.5345	0.3879	0.3769	0.000			
5	0.7559	0.6547	0.6646	0.5345	0.000		
6	0.6546	0.5346	0.5347	0.3789	0.6647	0.000	
7	0.6446	0.7569	0.7659	0.6556	**0.8451**	0.5345	0.000

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2-SDS protein analysis:

The results in table (5) and figure (3) demonstrate the SDS-protein profiles of seven squash genotypes used in this study. A maximum of 6 main bands were detected with molecular weights ranged from 159 to 12 KDa. Monomorphic bands were identified in all the cultivars at approximate molecular size of about 12, 16, 42 and 159 KDa and only two polymorphic bands at 83 and 99 KDa molecular weight. With regard to seed protein banding patterns, slight polymorphism has been identified among the seven genotypes under study and only Waltham genotype was given a distinctive fingerprint due to absence of a band at 83 KDa molecular weight . In this study, the obtained seed protein banding patterns could not distinguish the studied genotypes.In case of low level of protein polymorphism , these protein profiles could be used as a general biochemical fingerprint for the genotypes [13] .

The low level of protein polymorphism could be result from conservative nature of the seed protein [26].

Table 5: Summarized results of SDS- protein banding profile of seven squash genotypes

Fragment size range (KDa)	No. of main bands	No. of amplified bands	No. of monomorphic bands	No. of polymorphic bands	No. of unique bands	Polymorphism (%)	No.of identified genotypes
12-159	6	37	4	2	0	33.3	1

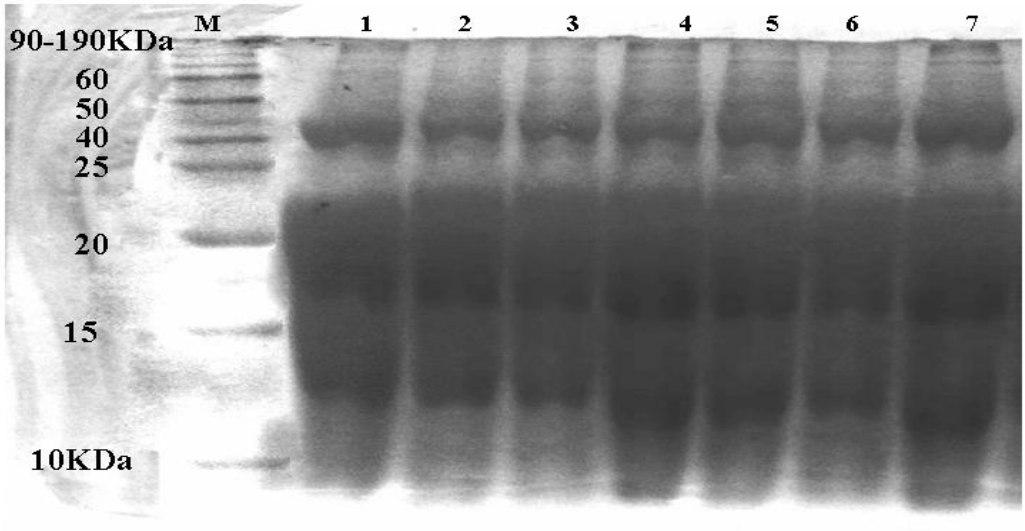

Fig 3 : SDS-protein banding pattern of total seed proteins of the seven squash genotypes

The efficiency of RAPD primers in fingerprinting *C.pepo* was confirmed in South Africa using seven *cucurbita* landraces and nine RAPD primers .The dendrogram mainly grouped the populations according to their mature fruit colour, and then according to their

geographical origin. All genetic parameters indicated that there was plentiful genetic diversity in C. *pepo* landraces [27].

In Egypt 27 RAPD primer used to discriminate among seven squash cultivar in which seven out twenty decamer arbitrary primer showed polymorphism in the RAPD profile .The results showed that some of amplified fragments were uniquely amplified in single cultivar ,thus they can be used as molecular marker for cultivar identification [28].

Tested molecular genetic evaluation of seven squash parents under Egyptian conditions using their leaves fingerprints as obtained through protein electrophoresis technique and RAPD-PCR technique using five random primers. Protein electrophoresis successfully generated reproducible polymorphic banding patterns. The generated profiles revealed high levels of polymorphism among parents . [29].

RAPD analysis revealed the presence of eight unique bands which identify the seven varieties,this indicatesthat using protein and RAPD markers for characterization and construction of genetic linkage maps and the molecular genetic diversity of parents support the use of marker-assisted selection (MAS) in squash cultivars breeding programs.

DNA(RAPD) used to identify the polymorphisms and the relationships between 14 genotypes, which belong to three different *Cucurbita* species (*C.pepo, C.moschata and C.maxima*) using six primers, and conclude, that information depend on polymorphism using RAPD markers is useful in the assessment o f genetic diversity and genetic relationships and could be useful in the breeding programs[7].

Conclusion:

In comparison between two makers ; RAPD technique produce a powerfull tool in squash varieties fingerprinting.

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البصمة الوراثية لبعض الانماط الوراثية للقرع الصيفي (الشجر) *Cucurbita pepo* باستخدام تقنيات جزيئية و كيموحيوية

أطياف جميل ثامر التميمي
كلية العلوم / جامعة الكوفة

الخلاصة :

اجريت الدراسة الحالية لتحديد البصمة الوراثية لسبعة من الانماط الوراثية للقرع الصيفي(الشجر) باستخدام تقنيتي واسمات التفاعل التضاعفي العشوائي المتعدد الأشكال لسلسلة الدنا RAPD والترحيل الكهربائي للبروتين الكلي للبذور . انتجت واسمات ال RAPD مستوى عالي من تعدد الاشكال polymorphism . اعطت البوادئ OPA-03, OPC-19 اعلى قيمة لكل من اعداد الحزم الرئيسة , المتضاعفة والفريدة ونجحت هذه البوادئ في اعطاء بصمة محددة لكل الانماط الوراثية بينما اعطى البادئ OPT-08 بصمة لنمط وراثي واحد.اعطت البوادئ OPD-13,OPN-06 اعلى قيمة لكفاءة البادئ وقدرته التميزية .

التحليل التجميعي (شجرة النشوء والتطور) جمعت الانماط الوراثية السبعة للقرع ضمن مجموعتين رئيستين الاولى صغيرة ضمت نمط وراثي واحد والاخرى كبيرة ضمت ستة انماط وقسمت الاخيرة بدورها الى مجموعتين فرعية الاولى صغيرة ضمت نمط واحد والاخرى كبيرة ضمت خمسة انماط وراثية .

اعلى قيمة للبعد الوراثي بلغت 0.84 بين Delicata وStraight neck بينما اقل بعد وراثي0.12 فكان بين Elite وFord hook .

انماط الترحيل الكهربائي لبروتين البذور الكلي اظهرت مستوى واطئ من تعدد الاشكال polymorphism من خلال انتاج اربع حزم متماثلة من اصل ستة حزم رئيسة. اعطت انماط البروتين بصمة محددة لواحد من الانماط الوراثية للقرع.

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اظهرت تقنية RAPD-PCR اعلى مستوى لتعدد الاشكال بلغ 63.6 بينما اعطت تقنية SDS electrophoresis قيمة اقل لتعدد الاشكال بلغت 33.3 . وبذلك تعد واسمات ال RAPD وسيلة ممتازة للدراسات المستقبلية لتحديد البصمة الوراثية للانماط الوراثية للقرع لاسيما باستعمال البوادئ OPA-03, OPC-19 .

الكلمات المفتاحية : القرع الصيفي (الشجر), واسمات التفاعل التضاعفي العشوائي المتعدد الأشكال لسلسلة الدنا,التحليل العنقودي ,البصمة, انماط البروتين .