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Genetic Diversity in Egyptian Snake Melon Accessions as Revealed by Inter Simple Sequence Repeat (ISSR) Markers

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ABSTRACT

Snake melon (*Cucumis melo* var. *flexuosus*) is native to the region from Egypt to Iran; therefore, local genotypes in Egypt may contain genes responsible for tolerance to biotic and abiotic stresses. Inter simple sequence repeat (ISSR) markers were used to examine genetic relations among 12 Egyptian snake melon accessions collected from different regions. Of the 10 markers tested, 6 produced 68 amplification products, of which, 23 were polymorphic (33.7% polymorphism). The average polymorphism information content (PIC) value was 0.68 over the 6 primers. Pairwise Jaccord's similarity coefficient ranged from 0.88 to 0.98, indicating a narrow diversity. Cluster analysis distinguished two clusters, one from Cairo, and the second represents the rest of accessions from different regions. It was shown that snake melon genotypes that were genetically close were not necessarily close in geographical distance. On a level of non-coding region using 6 ISSR primers, some differences were found among the Egyptian snake melon accessions, confirming the usefulness of the technique in detecting genetic diversity which may help in future genetic improvement programs in this underestimated crop.

Keywords: *Cucumis melo* var. *flexuosus*, ISSR, polymorphism, cluster analysis, genetic diversity.

INTRODUCTION

Snake melon *(Cucumis melo* var. *flexuosus*) (2n=2x=24) belongs to the family *Cucurbitaceae*. It was shown by Robinson and Decker-Walters (1997) that wild ancestors of melon seem to have been native to the region from Egypt to Iran and Northwest India. Melon can be divided into two subspecies as *C.melo* ssp. Agrestis and *C. melo* ssp. *melo. Cucumis melo* is also divided into six groups consist of *cantalopensis, inodorus, flexuosus, conomon, dudaiumchito and momordica.*

Vegetable production statics (Ministry of Agriculture, Egypt, 2016) revealed that the total cultivated area of snake melon was about 5700 feddans (feddan= 4200 m^2), with total production of 51186 tons. The production of snake melon represents only 0.22% of the total vegetable area in Egypt, and 0.26% of the total vegetable production. These data indicate that snake melon production is too small to meet the demands of the increasing population in Egypt (>100 million), and could be considered a threatened species due to the limited research efforts on its production and improvement. In addition, the cultivated area is scattered from the North to the Middle and Upper Egypt, with large phenotypic variations, mostly in fruit shape and colour.

Useful data for genetic studies have been relied mostly on morphological characters (Ali-Shtayeh *et al.*, 2017). However, this method has its limitation in the number of phenotypic features which can be affected by environmental factors (Al-Anbari *et al.*, 2015). The extent of genetic diversity among genotypes is important since hybridization between groups with maximum genetic divergence would be more responsible for their progenies (Meena and Bahadur, 2015).

Molecular markers are generally recognized as a reliable means for genetic identification among plant genotypes (Meng *et al.*, 2010). Inter simple sequence repeat (ISSR) markers is a method that combines most of the advantages of Simple sequence repeats (SSR) and Amplified fragment length polymorphism (AFLP) to Random amplification of polymorphic DNA (RAPD) (Henarch *et al.*, 2016). This system has a role for analysing genetic diversity by classification of genotypes. PCR techniques, such as ISSR analysis, can quickly identify varieties using materials from seeds to young leaves (Latha and Makari, 2011). In addition, the ISSR technique uses long primers (15-30 b p) which permit the subsequent use of high annealing temperature, leading to high reproducibility. This technique also allows the examination of genetic variation without prior knowledge of the DNA sequence (Zietkiewicz *et al.*, 1994).

The use of molecular markers can facilitate snake melon breeding by means of marker-assisted selection to improve agronomical important traits. The genetic diversity among snake melon accessions and landraces were previously studied in different countries using different molecular markers, *viz*; RAPD-PCR (Soltani *et al.*, 2010; Ismail, 2012; Abdel-Ghani and Mahadeen, 2014); SSR (Solmaz *et al.*, 2016), both RAPD and ISSR (Mallah, 2014), or ISSR (Dastranji *et al.*, 2017).

In Egypt, there are several different snake melon genotypes which show different fruiting characteristics, but data on genetic relationships among them are lacking. To assist in potential selection for future

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breeding programs, the aims of the current study was conducted to examine the genetic diversity and relationships among 12 snake melon accessions, collected from different regions in Egypt, using ISSR markers.

MATERIALS AND METHODS

Plant Materials

Seeds of 12 snake melon accessions (acc.) collected from different regions in Egypt were grown in a greenhouse until the emergence of seedlings. The 12 accessions include acc. No.3 (Damietta), acc. No. 5 (Cairo), acc. No. 7 (Bany Swif), acc. No. 8 (Fayoum), acc. No. 9 (Giza), acc. No. 11 (Menia), acc. No. 14 (Sohag), acc. No. 15 (Behaira, Wady Natron), acc. No. 17 (Ismailia), acc. No. 18 (Behaira, Badr), acc. No. 19 (Sharkia, Menia Al-kamh), and acc. No. 23 (Sharkia, Abo-Hamaad). Young leaf samples were collected from each genotype at 2-3 true leaf stage and directly frozen in liquid nitrogen.

DNA isolation

Extraction and purification of genomic DNA

High quality DNA is an essential requirement for developing DNA markers. DNA was extracted from leaves of the 12 accessions by DNeasy Plant Mini Kit (Qiagen Santa Clarita, CA), following the manufacturer's instruction as follows: Fresh young leaves (0.5 mg) were grinded in liquid nitrogen to a fine powder using a mortar and pestle. The tissue powder was transferred to 1.5 ml micro centrifuge tube after allowing the liquid nitrogen to evaporate. A volume of 600 µl of buffer AP1 and 2 µl of RNase A stock solution (100mg / ml) were added to a maximum of 0.15 g of ground plant tissue and vortexes vigorously. The mixture was incubated for 30-40 min at 65 ºC and mixed about 5 times during incubation by inverting tube. Buffer P3 190 µl was added to the lysate mixed and incubated for 10 min on ice. The lysate was applied to the QIA shredder mini spin column, placed in a 2 ml collection tube and centrifuged at 4 ºC for 5 min at 14000 rpm. Flow-through fraction from step 4 was transferred to a new tube without disturbing the cell-debris pellet. Buffer AW1/E (1.5 volumes) was added to the cleared lysate and mixed by inversion. A volume of 650 µl of the mixture from step 6, were applied to the DNeasy mini spin column sitting in a 2 ml collection tube then centrifuged for 1 min at 10000 rpm and flow-through was discarded. Step 7 was repeated with remaining sample. Both, the flow-through and collection tube were discarded. The DNeasy mini spin column was placed in a new 2 ml collection tube, 600 µl buffer AW2 was added to the DNeasy mini spin column and centrifuged for 3 min at 10 000 rpm. The flow-through was discarding. Step 9 was repeated twice. The DNeasy mini spin column was centrifuged for 5 min at 14000 rpm to dry the membrane. The DNeasy mini spin column was transferred to new 1.5 ml micro centrifuge tube and 100 µl of pre-heated (65ºC) AE buffer was added directly onto the DNeasy membrane. The micro centrifuge tube and then centrifuged for 5 min at 12000 rpm to elute. Step 11 was repeated once in another 1.5ml micro centrifuge tube to obtain the second elution.

Estimation of DNA concentration

DNA concentration was determined by diluting the DNA 1:5 in dH_2O. The DNA samples were electrophoresed in 1% agarose gel against 0.5ug of a DNA size marker. The concentration of genomic DNA was estimated by comparing the size and intensity of each sample band with those of a sizing standard marker (1Kb DNA plus ladder). This marker covers a range of DNA fragments size between 20000 bp and 75bp, and a range of concentration between 80 ng and 20 ng. The DNA samples were diluted to a concentration 10 ng/µl by Tris-EDTA (TE) buffer to be used as a working solution in molecular marker analysis (Smith *et al.*, 1989).

ISSR analysis

The genetic diversity of 12 snake melon accessions collected from different regions in Egypt was studied using 10 ISSR primers This was performed as described by Adawy *et al.* (2002). Out of all tested ISSR primers, only 6 primers (Table1) generated good, reproducible and scorable patterns, while other primers produced smears or fuzzy patterns that could not be scored. The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM Deoxy ribonucleotide triphosphates (dNTPs), 1 µM oligonucleotide primer, 25 ng genomic DNA and 1 unit of *Thermobacillus aquaticus (Taq)* DNA polymerase (Promega, USA). PCR amplification was performed in a Perkin-Elmer/Gene Amp PCR System 9700 (PE Applied Bio systems) programmed to fulfil 35 cycles after an initial denaturation cycle for 5 min at 94 ºC. Each cycle consisted of a denaturation step at 94 ºC for 1 min, an annealing step for each primer 1 min, and an elongation step at 72 ºC for 1.5 min. The primer extension segment was extended to 7 min at 72 ºC in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts, visualized, and documented using a gel documentation and image analysis system.

Data Analysis

Bands were scored as present (1) or absent (0), among the accessions and then data were used to generate a pair-wise similarity matrix using Jaccard's coefficient (Jaccard, 1908). The un-weighted pair-group method using the arithmetic average UPGMA was employed to create the clustering dendrograms using the NTSYS-PC software (Rohlf, 1998). Polymorphism information content (PIC) for molecular values was calculated according to Smith *et al.*, (1997), using the algorithm for all primer combinations as follows:

$$PIC=1-\sum^{n} pij^2$$

Where PIC is the polymorphism information content of marker i, pij the frequency of the j^{th} pattern for marker i, and the summation extends over n patterns.

Table (1): Sequence of the six primers that generated polymorphic products.

Primer	Sequence
ISSR- 1	5'-AGAGAGAGAGAGAGAGYC-3'
ISSR- 2	5'-AGAGAGAGAGAGAGAGYG-3'
ISSR- 3	5'-ACACACACACACACACYT-3'
ISSR- 4	5'-ACACACACACACACACYG-3'
ISSR- 5	5'-GTGTGTGTGTGTGTGTYG-3'
ISSR- 6	5'-CGCGATAGATAGATAGATA-3'

RESULTS

Of the 10 primers used in this study, 6 primers (Fig. 1) produced reproducible and polymorphic amplification products. Analysis with the 6 ISSR markers identified a total of 68 reproducible fragments in the snake melon accessions under investigation, with an average of 11.3 alleles per locus, among them a total of 23 bands were polymorphic (Table 2). Polymorphism ranged from 13% in ISSR4 to 53% in ISSR 1, and 45% in ISSR5. The 6 primers produce an average of 33.67% polymorphism per primer.

The most number of bands were produced by the two primers ISSR1 and ISSR4 (15), while the lowest numbers of bands were produced by primers ISSR 3 (8) and ISSR 6 (9). Bands molecular weight (MW) ranged from 207 to 1061 bp for ISSR1; 184 to 830 bp for ISSR2; 180 to 632 for ISSR3, and from 338 to 925 bp for primer ISSR6 (Table 2).

The highest band frequency was recorded by primers ISSR2 and ISSR4 (0.9), followed by primers ISSR3 and ISSR6 (band frequency= 0.8), while primers ISSR1 and ISSR5 produced lower band frequency (0.6 and 0.7, respectively) as shown in (Table 2). Polymorphism information content (PIC) indicates a high level of distinction for a primer on a genotype. In this respect, primer ISSR1 produced a high PIC value (0.86), while ISSR5 recorded the lowest PIC (0.55), with an average PIC value of 0.68 over the 6 primers (Table 2). Among the 6 primers, only ISSR1 and ISSR5 produced unique bands (4 and 2, respectively).

Analysis of the similarity matrix indicated genetic distance among the examined snake melon accessions, ranged from 0.88 to 0.98 (data not shown). The highest genetic distance was detected between acc. No. 7 and acc. No. 8 (0.98), and acc. No. 11 and No. 15 (0.97). The lowest similarity matrix (0.88) was found between acc. No. 5 and No. 15; acc. No. 5 and No. 19 and acc. No. 9 and No. 23.

Cluster analysis by UPGMA was done for the 12 Egyptian snake melon accessions (Fig. 2). This analysis grouped the 12 accessions into 2 main clusters. The first include only acc. No. 5 from Cairo, while the second cluster was divided into 2 groups. The first group included acc. No. 19 from Sharkia (ManiaAl-kamh) and acc. No. 23 from Sharkia (Abo-Hamaad), while group 2 included acc. No. 14 from Sohage and the rest of accessions were in separate group. This included two sub-groups (acc. No. 18 from Behaira (Badr) and No. 9 from Giza), while other subgroup branched into two branches. The first included the two accessions (acc. No. 11 from Mania and No. 15 from Behaira (Wady one branch and acc. No. 3 (Damietta), along with the two accessions (No. 7 from Bany Swif and No. 8 from Fayoum).

DISCUSSION

In the present study, 6 out of 10 ISSR primers were chosen as suitable markers to explore possible differences among 12 snake melon genotypes selected from different regions in Egypt on a level of non-coding regions. The 6 primers yielded a total of 68 scorable bands with an average of 11.3 alleles, a result close to those obtained by Dastranji *et al.,* (2017) with 16 Iranian snake melon accessions using ISSR analysis. However, other researchers obtained lower number of alleles using SSR techniques in several melon genotypes (Yildiz *et al*., 2014; Kacar *et al.,* 2012; Mon forte *et al.,* 2003; and Solmaz *et al.,* 2016 on Turkish snake melon). Of the resulting 68 fragments, 23 were polymorphic (with average 33.6% polymorphism). In our study, this value of polymorphism is far lower than that obtained among Iranian accession (82.95%) as reported by Dastranji *et al.,* (2017) and within Turkish accessions (100%) according to Solmaz *et al.,* (2016). However, Raghami *et al.,* (2014) found low variability within Iranian melon accessions, suggesting the lack of inter-crossing between accessions, or a high rate of self-pollination. High polymorphism was found between Meanwhile, studies done on Tunisian melon and snake melon recorded variation (91.6%) either using SSR markers (Henane*et al.,* 2015) or using RAPD markers (Soltani *et al.,* 2010).

Regarding to the genetic parameters of the ISSR markers, results showed that PIC value ranged from 0.55 (ISSR 3) to 0.86 (ISSR1) with an average of o.68. Similar to our finding, Henane *et al*., (2015), reported a PIC range of 0.43 to 0.92, close to those obtained by Solmaz*et al.,* (2016) (0.57 to 0.9) when using SSR markers. However, Dastranji *et al*., (2017), using ISSR markers, found a mean PIC value of 0.34 in Iranian snake melon accessions, with high degree of polymorphism among them.

Results of the similarity matrices according to Dice coefficient indicated genetic similarity degree ranged from 0.88 to 0.98 among all possible pairs of accessions (ave.92), which indicated a narrow diversity among the studied snake melon genotypes, perhaps due to the dominant nature of the ISSR markers. This was in agreement with the finding of Singh *et al*., (2015) on snap melon. High degree of similarity was found among most accessions, which means that these accessions are genetically related, despite of the wide distance between the regions from which they were collected. However, lower degree of similarity (0.86) was recorded between acc. No.5 (middle Delta) and acc. No.7 and No.8 (from Upper Egypt), indicating that they could genetically be diverse. In accordance with

our results, Dastranji *et al.*, (2017) found two snake melon accessions genetically very similar, despite the high geographical distance between them, which may be attributed to the movement of seeds between distance regions. In other direction, results of cluster analysis indicated that some accessions in a certain cluster were close to each other geographically (i.e., acc. No.7 from Bany Swif, acc. No.8 from Fayoum; acc. No.19, and No.23 from Sharkia). Therefore, grouping of accessions based on molecular markers (ISSR) was not consistent with the geographical distribution or genetic distances, and accessions that were genetically close together were not necessarily with the study of Dastranji *et al.*, (2017).

In conclusion, on a level of noncoding regions using six ISSR markers, some differences have been recorded among Egyptian snake melon genotypes. These result are confirming the usefulness of this technique for detecting genetic diversity among the studied snake melon accessions, which may help in future genetic improvement and breeding programs in snake melon.

Table (2): Genetic features of the 6 ISSR markers used in the study.

Marker	Amplified Bands (No.)	Polymorphic bands (No.)	Polymorphism (%)	Mean band frequency	PIC %	Major allele frequency
ISSR1	15	8	53	0.6	0.86	0.17
ISSR2	10	2	20	0.9	0.65	0.33
ISSR3	8	3	38	0.8	0.55	0.58
ISSR4	15	2	13	0.9	0.58	0.42
ISSR5	11	5	45	0.7	0.78	0.25
ISSR6	9	3	33	0.8	0.68	0.42
Total	68	23	Ave.=33.67		Ave.= 0.68	0.36

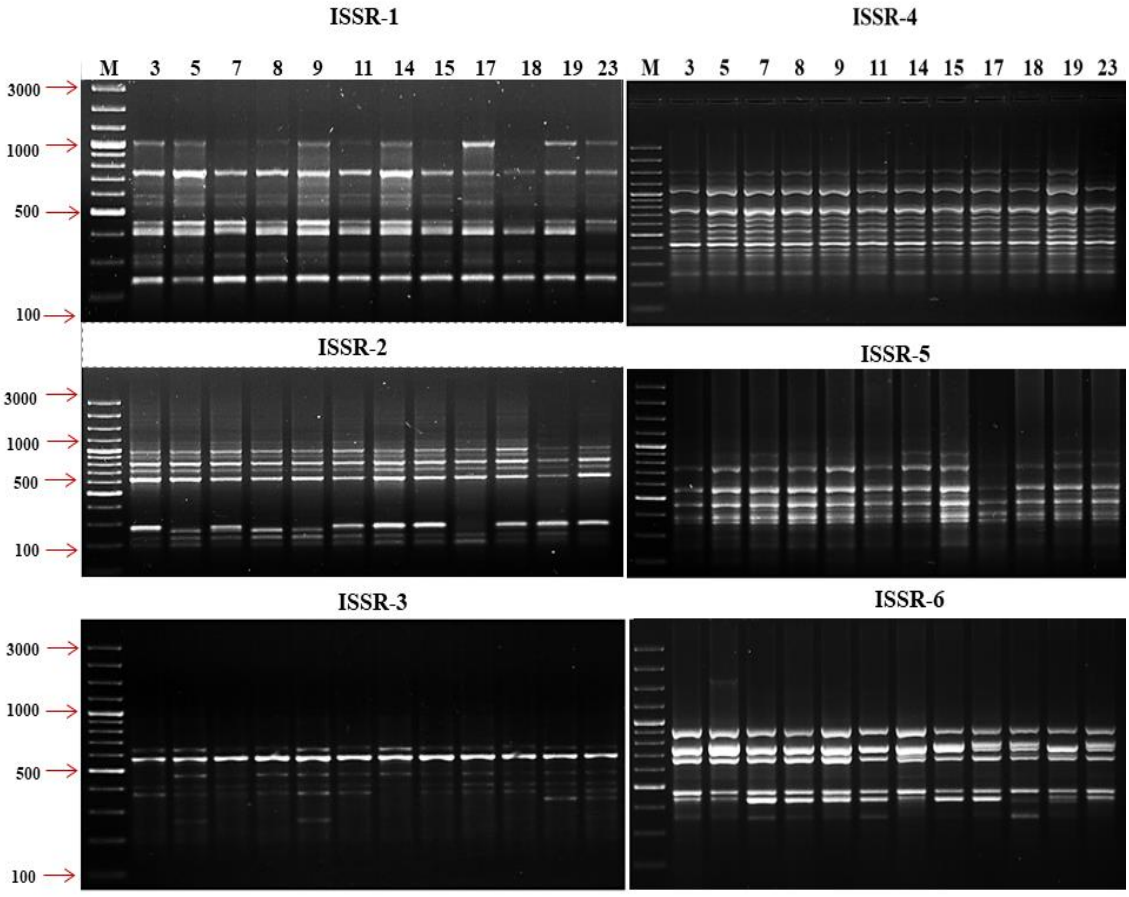

Figure (1): Agarose gel image showing amplification profiles generated by ISSR primers (1-6) with 12 snake melon accessions; lanes 1-12 correspond to the snake melon acessions (3-23); lane M is 1Kb DNA marker.

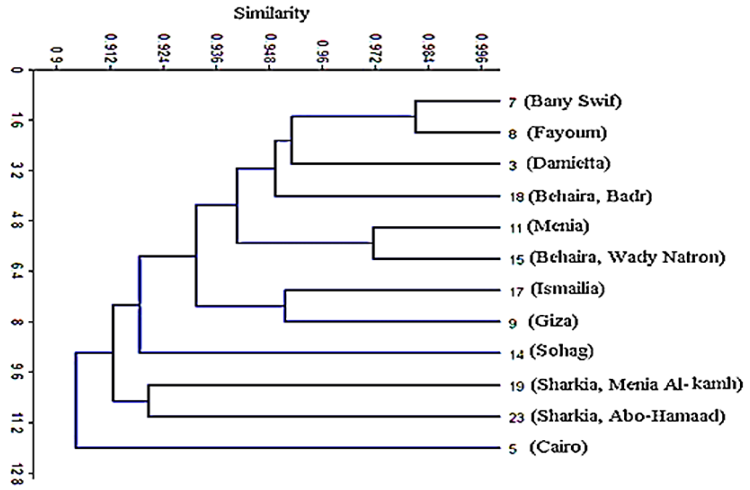

Figure 2: Cluster analysis of 12 Egyptian snake melon accessions using ISSR data. Accession regions are shown in bracts for each accession number.

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التنوع الوراثي في القثاء المصري الذي تم الكشف عنه بواسطة علامات تكرار التسلسل البسيط (ISSR)

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الملخص العربي

يعود الموطن الأصلي لنبات القثاء (*Cucumis me*lo var. *flexuosus*) للمنطقة الممتدة من مصر إلى إيران؛ لذلك قد تحتوي التراكيب الوراثية المحلية في مصر على جينات قد تكون مسؤولة عن تحمل الإجهادات الحيوية وغير الحيوية. ولذلك اهتمت هذه الدراسة باستخدام الدلائل الوراثية من نوع ISSR لفحص القرابة الوراثية بين 12 سلالة محلية من القثاء تم جمعها من مناطق مختلفة من مصر. من بين 10 واسمات Markers التي تم اختبارها، أنتجت 6 منها 23 منتج متعدد الأشكال Polymorphic (بنسبة 33,7٪ Polymorphism). وكان متوسط محتوى معلومات تعدد الأشكال (PIC) بقيمة 0,68 عبر الواسمات ال 6. تراوح معامل تشابه Jaccord بين 0.88 و 0.98، مما يشير إلى تنوع ضيق. ميز التحليل العنقودي مجموعتين، واحدة تحتوي السلالة رقم (5) من القاهرة، والمجموعة الثانية تمثل بقية السلالات من المناطق المختلفة. وقد تبين أن التراكيب الوراثية للقثاء ذات القرابة الجينية ليست بالضرورة قريبة من الناحية الجغرافية. على مستوى المناطق غير المشفرة باستخدام 6 بادءات ISSR , تم العثور على بعض الاختلافات بين سلالات القثاء المصرية، مما يؤكد فائدة هذه التقنية في اكتشاف التنوع الوراثي الذي قد يساعد في برامج التحسين الوراثي المستقبلية لهذا المحصول.