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Molecular Characterization of Some Syrian Pomegranate (*Punica granatum* L.) Genotypes

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ABSTRACT

Considering the high level of morphological diversity in Syrian pomegranate (*Punica granatum* L.) genotypes, this study was conducted to evaluate the genetic variation among five pomegranate genotypes using twenty inter simple sequence repeat (ISSR) markers. Twelve ISSR primers were successfully used as fingerprinting tools and amplified 137 DNA fragments out of which 78 were polymorphic (56.93%). Primers (17, NLSSR3, 16) showed the highest polymorphism percentage (90.41, 80.00, 76.47%, respectively) with the highest number of unique bands (6, 6, 9 respectively). Genotype C amplified the highest number of DNA fragments (115) and unique bands (13). Polymorphism information content (PIC) ranged from 0.449 to 0.768, while genetic diversity (GD) ranged from 0.56 to 0.80. The genetic distance ranged from 0.26 to 0.37. Some genotypes showed wide divergence (C and A; C and D), while genotypes A and B were closely related. The unweighted pair-grouped method with arithmetic average (UPGMA)dendrogram grouped the genotypes into two clusters. These results indicated that the ISSR technique was sufficiently informative and powerful to assess genetic variability in pomegranate.

Keywords: Pomegranate, Syrian genotypes, genetic diversity, ISSR.

INTRODUCTION

The pomegranate is one of the oldest known edible fruits. Its history dates back to very ancient times. Botanically, pomegranate belongs to the family *Punicaceae* which has a single genus *Punica* and two species; *P. Protopunica* Balf and *P. granatum* L. with 2n = 2x= 16 or 18 (Mars, 2000).

Pomegranate is thought to be indigenous to the region of Iran where it is native (Stover & Mercure, 2007) and it is also thought to be native to Turkey (Ercisli et al., 2007). It is now widely cultivated in the Mediterranean, tropical, and subtropical areas (Mars, 2000). Pomegranate is considered an excellent tree for growing in arid zones for its resistance to drought conditions. In addition, the tree is also cultivated for its pharmaceutical and ornamental usages (Ismail et al., 2014).

The edible fruit is a berry which is about 5-12 cm in diameter with a rounded hexagonal shape, thick reddish skin, and around 600 seeds, each surrounded by a water-laden pulp (aril) ranging in color from white to deep red or purple, the aril is the edible part of the fruit (Stover & Mercure, 2007). Pomegranate contains some species of flavonoids and anthocyanidins (delphinidin, cyaniding,

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and pelargonidin) in its seed oil and juice and has shown antioxidant activity three times greater than tea extract (Seeram et al., 2006).

Genetic diversity at morphological, as well as biochemical levels, has limitations due to the influence of the environment on the performance of genotypes (Awamleh et al., 2009). DNA-based molecular markers provide a good and informative approach to estimating the genetic diversity and genetic relationships of horticultural plants. They are abundant, highly polymorphic, and independent of environment or tissue type. Consequently, DNA-based techniques such as Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) markers are effective in assessing genetic diversity among the cultivars, because they provide unlimited potential markers to reveal differences at the molecular level (Shrikent, 2015).

In recent years, an increasing number of works have been focusing on molecular characterization and genetic diversity assessment of *Punica granatum* by using different classes of markers such as Amplified Fragment Polymorphism (AFLP) (Moslemi et al., 2010), Restriction Fragment Length Polymorphism (RFLP) (Melgarejo et al., 2009), Random Amplified Polymorphic DNA (RAPD) (Singh et al., 2013), SSR (Jbir et al., 2010; Hasnaoui et al., 2012), and ISSR (Bedaf et al., 2011; Ismail et al., 2014).

High genetic diversity is expected to occur in pomegranate genotypes cultivated in Syria due to long historical cultivation and different environmental conditions in which, these cultivars are growing. Most of the pomegranate trees are grown in the governorates of Aleppo (40%) and Idlib (25%0) (Syrian Statistic Group, 2010), which have been threatened and out of control. To avoid the loss of its genetic resources, which have been evolved over thousands of years, genetic material available in Syria must be collected, identifie,d and described to establish a starting point for further studies. Some studies based on morphometric and phytochemical criteria have recently been performed to determine the degree of polymorphism within local material, but very little work have been done on molecular characterization of the pomegranate genotypes in Syria. Therefore, our study is concerned with the identification of genetic diversity in some pomegranate genotypes growing in Syria using the ISSR marker technique, as these data may be of further use in hybridization and selection programs.

Material and Methods

This study was conducted during 2016-2018 at Molecular Genetics Laboratory, Department of Biotechnology, General Commission for Scientific Agricultural Research, Damascus, Syria.

Plant material: Five Syrian pomegranate genotypes (A, B, C, D, E) described in Table (1) were collected from the Tartus governorate in Syria. Fully-grown fresh leaves free of apparent pest and disease from each genotype were washed with distilled water, dried, and kept in labeled falcon tubes at (-80°C).

Table1. Name and morphological characterization of studied genotypes.

Code	Characterization
A	The peel of the fruit is the darkest red in color, the juice is red and had a sweet-sour taste
B	The peel of the fruit is red in color, the juice is red and had a sweet-sour taste
C	The peel of the fruit is pinkish-white in color, the juice is pinkish and had a sweet-sour taste
D	The peel of the fruit is pinkish-red in color, the juice is pinkish and had a sweet taste
E	The peel of the fruit is pinkish-white in color, the juice is pinkish-white and had a sour taste

Isolation and detection of genomic DNA: For each genotype, genomic DNA was isolated from the collected leaves using the cetyl trimethyl ammonium bromide (CTAB) based method described by Awamleh et al.

(2009). The DNA was then kept at -80°C.

DNA quality and integrity were checked on 1% agarose gel stained with Red-Gel® dye solution and spectrophotometer. The purity DNA was estimated by calculating the ratio of absorbance at 260 and 280 nm, with a value of approximately 1.8-2.0 indicating a good quality DNA. All genomic DNA samples were uniformed to a final concentration of 50 ng.μl^{-1} and used for PCR amplification reactions.

DNA amplification and visualization by ISSR analysis: Molecular polymorphism was assessed by a set of 20 ISSR primers described in Table (2). These primers were synthesized by Vivantis Corporation. Malaysia.

The amplification reaction was carried out separately for each primer in a total reaction volume of 25µl containing 2X ready master mix (Vivantis Corporation), 20 pM oligonucleotide primer, and 100 ng of template DNA. PCR reaction was performed on Eppendorf Mastercycler according to the following protocol: initial denaturation of the template DNA at 94°C for 5 min, followed by 37 cycles of 1 min denaturation at 94°C, 1 min annealing at (Ta) according to the primer (Table, 2), and 1.30 min extension at 72°C, and finally followed by 10 min of additional extension at 72°C. Then, amplification products were visualized by horizontal electrophoresis in 2% agarose gel and photographed by a gel documentation system (Cleaver, England). A 100 bp ladder (Fermentase, Germany) was used.

Table 2. Name, sequence, and annealing temperature (Ta) of the primers used in ISSR analysis.

No.	Primer Name	Sequence	Ta
1	4	CAC ACA CAC ACA CAC AAC	48
2	A830241	ACT GAC TGA CTG ACT GAC TG	44
3	813	CTC TCT CTC TCT CTC TT	50
4	807	AGA GAG AGA GAG AGA GT	50
5	8565	GTC (ACC)6 AC	64
6	866	(CTC)6	53
7	W814	CTC TCT CTC TCT CTC TTG	44
8	8	CAC ACA CAC ACA CAC AGA C	49
9	862	(AGC)6	53
10	17899B	CAC ACA CAC ACA GG	44
11	231	GAG TCT CTC TCT CTC TCT C	51
12	8082	CTC TCT CTC TCT CTC TCT G	50
13	NLSSR3	(CAG)5	54
14	17	CAG CAC ACA CAC ACA CAC	51
15	5	CAC ACA CAC ACA CAC AGT	48
16	830	TGT GTG TGT GTG TGT GG	45
17	811	GAG AGA GAG AGA GAG AC	44
18	812	GAG AGA GAG AGA GAG AA	49
19	8564	(CAC)7 C	48

20	16	CGT CAC ACA CAC ACA CAC	49

Data analysis: ISSR bands were scored in a 0-1 binary format (1 for present, 0 for absent). The molecular results were analyzed using the Total Lab 1D software. The Polymorphism Information Content (PIC) and Genetic Diversity (GD) for each primer were calculated using the Power Marker software program.

Cluster analysis and building of the dendrogram were performed by using XLSTAT software, based on the UPGMA (unweighted pair group method with arithmetical averages) algorithm.

Results

A set of twenty ISSR primers were used to estimate the genetic variability among five pomegranate genotypes. Only twelve primers were amplified successfully (Fig, 1).

A total of 137 amplified DNA bands, with an average frequency of 11.42 bands per primer were obtained. The total number of scorable bands produced per individual primer ranged from 5 in primer (5) to 17 in primer (16). The size of the amplified band ranged from 65 bp in (807) to 1568 bp in (16) (Table, 3).

Seventy-eight bands were polymorphic (56.93%) with an average of 6.5 bands/ primer, while 59 bands (43.07%) were monomorphic with an average of 4.92 bands/ primer. The average percentage of polymorphism obtained on ISSR markers was 53.66% ranging from 90.91% in primer (17) to 22.22% in primer (17899B). On the other hand, primer (231) did not show any unique bands as all amplification bands were monomorphic (Table, 3).

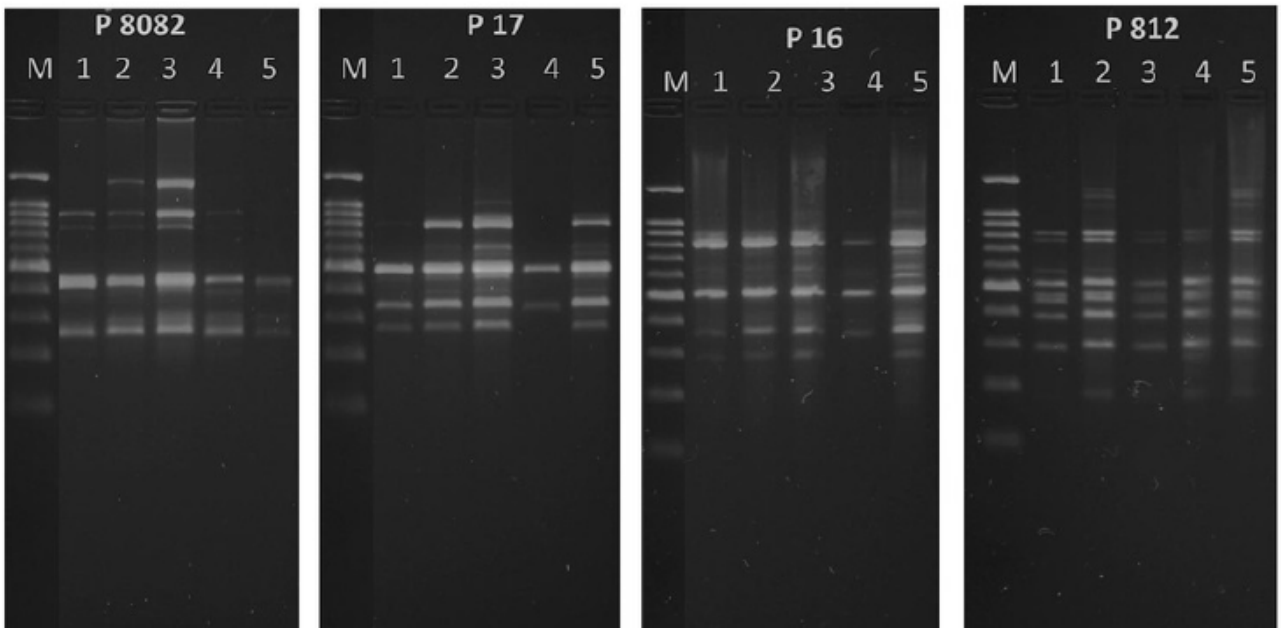

Fig 1. ISSR profile of the five pomegranate genotypes amplified with the ISSR primers (8082, 17, 16, 812); M: 100 bp Iadder marker, Lane 1 through 5 refer to pomegranate genotypes: A, B, C, D, E, F.

As we can see in Table (3), the PIC values obtained ranged from 0.499 in primers (17899B, 8082, 5) to 0.768 in primers (807, 8. NLSSR3, 17, 812, 16) with mean value (0.62). The GD values obtained ranged from 0.56 in

primers (17899B, 8082, 5) to 0 80 in primers (807, 8, NLSSR3, 17, 812, 16) with mean value (0.66).

Table 3. Statistics of the ISSR fragments for five Syrian pomegranate genotypes.

Primer	MW size (bp)	AF	MB	PB	P%	GD	PIC
807	64.72-838.03	14	3	11	78.57	0.80	0.768
8	237.43-1484.51	10	6	4	40.00	0.80	0.768
17899B	249.75-1321.20	9	7	2	22.22	0.56	0.499
231	273.80-1061.69	11	11	0	0.00	0.00	0.000
8082	254.26-1370.88	7	4	3	42.86	0.56	0.499
NLSSR3	251.58-1109.64	15	3	12	80.00	0.80	0.768
17	273.03-1148.72	11	1	10	90.91	0.80	0.768
5	429.26-796.88	5	2	3	60.00	0.56	0.499
811	262.26-1500	15	6	9	60.00	0.72	0.672
812	176-1300	14	8	6	42.86	0.80	0.768
8564	327.38-1072.48	8	4	4	50.00	0.72	0.672
16	291.40-1567.57	17	4	13	76.47	0.80	0.768
Total		**137**	**59**	**78**			
Average	**64.72-1567.57**	**11.42**	**4.92**	**6.50**	**53.66**	**0.66**	**0.62**

AF: amplification fragments, MB: monomorphic bands, PB: polymorphic bands, P: Polymorphism, GD: genetic diversity, PIC: polymorphism information content.

The highest number of amplicons were generated from genotype C (115), while genotypes A and D generated the lowest number of amplicons (85 and 84, respectively). The highest number of unique bands (13) was scored for the C genotype, while genotype B scored the lowest number of unique bands (2).

Table 4. Number of amplified fragments and unique bands generated from five Syrian pomegranate genotypes based on ISSR analysis

Primer Name	A		B		C		D		E		TUB
	AF	UB	AF	UB	AF	UB	AF	UB	AF	UB	
807	5	1	10	0	13	2	8	0	5	1	4
8	7	0	6	0	10	1	7	0	9	0	1
17899B	7	0	7	0	8	1	7	0	8	1	2
231	11	0	11	0	11	0	11	0	11	0	0
8082	6	0	7	0	7	0	7	0	4	2	2
NLSSR3	11	0	7	0	10	5	9	1	11	0	6
17	6	0	9	0	11	1	2	4	7	1	6

Primer Name	A		B		C		D		E		TUB
	AF	UB	AF	UB	AF	UB	AF	UB	AF	UB	
5	4	2	3	1	4	0	4	0	4	0	3
811	7	0	9	1	13	2	10	0	10	0	3
812	9	0	13	0	8	0	9	1	12	0	1
8564	5	1	7	0	8	1	5	1	7	0	3
16	7	1	9	0	12	0	5	3	17	5	9
Total	**85**	**5**	**98**	**2**	**115**	**13**	**84**	**10**	**105**	**10**	**40**

AF: amplification fragments, UB: unique band (either present or absent), TUB: total number of unique bands, **A through E: Pomegranate genotypes.**

Primer (16) scored the highest number of unique bands (9), primers (8 and 812) scored the lowest number of unique bands (1), while primer (231) did not show any unique band (Table, 4).

As can be seen in Table (5), a total of 40 amplicons were unique bands, in which 19 of them were scored for the presence of a unique band for the five genotypes (positive marker), while 21 were scored for the absence of a common band (negative marker). The highest number of positive markers (10) were registered in genotype C, while the lowest number of negative markers (9) were registered in genotype D.

Table 5. Genotype unique-bands resulting from ISSR analysis

Pomegranate genotype	Positive bands (presence)	Negative bands (absence)	Total
A	1	4	5
B	1	1	2
C	10	3	13
D	1	9	10
E	6	4	10
Total	**19**	**21**	**40**

The dissimilarity matrix of pomegranate genotype based on ISSR analysis

According to the results of ISSR amplification, as can be seen in Figure (2), the genetic distance ranged from 0.26 to 0.37 with a mean value of 0.328 The lowest genetic distance (0.26) was observed between genotypes A and B, suggesting their close relatedness. Whereas genotypes D and C, and genotypes C and A seemed to be the most divergent since they had exhibited the highest genetic distance (0.37)

Cluster analysis as revealed by ISSR

ISSR dendrogram obtained from UPGMA cluster analysis of genetic dissimilarity is presented in Fig (2). Two main clusters could be identified separating at 0.34 genetic distance.

The first cluster is made up of two genotypes: C and E at 0.31 genetic distance. The remaining genotypes were divided into two sub-clusters: the first sub-cluster is made up of genotype D at 0.33 genetic distance and the second sub-cluster is made up of genotypes A and B at 0.26 genetic distance.

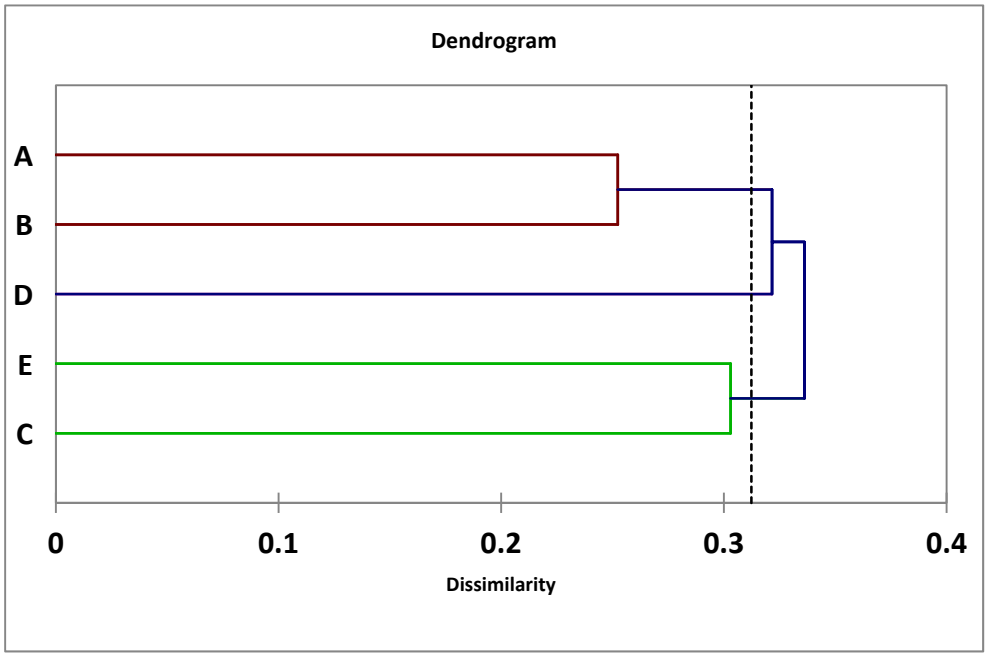

Fig 2. Cluster analysis as revealed by ISSR data for five pomegranate genotypes collected from Tartus governorate

Discussion:

The success of any genetic conservation or breeding program depends on understanding the amount and distribution of the genetic variation present in the genetic pool (Awamleh et al., 2009). Morphological traits were used to describe such genetic variation in pomegranate varieties.

Inter Simple Sequence Repeat ISSR is a class of molecular markers based on inter-tandem repeats of short DNA sequences.

Our data portrayed the advantages of the ISSR method in the exploration of pomegranate polymorphism at the DNA level. This is strongly supported by recent works dealing with the usefulness of ISSR to investigate pomegranate diversity (Ismail et al., 2014, Zahra et al., 2012: Zhao et al., 2011). ISSR has been used in discriminating wild species as well as cultivars and have been introduced as a useful tool in several DNA marker studies Nazary et al. (2009) described genetic diversity across natural populations of Indian pomegranate based on ISSR markers, Bedaf et al. (2011) suggested that the ISSR markers produced much better reproducible bands and were efficient in grouping Iranian pomegranate cultivars comparing to RAPD markers.

The high percentage of polymorphic markers, obtained from ISSR primers in this study, indicated that pomegranate is a highly polymorphic fruit tree which agrees with other works done by Awamleh et al., (2009) and Zhao et al., (2011).

Primers (17, NILSSR3, 16) showed the highest polymorphism percentage (90.41, 80.00, 76.47%, respectively) with the highest number of unique bands (6, 6, 9 respectively). These primers are recommended to be used for the identification of pomegranate genotypes with less time and cost. These primers are based on (CA)n and (CAG), repeats. This result is closely related to those of Ismail et al. (2014) who found that primers with (CA)n repeat produced the highest number of amplification fragments, while this disagrees with the findings of Wang et al. (1994) who reported that (AT)n was the most

abundant microsatellite in plant nuclear genome.

The ISSR based PCR amplification products allowed to distinguish each pomegranate genotype and to study the genetic relationship among them. Some genotypes showed wide divergence (C and A; C and D), and their favorable characters should be taken into consideration in future breeding programs.

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التوصيف الجزيئي لبعض طرز الرمان السورية (*Punica granatum* L.)

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ملخص

أجريت هذه الدراسة لتقييم التنوع الوراثي لخمس طرز وراثية من الرمان (*Punica granatum* L.) باستخدام 20 بادئ لتقنية التكرارات التتابعية البسيطة الداخلية (ISSR) وذلك بسبب المستوى العالي للتنوع المورفولوجي للطرز الوراثية للرمان في سوريا. تم استخدام 12 بادئ ISSR بنجاح لدراسة البصمة الوراثية. بلغ عدد قطع الحامض النووي الكلية الناتجة عن عملية التضاعف 137 قطعة، منها 78 قطعة دلت على التعددية الشكلية (56.93%). أعطى كل من البادئات (17، NLSSR3، 16) أعلى نسبة تعددية شكلية (90.41، 80، 76.47% على التوالي)، بالإضافة لأكبر عدد من القطع الفريدة (6، 6، 9 على التوالي). أعطى الطراز الوراثي C أكبر عدد من القطع (115) قطعة وأكبر عدد من القطع الفريدة (13) قطعة. تراوح محتوى المعلومات التعددية الشكلية (PIC) بين 0.449 و 0.768، بينما تراوحت قيمة التنوع المورثي (GD) بين 0.56 و 0.8. في حين تراوح البعد الوراثي بين 0.26 و 0.37. أظهرت الطرز الوراثية (C و D) و (C و A) تباين وراثي كبير، بينما كان أقرب طرازين لبعضهما البعض هما (A و B). بينت شجرة القرابة الوراثية انقسام الطرز الوراثية إلى مجموعتين باستخدام طريقة الأزواج المتوازنة لحساب المتوسطات (UPGMA). كما بينت النتائج أن استخدام تقنية ISSR ناجحة وفعالة لتقييم التنوع الوراثي في الرمان.

الكلمات الدالة: الرمان، طرز وراثي سورية، تنوع وراثي، تكرارات تتابعية بسيطة داخلية ISSR.

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