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Molecular characterization of some Syrian pears (*Pyrus syriaca* Boiss) genotypes using SSR marker

***Bayan Mohammed Muzher*[1] ✉ *and Ahmed Naguib Sharaf*[2]**

ABSTRACT

The present investigation was carried out to study the genetic variability and the relationship among six Syrian pears; 3 wild type genotypes ($W.T_1$, $W.T_2$, and $W.T_3$) of *pyrus syriaca* Boiss, 3 Syrian *pyrus* cultivars (Meskawi, Romi and Abu- satel), and Liconte cultivar by using fifteen SSR primer-pairs. Fourteen primer pairs of them were able to characterize the studied pear genotypes, they revealed 47 alleles, of which 44 were polymorphic (93.6%) among all genotypes, when focusing on the Syrian pear genotypes only 42 of 45 alleles were polymorphic (93.3%). The number of alleles per primer-pairs ranged from 1 to 6 alleles with an average 3.4. Liconte revealed low genetic relationship with Syrian pear genotypes which indicate that the two groups belong to different species or subspecies. On the other hand, Meskawi cultivar revealed the highest genetic similarity with $W.T_3$ (65%). The dendrogram clustered the seven pear genotypes into two clusters where Liconte and Romi genotypes grouped in the first cluster, and the other genotypes grouped in the second cluster. A total of 12 SSR primer-pairs out of 14 characterized the seven pear genotypes by 20 unique SSR alleles (17 positive and 3 negative). The average of expected heterozygosity (H_e) was 0.263 and the marker index was 11.57. Consequently, the obtained data reflected the ability of SSR marker to identify the genetic variability among the seven pear genotypes.

Keywords: *Pear , Pyrus syriaca* Boiss, SSR, genetic relationship.

INTRODUCTION

The genus *pyrus* belongs to the subfamily *Pomoedeae*of the *Rosaceae.*The basic chromosome number of *pyrus* (X=17) is high compared to other *Rosaceae* subfamilies, suggesting a polyploid origin. Botanical data and chemotaxonomic studies based on Phenolic compounds and isozymes support the allopolyploid theory. The *Pomoedeae* probably arose as an amphidiploid of two ancestors from the *Spiraeoideae* (X=9) and *Prunoideae* (X=8) subfamilies (Chevearau*et al.*,1989).

*PyrusSyriaca*Boiss. is widely distributed in Lebanon, Palestine, Turkey, Iraq, Jordan and Syria (Mouterde, 1966). Many genotypes are distributed in Syria in different altitudes from 200m to 1800m, and in different environmental regions from semi-arid to humid areas which give them a good tolerance for biotic and abiotic stresses (Muzher, 1998).

Biochemical markers such as isozymes have proved to be useful for cultivar identification in pear. The first study of pear isozyme was achieved at 1980 which identified 6 ornamental of *P.calleryana*by peroxidase (Chevereau*et al.,* 1997).

Recently, DNA based markers were developed as a

[1] Ph.D. Researcher in the General Commission for Scientific Agriculture Research (GCSAR) Sweida-Syria.
✉ bmuzher@hotmail.com
[2] Prof. Dr. in Cairo University, Faculty of Agriculture
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second generation of markers to overcome the drawbacks of morphological and biochemical markers. Moreover, molecular markers became an extension and an integral part of classical breeding, contributing successfully to shortening breeding and selection cycles (Altman, 1999). Random amplified polymorphic DNA (RAPD) established by Williams *et al*.(1990) have been used for the cultivar characterization and identification in pear (Banno*et al.,*2000; Ritschel*et al*., 2008;Lisek and Rozpara, 2010). Also, Amplified fragment length polymorphism (AFLP) technique was used to study the genetic diversity of pear (Pan *et al.*, 2002; Shenghua*et al.*, 2002).

Simple sequence repeat (SSR) or microsatellite markers have become the marker of choice for genotype identification (Bassil, 2005). SSRs were implemented for identification, genetic diversity, genetic relationship and for breeding programs among different fruit species like Peach (Ahmed *et al*., 2004), Apricot (Perez *et al*., 2005), Cherry (Ganopoulos *et al*., 2011), Almond (Amirbakhtiar *et al*., 2006), Apple (Muzher *et al.,* 2007; El-Halabi *et al.,*2009) and Pear (Yu-fen *et al*.,2007; Van Dyk *et al.,*2008; Tian *et al.,* 2012). On the other hand, SSR technique was used for diversity studies of cultivated and wild *P. communis* genotypes (Volk *et al*., 2006; Brini *et al.,* 2008), to identify incorrect pear cultivars and cultivars with multiple names to establish credible gene bank to create a resource for breeding, plant nurseries and quality control (Xuan, 2008), identify historic pear trees in U.S. National Parks as an important plan to conserve historical orchards within National Park boundaries (Bassil *et al*., 2008). In addition to study the genetic diversity and relationship among accessions related to deferent pear species (Erfani *et al*., 2012).

The present investigation was carried out to study the genetic relationship between the Syrian wild types and related cultivars based on the genetic distances obtained by SSR molecular marker data and to identify the different pear genotypes by unique DNA markers.

MATERIALS AND METHODS

Plant material:

The study included six Syrian pears ; 3 wild type genotypes ($W.T_1$, $W.T_2$, and $W.T_3$) of *pyrus syriaca* Boiss, 3 Syrian pear cultivars (Meskawi, Romi and Abu Satel), which characterized morphologically, anatomically and biochemically using isozyme analysis by Elshihy *et al*.(2004), in addition to Liconte cultivar which derived from the hybridization between *Pyrus communis* and *Pyrus pyrifolia* (Table 1).

Table 1. List of pear cultivars and wild type genotypes used in the study.

Code number	Cultivars and wild types	Climatic region	End Use
1	Meskawi	South of Syria	Cultivar
2	***W.T1* (P. syriaca *Boiss)***	South of Syria	Seedling rootstock
3	***Abu satel***	South of Syria	Cultivar
4	***W.T2* (P. syriaca *Boiss)***	South of Syria	Seedling rootstock
5	***W.T3* (P. syriaca *Boiss)***	South of Syria	Seedling rootstock
6	Romi	South of Syria	Cultivar
7	Liconte (*P.communis* x *P.pyrifolia)*	North of Egypt	Cultivar

DNA Extraction:

Extraction of total DNA from young green leaves was performed using CTAB protocol according to (Porebski *et al*., 1997). DNA estimation was carried out depending on visual method by electrophoreses the DNA samples in 0.8% agarose gel against 5 µl of a 100 bp DNA ladder (Promega, U.S.A).

Direct amplification of Simple Sequence Repeats (SSR):

Fifteen SSR primer-pairs were used, fourteen SSR primers derived from apple; two SSRs developed in apple depending on apple genomic library designed using PRIME from the GCG package (Guilford *et al.*, 1997), and twelve SSRs were developed using the program primer version 0.5 available from the Whitehead institute for Biomedical Research. Cambridge, Massachusetts, USA (Gianfranceschi *et al*., 1998). In addition, SSR primer pairs (BN) were designed by a repeat- enriched pear genomic library (Table 2).

The PCR reaction was carried out using hot start Taq polymerase (Roche Diagnostics GmbH) in 25 µl reaction volume containing 1X PCR buffer (200mM Tris-HCl, pH= 8.4, 500M KCl). 2 mM of each dNTPs, 10 P mol of forward primer, 10 P mol of reverse primer, 50 ng genomic DNA, 5 x GC solution, and 1 unit hot start Taq DNA polymerase. Hot start and touchdown profile was used as follows: An initial hot start and denaturation step at 95°C for 5 minutes followed by 20 cycles at 95° C for 1 min, annealing temperature (Table 2) for 1 min, and a primer elongation at 72°C for 1 min was performed, then followed by twenty three cycles at 95°C for 45 seconds, annealing temperature at (55°C or 45°C) for 45 seconds, and a primer elongation at 72° C for 45 seconds and an extension cycle for 7 minutes.

The products of the microsatellites based PCR were detected by electrophoresis on agarose gel as follows: The PCR products was mixed with loading dye (5:1) and 10 µl was loaded in ethidium bromide – stained 2% agarose gel in 1 X TBE buffer. Bioprofil- Bio- 1D software was used to detect Alleles size by using 100bp DNA Ladder (Promega, U.S.A).

The banding patterns generated by SSR marker were compared to determine the genetic relationship of the seven pear genotypes. The amplified fragments were scored either as present (1) or absent (0). Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between two genotypes was estimated according to Jaccard coefficient (Jaccard, 1908). Dendrogram was clustered by cluster analysis using the unweighted pair-group method with arithmetic averages (UPGMA) on the basis of SSR data. The expected heterozygosity of the polymorphic loci (He) was calculated depending on allele frequency (Lorenzo *et al*., 2007). The marker index (MI) calculated from the formula:

MI= He * E, where E is the effective multiplex ratio and calculated from the formula: E= nB, which B is the fraction of polymorphic loci (Powell *et al*., 1996). The software's used through this study were Microsoft EXCEL, SPSS, Bioprofil- Bio- 1D, and Gene stat.

Table 2. List of SSR flanking primers. The expected product length, and annealing temperature

SSR Primer	Primer Sequence (5'------ 3')	Allele size range/bp	Annealing temperature
CH02B10	CAA GGA AAT CAT CAATTA AAG CAA GTG GCT TCG GAT AGT TG	114-157	55-45
CH01F02	ACC ACA TTA GAG CAG TTG AGG CTG GTT TGT TTT CCT CCA GC	168-222	55-45
CH01G12	CCC ACC AAT CAA AAA TCA CC TGA AGT ATG GTG GTG CGT TC	107-186	55-45
CH02C06	TGA CGA AAT CCA CTA CTA ATG CA GAT TGC GCG CTT TTT AAC AT	216-254	55-45
CH01H01	GAA AGA CTT GCA GTG GGA GC GGA GTG GGT TTG AGA AGG TT	107-141	55-45
CH02D12	AAC CAG ATT TGC TTG CCA TC GCT GGT GGT AAA CGT GGT G	175-205	55-45
CH01E12	AAA CTG AAG CCA TGA GGG TTC CAA TTC ACA TGA GGC TG	243-248	55-45
CH01F09	ATG TAC ATC AAA GTG TGG ATT GGC GCT TTC CAA CAC ATC	112-139	55-45
CH02B03b	ATA AGG ATA CAA AAA CCC TAC ACA G GAC ATG TTT GGT TGA AAA CTTG	77-109	55-45
CH02B12	GGC AGG CTT TAC GAT TAT GC CCC ACT AAA AGT TCA CAG GC	124-142	55-45
CH01H02	AGA GCT TCG AGC TTC GTT TG ATC TTT TGG TGC TCC CAC AC	226-252	55-45
CH01E01	GGT TGG AGG GAC CAA TCA TT CCC ACT CTC TGT GCC AGA TC	104-138	60-50
23g4	TTT CTC TCT CTT TCC CAA CTC AGC CGC CTT GCA TTA ATT AC	112	55-45
02b1	CCG TGA CAA AGT GCA TGA ATG AGT TTG ATG CCC TTG GA	238	55-45
NB	AGC TCT CGG CTT CAA TGG TTG TTA GCA TGT GAA ATG TCC GTA AAG TA	326	60-50

RESULTS AND DISCUSSIONS:

Polymorphism detected by simple sequence repeats(SSRs):

In the present study Fourteen primer pairs of the fifteen were able to characterize the studied pear genotypes, while the primer pairs CH02C06 couldn't reveal any allele, the fourteen SSR primer sets revealed 47 alleles, of which 44 were polymorphic (93.6%) among all genotypes. However, when focusing on the Syrian pear genotypes only 42 of 45 alleles were polymorphic (93.3%). CH01G12 primer- pair produced one monomorphic allele per genotype, and the heterozygous locus CH01H02 revealing two monomorphic alleles per genotype, while the twelve primer- pairs revealed polymorphic patterns (Table 3). The size of all alleles exhibited by these polymorphic loci were different than the expected size as shown in Table (2) except the primer pair BN. Yamamato *et al*.(2001) reported that the differences in the size of the fragments among pear cultivars were mainly due to differences in the repeat number of the AG unit. The number of alleles per primer-pairs ranged from 1 to 6 alleles (figure 1) with an average 3.4. As well, when Liconte was not included the average was 3.2. However, the average number of alleles depends on the primers and the number of accessions. Erfani *et al.* (2012) stated that the number of observed alleles for each locus was 3 to 12 with an average 6.21 per locus when they studied the genetic diversity and relationship among 47 pear cultivars and genotypes using 28 SSR primer pairs.

Table 3. Total number of allelic forms obtained among the seven pear genotypes under this study and their polymorphism estimation and their correspondence among the 6 Syrian genotypes (Values between brackets)

Primers	Total allelic forms	Polymorphic alleles	% polymorphism	Allele size/bp
CH02B10	4	4	100	150,140,125,115
CH01F02	4	4	100	200,195,185,175
CH01G12	1	0	0	125
CH01H01	3	3	100	135,120,100
CH02D12	4	4	100	240,230,218,200
CH01E12	2	2	100	240,230,223,200
CH01F09	5	5	100	265,253
CH02B03b	6	6	100	130,120,110,100,92,80
CH02B12	5	5	100	160,150,137,125,100
CH01H02	2	0	0	275,260
CH01E01	4	4	100	285,275,258,200
23g4	2	2	100	124,117
02b1	2	2	100	260,235
BN	3	3	100	366,355,325
14 Loci	**47 (45)**	**44 (42)**	**93.6(93.3)**	
Average	**3.35 (3.2)**	**3.14 (3)**		

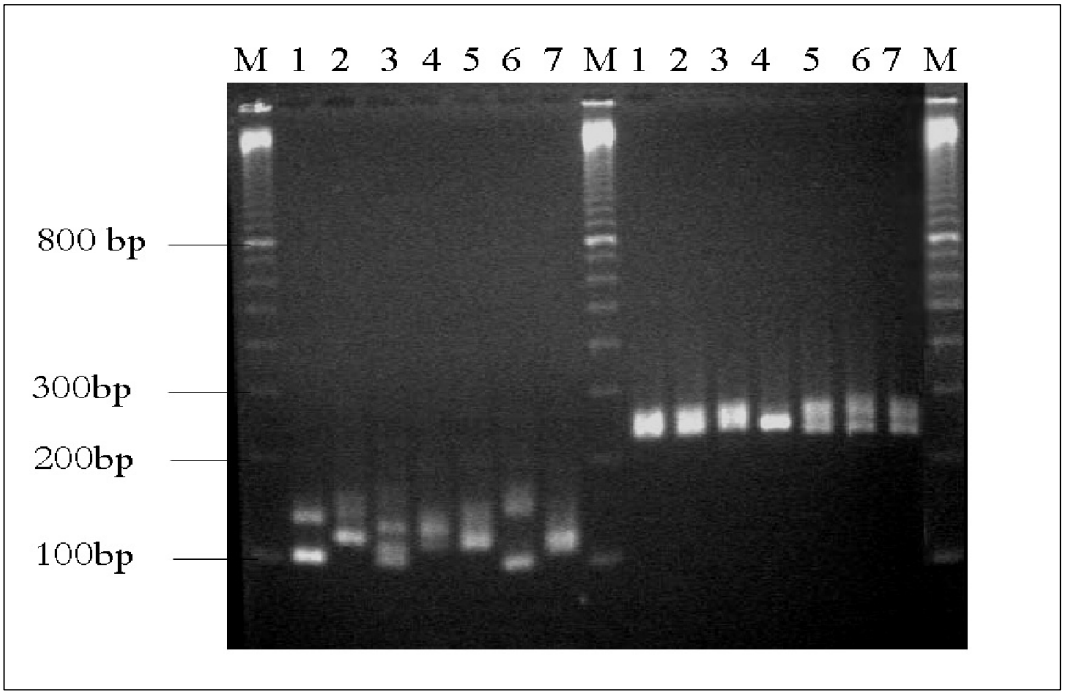

Figure 1. The separation pattern of SSR- PCR products on 2% agarose gel. The amplification products using CH02B12"Left" and CH01H02 "Right"flanking primer sets,.M: DNA molecular wight 100bp ladder.Lane's 1 through 7 refers to pear genotypes: Meskawi, (1) W.T_1, (2) Abu-Satel, (3) W.T_2 (4), W.T_3 (5), Romi (6) and Liconte (7)

Genetic Relationship as Revealed by SSR Data:

The genetic similarity based on Jaccard coefficient for the seven pear genotypes ranged from 65% to 26% between Meskawi with W.T_3, and Abu-satel with Liconte, respectively (Table 4). Also, Romi revealed the lower genetic similarity with W.T_2 (26%). So, it differs than the obtained results by biochemical analysis "isozymes" for the same genotypes which revealed the highest relatedness between Romi and W.T2 due to the high number of bands revealed by Romi cultivar "6 bands" when using Peroxidase isozyme (Elshihy *et al*.2004). Liconte Cultivar revealed the highest relationship with Romi (41%) ,while revealed the lowest genetic relationship with Abu-satel (26%). According to the obtained results, the lower genetic relationship between Liconte and Syrian pear genotypes indicated that the two groups belong to different species, except the genetic relationship between Liconte and Romi. Concerning the Syrian pear genotypes, the highest genetic relationship was between Meskawi and W.T_3 (65%), also Abu-Satel revealed efficient genetic relationship with W.T_2 (46%), while Romi revealed low genetic similarity with all Syrian pear genotypes which need more genetic studies to verify the origin of Romi cultivar. The obtained data reflected the ability of SSR marker to differentiate between individuals. Kimura *et al*. (2002) used eleven SSR primers to identify the genetic diversity of 57 pear varieties, they observed the genetic relationship between the genotypes within species as well as among species, and suggested that the use of 11 SSR markers was sufficient to evaluate the genetic relationship between all pear varieties.

Table 4. Genetic similarity matrices computed according to Jaccard coefficient from SSRs data.

	Meskawi	**W.T$_1$**	**Abu-Satel**	**W.T$_2$**	**W.T$_3$**	**Romi**	**Liconte**
Meskawi	1						
W.T$_1$	0.5	1					
Abu-Satel	0.44	0.29	1				
W.T2	0.32	0.27	0.46	1			
W.T3	0.65	0.56	0.39	0.43	1		
Romi	0.36	0.30	0.30	0.26	0.27	1	
Liconte	0.36	0.30	0.26	0.31	0.31	0.41	1

Cluster Analysis as Revealed by SSR Data:

The dendrogram clustered the seven pear genotypes into two clusters where Liconte and Romi cultivars formed the first cluster indicating the linkage distance between the two genotypes (Figure 2), W.T$_2$ formed with Abu-satel cultivar the first subcluster, while the second subcluster formed two groups, one contains only one genotype "W.T$_1$" and the second group contains Meskawi and W.T$_3$ which revealed the highest genetic linkage. The obtained data showed that Liconte genotype is separated in a distinct cluster with Romi cultivar which suppose that Romi may not belong to the Syrian local pear cultivars and needs deeply genetic studies to prove this hypothesis. Moreover, Meskawi clustered with the two wild type genotypes W.T$_1$ and W.T$_3$, and Abu- Satel with W.T$_2$, which reveal a high genetic relatedness with the Syrian pear wild types. The obtained result by SSR marker confirmed the results obtained by using Biochemical analysis "Peroxidase and Esterase isozymes" for the same Syrian pear genotypes which revealed the high relatedness between Meskawi,W.T1 and W.T3. Peroxidase analysis characterized W.T3 with 5 bands which contributed with the two revealed bands of W.T1 and with two out of three bands of Meskawi cultivar, while Esterase analysis gave monomorphic bands of all Syrian pear genotypes (Elshihy *et al.,*2004).

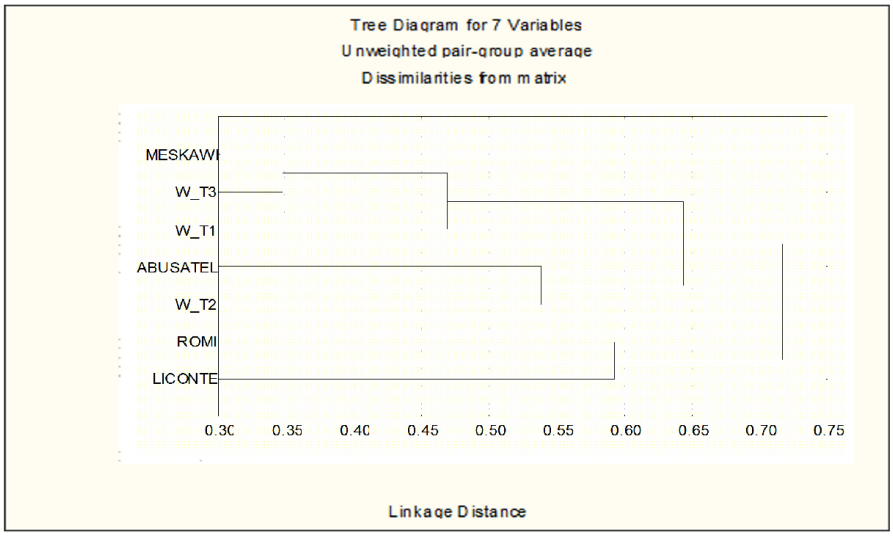

Figure 2. Cluster analysis as revealed by SSRs data

Genotype identification by unique SSR alleles:

Unique DNA markers obtained by SSR marker was used in the present study to characterize the seven pear genotypes. A total of 12 SSR primer-pairs out of 14 revealed 20 unique SSR alleles (17 positive and 3 negative) characterizing the seven pear genotypes (Table 5). $W.T_1$ was characterized by the highest number of unique SSR alleles which were 5 unique alleles (4 positive and 1 negative), while Meskawi and $W.T_3$ genotypes characterized by only one unique allele for each genotype. Liconte genotype was characterized by 3 unique alleles; CH01F09 and CH01H02 (positive alleles), and CH01F01(negative allele), their sizes were 180 bp, 230bp, and 100 bp, respectively . The primer- pair CH02B03b was able to characterize 5 out of the seven pear genotypes by unique alleles. Moreover, the seven pear genotypes can be characterized by the SSR primer-pairs CH02B03b, CH01F09, and CH01H01 which derived from apple genomic library. The results were in agreement with Yamamoto *et al.* (2001) as they found that SSRs derived from apple are highly conserved in pear.

Table 5. Genotype identification by unique SSR alleles, primer-pairs revealed unique alleles, the size of unique alleles, marker type (positive or negative), and number of unique alleles among each of the seven pear genotypes.

Genotype	Primer- pair	Allele size (bp)	Marker type	Number of unique alleles
Meskawi	CH01H01	144	+	1
$W.T_1$	CH01G12	125	+	
	CH01F09	275	+	
	CH02B03b	130	+	5
	O2b1	272	+	
	O2b1	238	-	
Abu-Satel	CH02D12	200	+	
	CH02B03b	90	+	3
	BN	390	+	
$W.T_2$	CH01H01	117	+	
	CH01H01	100	-	3
	CH02B03b	118	+	
$W.T_3$	CH02B03b	108	+	1
Romi	CH02B10	115	+	
	CH01F02	190	+	
	CH02B03b	100	+	4
	23g4	100	+	
Liconte	CH01F09	180	+	
	CH01H02	230	-	3
	CH01E01	100	+	

+: positive, -: negative

The efficiency of SSR marker in pear genome analysis:

The average of expected heterozygosity (H_e), the effective multiplex ratio (E), and the marker index (MI) were computed for SSR marker type .

The microsatellite based marker (SSRs) revealed 47 alleles among the seven pear genotypes, the maximum number of alleles was revealed by $W.T_1$ and Abu-Satel genotypes (20), while the lowest number was revealed by $W.T_2$ (18). The Liconte genotype and the rest of the Syrian pear genotypes revealed 19 loci. The multiplex ratio of SSR technique was (3.14) and the percentage of polymorphic alleles was (6.69**).** On the other hand, SSR markers are produced from amplification by primers designed depending on the conserved flanking regions and hence, revealed the lowest multiplex ratio. These results are in agreement with those of Al-Said (2001).

The average of expected heterozygosity (H_e) for SSR marker was used to evaluate the efficiency of this marker for polymorphism detection. SSR technique revealed 0.263. The obtained results in this investigation agreed with those of Powell *et al.* (1996); they reported that SSR revealed the high H_e, followed by RAPD and AFLP. While, Wunsch and Hormaza (2007) found that the expected heterozygosity **(**He**)** was 0.68 when they compared between 63 European cultivars by using 7 SSR primer pairs.

Marker index (MI) was calculated based on the experimental data. The marker index was (11.57) due to the effective multiplex ratio (44).

This investigation has concluded that the Leconte cultivar revealed the highest similarity with Romi cultivar, while the highest level of genetic similarity between Syrian genotypes was between Meskawi and $W.T_3$, the fourteen primer- paires derived from apple genomic library were very useful to discriminate the seven pear genotypes by 20 unique SSR markers. Consequently, SSR marker is very efficient tool to identify the genetic variability between the seven pear genotypes and to discriminate between individuals.

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التوصيف الجزيئي لبعض طرز الأجاص السورية *Pyrus syriaca* Boiss باستخدام تقنية الـــ SSR

بيان محمد مزهر[1] و أحمد نجيب شرف[2]

ملخـــص

أجري هذا البحث لدراسة القرابة الوراثية والعلاقة بين ستة طرز وراثية سورية من الأجاص " ثلاثة طرز برية من الأجاص السوري *Pyrus syriaca* Boiss وثلاثة أصناف محلية مسكاوي ورومي وأبوسطل " بالإضافة للصنف Liconte باستخدام 15 زوجاً من بادئات الـــ SSR. تمكن 14 زوجاً من البادئات من تمييز طرز الأجاص المدروسة، حيث أعطت 47 أليلاً منها 44 متعددة شكلياً (93.6%)، وبالتركيز على الطرز الوراثية السورية فقد أعطت 45 أليلاً منها 42 متعددة شكلياً (93.3%). تراوح عدد الأليلات لكل زوج من البادئات بين1– 6 أليلات بمتوسط 3.4 أليلاً. أظهر الصنف Liconte قرابة وراثية منخفضة مع طرز الأجاص السورية مما يدل على أن المجموعتين تتبعان لأنواع أو تحت أنواع مختلفة. ومن جهة أخرى فقد أظهر الصنف مسكاوي أعلى درجة تشابه وراثي مع الطراز البري السوري $W.T_3$ (65%). لقد قسم التحليل العنقودي الطرز المدروسة في مجموعتين، حيث وقع الصنفان ليكونت و رومي في مجموعة واحدة، وباقي الطرز في المجموعة الثانية. تمكن 12 من 14 زوجاً من البادئات من تمييز طرز الأجاص السبع بـــ 20 أليلاً فريداً (17 موجبة و3 سالبة). كانت نسبة التغاير المورثي المتوقع 0.263، ودليل المؤشر 11.57. وهذا يعكس قدرة تقنية الـــ SSR في تحديد التباين الوراثي بين تراكيب الأجاص السبع.

الكلمات الدالة: أجاص، SSR ،*Pyrus syriaca Boiss* ، القرابة الوراثية.

1) دكتور باحث في الهيئة العامة للبحوث العلمية الزراعية– السويداء
✉ bmuzher@hotmail.com

2) أستاذ دكتور في جامعة القاهرة/ كلية الزراعة

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