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IDENTIFICATION OF MALE GENOTYPES IN *Pistacia vera* L. SPECIES USING SSR MARKERS

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Abstract

Genetic identification among different 15 male genotypes of *Pistacia vera* L. species in comparison with five main commercial male cultivars accredited by Ministry of Agriculture (Adam, Jamil, Ebrahim, Elyas and Khalifa) was achieved using SSR markers. Seventeen primer pairs out of Twenty SSR primers were able to amplify PCR products. SSR segregation produced 44 putative alleles, of which 34 were polymorphic (77.27%). Genetic similarity among all studied genotypes ranged from 0.45 between Jamil cultivar and MAS1 genotype to 1 between Ebrahim cultivar and MA1 genotype which means that they are identical. The UPGMA cluster analysis based on Jaccard's coefficient grouped all genotypes into three main clusters. The number of alleles revealed by each SSR analysis ranged from 1 to 5, with a high level of expected heterozygosity (He) 0.507. Co-dominant SSRs loci were observed in some studied genotypes giving a value 0.235 of observed heterozygosity (Ho). According to the polymorphic allele's number and the expected heterozygosity; Marker Index (MI) was 23.97. Our results concluded that SSR marker is an informative technique, which revealed high ability to differentiate individuals, and played an important role as a genetic marker for identification and evaluation studies within *P. vera* species.

Keywords: *Pistacia vera* L., SSR markers, genetic similarity, expected heterozygosity, observed heterozygosity.

Introduction

The genus *Pistacia* belongs to *Anacardiaceae* family comprises at least eleven species, some of which are of high economic and cultural importance in Mediterranean and Asian countries (Isfendiyaroglu, 2007). The pistachio tree is a deciduous, dioecious and wind-pollinated woody tree. Pistachio varieties are usually self-sterile. The reasons for this sterility are the difference of anthers dehiscence and the receptive ability of the stigmata of the female organs, and hormonal or genetic incompatibility (Kebour *et al.*, 2013). This semi-accordance directly affects the productivity and rises the percentage of blank nuts, whether it is the parthenocarpy or abortion phenomena. While the main information of the behavior of female pistachio cultivars are recently convenient, little studies are carried out about male clones and genotypes as pollinators (Acar and Eti, 2007). Many literatures reviewed the effectiveness of male pistachio cultivar on productivity process (Hormaza and Herrero, 1998; Ozeker *et al*., 2006; Atli *et al*., 2006; Abu-Zahra and Al-Abbadi, 2007; Kebour *et al*., 2013 Guerrero *et al*., 2014). Although the number of varieties constituting the species *Pistacia vera* L. is considerable; evaluation and identification processes are facing miss-genetic relatedness of taxonomic classifications (Kebour *et al*., 2012). The pistachio production of many orchards in South of Syria has long been suffering from an inappropriate pollination due to several reasons. Any investigation on pollinator selection and identification has not been achieved in this area up to now. Moreover, the morphological markers are influenced by environmental conditions and developmental stages (Al-Sousli *et al*., 2014). SSR markers are multi-allelic, co-dominant genetic markers with a very high reputability, and so are particularly suitable for phylogenetic studies because of their high polymorphism and abundance (Gupta *et al*., 1996; Vos *et al*., 1995; Senior and Heun, 1993). The current investigation was conducted to identify and screen in situ male *P.vera* genotypes using specific primer pairs of SSRs molecular markers in comparison with the accredited male cultivars in the South of Syria, in the aim to assess the genetic relationships among all existed samples.

Material and Methods

Plant Material:

The study included 15 *P.vera* male genotypes from different pistachio fields in the south of Syria (MK1, MK2, MK3, MA1, MA2, MAA3, MAA4, MAH1, MAH2, MAH3, MAS1, MS2, MES3, MKS4, and MM1) in the comparison with five commercial cultivars namely (Elyas, Jamil, Khalifa, Adam and

Ebrahim) accredited by Ministry of Agriculture, using SSR markers. All male genotypes are planted at the same location (4 sites) at altitude of 900-1000 m. The genotypes were grouped into three groups according to their flowering date:

Early blooming genotypes (3-15 March): MAA4, MAA3, MAH1, MAH2, MAS1 and Adam variety

Mid blooming genotypes (15-25 March): MK1, MK2, MAH3, MES2, MA1, MA2 and Elyas variety

Late blooming genotypes (25 March- 6 April): MS2, MKS4, MK3 and Khalifa variety

Genotypes of two full continued flowering stages (10-22 March): MM1

Adam, Elyas and Khalifa cultivars: the most spread *P.vera* male cultivars in the mentioned area.

Jamil and Ebrahim cultivars: New cultivated cultivars, most of the planted trees are still young less than four years and it does not undergo initial phonological stages yet.

DNA Extraction:

Samples of young leaves of studied male genotypes and cultivars were collected (a half gram of each sample) and DNA extraction was done by using CTAB protocol as used by Porebski *et al*. (1997). DNA quantity and quality were estimated using Biophotometer (Eppendorf, Germany) by measuring the absorbencies at A260 and A280 nm.

SSR markers:

Extracted DNA was PCR-amplified using 20 SSR primer pairs which were previously cloned and sequenced in *P.vera* according to (Ahmad *et al*., 2003; Vendramin *et al*., 2010). Amplification reactions were done in a 25μL volume. The cycling parameters were as following: one cycle of 95 for 4 min; 35 cycles of 94°C for one min, annealing temperature of the different primers for one min, and 72 °C for one min; followed by 4 minutes at 72 °C. Amplified PCR products were separated based on size differences of the segregating alleles in 1.8% agarose gel electrophoresis stained with ethidium bromide (0.5g/ml), and visualized by Geldocumentation (VILBER LOURMOT Germany).

Data analysis:

The amplified alleles were scored either as present (1) or absent (0). Genetic similarity (GS) between any two genotypes was calculated from the alleles across the 20 SSR loci using Jaccard's similarity coefficients (Jaccard, 1908). Polymorphism percentage was calculated according to the equation: the number of polymorphic alleles / the total number of alleles x 100. A dendrogram was constructed using data

obtained from the revealed SSR loci, with the UPGMA method using arithmetic averages (Sneath and Sokal, 1973). Expected Heterozygosity (He) was estimated according to Lorenzo *et al*. (2007) depending on allele frequency. Marker Index (MI) was calculated according to the equation MI= E*He whereas E is the effective multiplex ratio (Powell *et al*., 1996). Observed Heterozygosity (Ho) was calculated as the number of genotypes which were heterozygous at a given locus divided by the total number of genotypes surveyed at that locus (Wunch and Hormaza, 2007). The software used through this study were Microsoft EXCEL, SPSS17, and Past.

Results and discussion

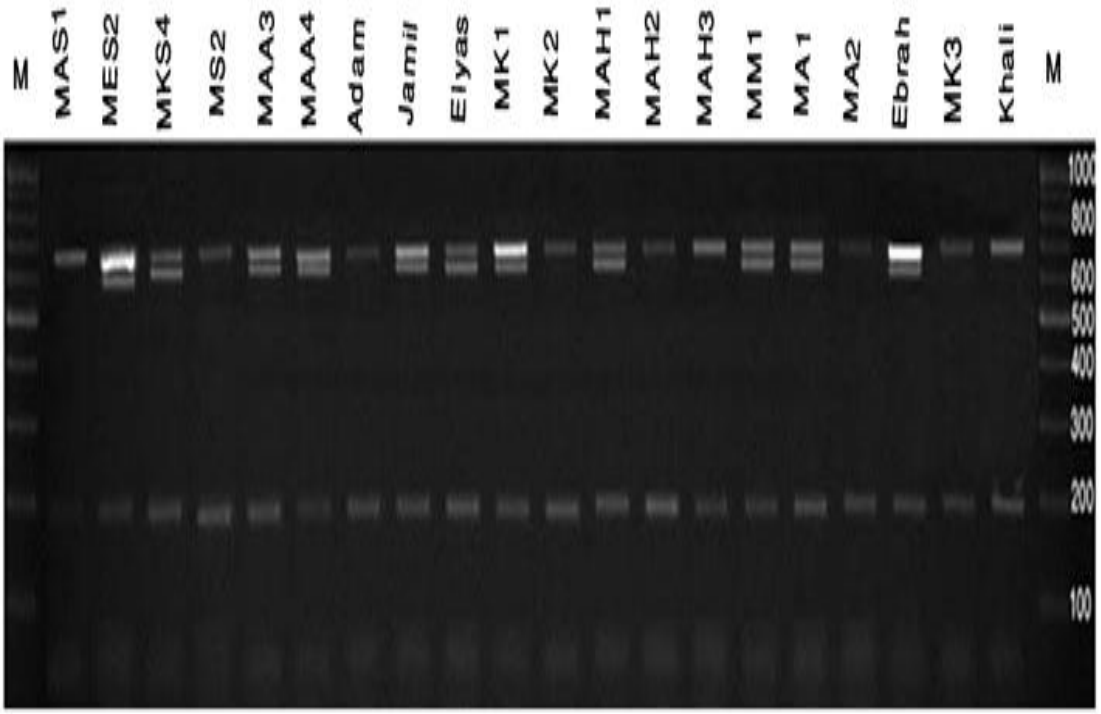
Levels of Polymorphism and Discriminating of the Assay:

Twenty SSR primer pairs were applied, 17 of them amplified unambiguous and scorable PCR products in all studied genotypes, and revealed 44 alleles, of which 34 alleles were polymorphic (77.27%) reflecting a high informative of SSR technique. Ahmad *et al.* (2005) as stated in their study on *P. atlantica* and *P. integerrima* and two hybrids, that 35 putative alleles were revealed by 12 SSR primer pairs. All primer pairs produced 1-5 putative alleles each with an average 2.6 alleles per locus, and the highest number of alleles was revealed by Ptms-7 and EPVF019 primer pairs (Table 1). Our results were in resemblances with Arabenzhad *et al.* (2011) as they got an average number of presumed alleles per locus 2.8, ranging from one to six. Baghizadeh *et al.* (2010) reported that in SSR population analysis, four SSR primers produced 11 alleles among 31 pistachio genotypes with average value 2.75 alleles. However, Primer pairs (Ptms-9, Ptms-11, and EPVM-21) revealed monomorphic alleles, whereas the other primer pairs revealed polymorphic alleles. The highest number of alleles were detected in MM1 genotype (38 alleles), whereas the lowest allelic number was revealed in Khalifa male cultivar (20). SSR primer pairs EPVF013 (Figure 1A) and EPVM016 were able to detect the heterozygosity in some individuals, while the primer Ptms-7 revealed duplicated loci in all studied genotypes and cultivars except (MAS1, MAA3, MM1, MAH3, and Adam cultivar). Allele size ranged from 78 to 690 bp. Vendramin *et al.* (2010) indicated that the allele size ranged from 206 to 609 bp in pistachio, while it ranged from 213 to 766 bp in *P. integerrima*, and 219-766 bp in *P. terebinthus*. In the current study, the primer Ptms-7 amplified 5 polymorphic alleles of allelic size 177- 203- 213- 225 and 236 bp, which was in accordance with Ahmad *et al.* (2003) as the same primer revealed 5 alleles of similarly sizes ranged between 189-225 bp. The genotype MM1 characterized

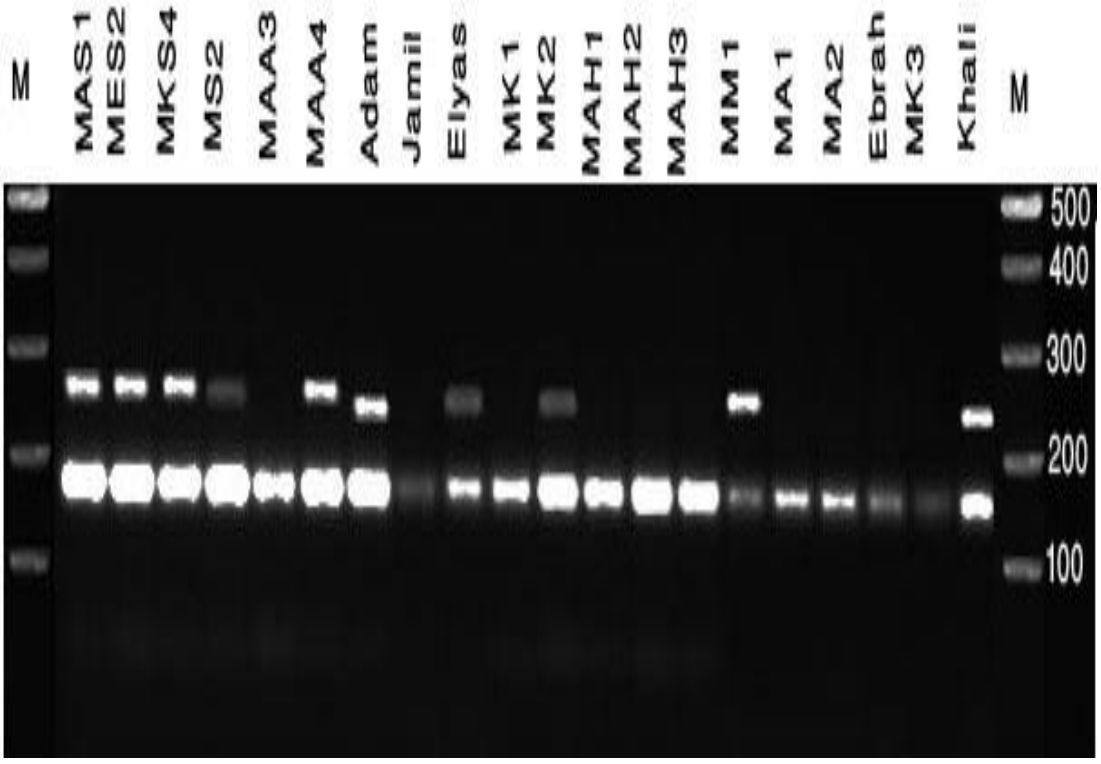
by giving two continued periods of flowering stage that cover mostly of receptive stigma, and it is also labeled by unique alleles using primer pairs Ptms-33, Ptms-40, EPVM016 and EPVM058. Effectively, both of genomic EST-SSRs and Ptms SSR primer pairs revealed useful polymorphous loci (Figure1B) in all male *P. vera* genotypes and varieties detecting a high genetic miscibility among pistachio male genotypes.

Table 1: Total number of alleles produced by 17 SSRs primer pairs, number of polymorphic alleles, polymorphism percentage and allele size (bp)

PRIMER	TOTAL NO. OF ALLELES	NO. OF POLYMORPHIC ALLELES	POLYMORPHISM %	ALLELE SIZE (bp)
Ptms-3	2	2	100	135-140
Ptms-7	5	5	100	177-203-213-225-236
Ptms-9	1	0	0	133
Ptms-11	1	0	0	399
Ptms-14	2	2	100	78- 104
Ptms-31	2	2	100	131-142
Ptms-33	4	4	100	169-179-195-229
Ptms-40	2	2	100	166-180
Ptms-42	2	2	100	167-175
Ptms-45	4	3	75	152-212-220-235
Ptms-10	2	1	50	196-438
EPVF021	2	0	0	115-333
EPVF013	3	1	33.3	198-633-690
EPVF016	2	1	50	503-524
EPVF056	2	2	100	401-447
EPVF019	5	4	80	176-234-250-420- 480
EPVM058	3	3	100	249-273-294
SUM	44	34	77.27	
AVE	2.6	2		



1A: EPVF013 SSR Primer Pairs



1B: Ptms-45 SSR Primer Pairs

Fig (1A, 1B): putative alleles produced by using Ptms-45 and EPVF013 SSR primer pairs on studied Genotypes and cultivars M: 100 bp ladder

Genetic similarity ranged from 0.45 (between Jamil cultivar and MAS1 genotype), to 1 (between Ebrahim cultivar and MA1 genotype), indicating that the genotype MA1and Ebrahim cultivar are identical, Table (2). On the other hand MKS4 revealed high genetic similarity with MS2 and MES2 (0.96), also Khalifa male cultivar revealed high genetic similarity with MK3 (0.96) which reflected high closely relatedness between these genotypes, and leads to the hypothesis that there is an incorrect use or knowledge by dealing with pistachio male cultivars. The average of genetic similarity between all cultivars (Adam, Jamil, Ebrahim, Elyas and Khalifa) was 0.62., while the highest genetic similarity among cultivars was 0.88 between Ebrahim and Khalifa cultivars. The highest genetic similarity of MM1 genotype (which gives two full continued blooming racemes) was 0.67 with MA1 genotype and Ebrahim cultivar. The genetic distance among early blooming genotypes was 0.59, while it was 0.62 among mid blooming genotypes, and 0.69 among late blooming genotypes. This genetic variation may relate to some male specimens which were generated from seeds without grafting by accredited cultivars or due to miss transferability processes between nurseries. Pazouki *et al.* (2010) reported that genetic similarity within *Pistacia* spp. ranged from 0.03 to o.8. While Kafkas *et al*. (2008) revealed a genetic similarity ranged between 0.08- 0.98 among 46 *Pistacia* genotypes belong to 12 species.

Table 2: The distance of genetic similarity among all studied mail genotypes and cultivars using Jaccard coefficient.

	MAS1	MES2	MKS4	MS2	MAA3	MAA4	Adam	Jamil	Elyas	MK1	MK2	MAH1	MAH2	MAH3	MM1	MA1	MA2	Ebrah	MK3	Khalif
MAS1	1																			
MES2	0.73	1																		
MKS4	0.76	0.96	1																	
MS2	0.79	0.92	0.96	1																
MAA3	0.68	0.8	0.83	0.79	1															
MAA4	0.63	0.81	0.84	0.8	0.83	1														
Adam	0.62	0.67	0.69	0.72	0.83	0.69	1													
Jamil	0.45	0.61	0.63	0.59	0.68	0.76	0.62	1												
Elyas	0.55	0.55	0.57	0.53	0.61	0.68	0.55	0.55	1											
MK1	0.61	0.55	0.57	0.53	0.5	0.57	0.45	0.5	0.66	1										
MK2	0.52	0.57	0.59	0.61	0.63	0.70	0.63	0.63	0.68	0.62	1									
MAH1	0.55	0.5	0.52	0.48	0.55	0.62	0.5	0.67	0.6	0.66	0.57	1								
MAH2	0.52	0.47	0.48	0.5	0.57	0.59	0.52	0.57	0.57	0.47	0.48	0.68	1							
MAH3	0.65	0.64	0.61	0.63	0.72	0.73	0.65	0.59	0.64	0.53	0.61	0.64	0.73	1						
MM1	0.52	0.52	0.53	0.5	0.57	0.59	0.57	0.63	0.62	0.57	0.53	0.68	0.59	0.61	1					
MA1	0.54	0.59	0.61	0.57	0.65	0.73	0.54	0.72	0.64	0.53	0.55	0.70	0.67	0.69	0.67	1				
MA2	0.52	0.52	0.54	0.56	0.58	0.65	0.58	0.71	0.57	0.52	0.59	0.69	0.65	0.68	0.65	0.91	1			
Ebrah	0.54	0.59	0.61	0.57	0.65	0.73	0.54	0.72	0.64	0.53	0.55	0.70	0.67	0.69	0.67	1	0.91	1		
MK3	0.54	0.59	0.55	0.57	0.59	0.67	0.54	0.65	0.59	0.48	0.55	0.64	0.67	0.76	0.61	0.91	0.91	0.91	1	

Khalif	0.52	0.57	0.53	0.55	0.57	0.64	0.52	0.63	0.62	0.47	0.59	0.62	0.64	0.73	0.64	0.88	0.87	0.88	0.96	1

Clustering and Genetic Relationships Among all Studied Genotypes:

The pattern of cluster analysis based on Jaccard's coefficient and UPGMA algorithm clustered the genotypes into three main clusters, Figure (2). The first cluster was divided into two sub-clusters, the first sub-cluster contained (MK2 genotype and Elyas cultivar), while the second sub-cluster contained only one genotype (MK1).The second cluster divided into three sub-clusters, the first sub-cluster branched into two groups, the first group contained Jamil cultivar, whereas the second group divided into two sub-groups, the first sub-group comprised Ebrahim cultivar with the MA1 genotype with100% genetic similarity, while the second sub-group contained Khalifa cultivar and MK3 genotype with genetic similarity 0.96. The second sub-cluster comprised of the genotypes MAH2 and MAH3, and the third sub-cluster contained MAH1 and MM1 genotypes. The third cluster divided into four sub-clusters, the first sub-cluster included MES2, MKS4 and MS2 genotypes, and the second sub-cluster contained MAA3 and MAA4 genotypes, while each of Adam cultivar and MAS1 were independently located in the third and fourth sub-clusters, respectively. Carefully studying the cluster plots, we inferred that the early genotypes MAS1, MAA3 and MAA4 were clustered closer to Adam cultivar which is an early cultivar, while the med blooming genotypes MK1 and MK2 were located closer to medial flowering cultivar (Elyas), and the late blooming genotype MK3 was closely located to the late cultivar (Khalifa).

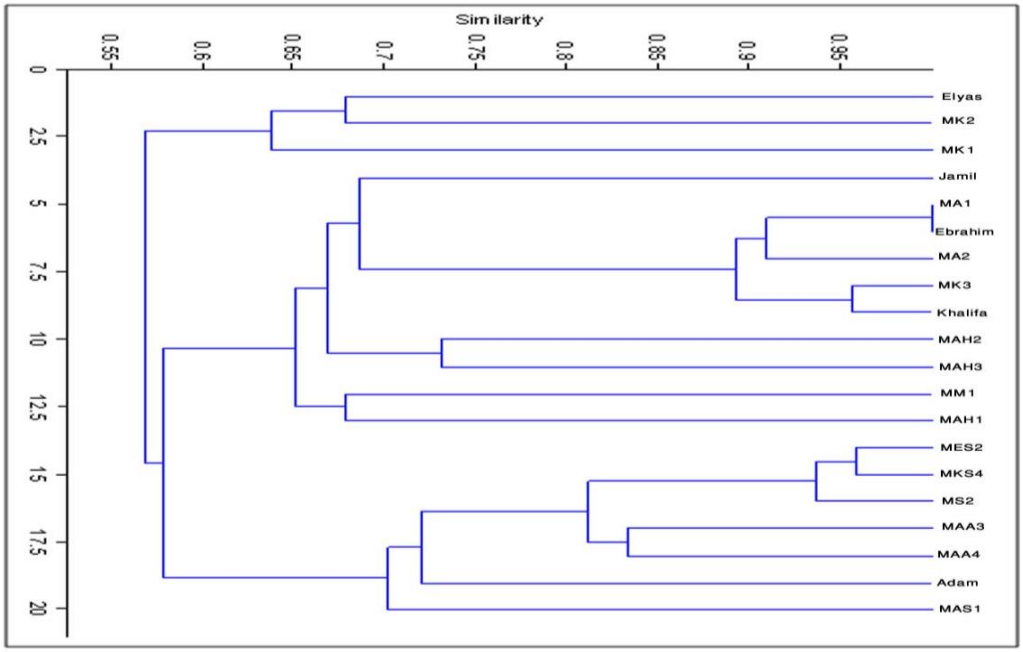


Fig 2: Cluster analysis for 15 male pistachio genotypes and 5 commercial cultivars (Elyas, Ebrahim, Jamil, Adam, Khalifa) using Jaccard's coefficient

To evaluate the efficiency of SSR microsatellite markers for polymorphism detection; Observed and Expected Heterozygosity (Ho and He), in addition to Marker Index (MI) were calculated. Observed heterozygosity (Ho) was appeared in 3 of 17 SSR loci giving an

average value 0.235. Expected heterozygosity depending on allele frequency was 0.705 in all polymorphic tested loci. Marker Index (MI) was estimated depending on the effective multiplex ratios which was 23.97. Albaladejo *et al.* (2010) revealed that the Expected Heterozygosity (He) ranged from 0.139-0.895 per locus using eight polymorphic nuclear microsatellite markers in two *P. lentiscus* L. populations. Whereas Zhizhuang *et al.* (2010) study revealed an Expected Heterozygosity of 0.472 among 8 populations of *Pistacia Chinensis* using 9 SSR primer pairs.

Consequently, this perusal demonstrated a high genetic diversity among male *P. vera* genotypes which used as pollinators for different female cultivars. On the other hand, our results concluded that SSR marker is an informative, robust and reliable technique which revealed high ability marker systems for genome analyses in pistachio to differentiate individuals and played an important role as a genetic marker for identification and evaluation studies within *P. vera* species.

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