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Association Analysis for Morphological Traits in Pomegranate (*Punica geranatum* L.) Using Microsatellite Markers

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Abstract: Microsatellite markers were used to identify informative markers associated with traits Sunburn sensitivity, Hull cracking sensitivity, Fruit height, Fruit diameter, Fruit shape index (Fruit height/Diameter ratio), Calyx height, Calyx diameter, Calyx height/Diameter ratio, Fruit shape, Calyx shape, Calyx type, Fruit taste, Flower height, Flower diameter, Flower height/diameter ratio and Style height. From 30 primers that were used, 7 pairs were polymorph and product 23 alleles in 202 pomegranate genotypes. The mean number of alleles were 3.28 alleles for each microsatellite locus. Polymorphic information content ranged from 0.01 (Locus MP07) to 0.56 (Locus MP39), with an average of 0.34. Stepwise regression analysis between molecular date as independent variables and morphological data as dependent variables was performed to identify informative markers associated with the studied traits.Each of the 14 traits (excluding fruit shape, calyx type, Hull cracking sensitivity and skin color) showed significant association on a total of 14 of the 23 polymorphic SSR bands.The association markers explained 2% to 29% of the variation for individual traits. The most variation of calyx height (0.29) was accounted by MP30-11, MP51-13, MP51-16 and MP07-21. SSR loci associated with fruit diameter, calyx shape and fruit size was the same. The results showed there is a significant and positive correlation among these traits. MP26 marker was linked with most traits studied in this research. Since all the used SSR loci particularly MP26 showed significant association with the studied traits, therefore, it is possible to use these markers along with morphological traits in pomegranate breeding programs for identification of suitable parents to produce mapping populations and hybrid varieties.

Key words: Association analysis · Morphological traits · Microsatellite markers · Pomegranate

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae which has a single genus *Punica* and two species *P. protopunica* Balf. and *P. granatum* L. [1] According to smith [2],*P. granatum* L. has 2n= 2x= 16, 18 chromosomes. Pomegranate thought to be indigenous to the region of Iran where it is native [3] and it is also thought to be a native in Turkey [4]; It was spread to Mediterranean countries at a very early date. The pomegranate and its use are deeply embedded in human history with references in many ancient cultures of its use in food and medicine [5]. Pomegranate produces fruit that is valued for its juice-containing arils, health benefits and decoration and is consumed and marketed as whole fresh fruit,juice, seed oil and other products [6]. Despite the long history of pomegranate culture as a fruit crop, its economic importance and high morphological diversity in pomegranate germpelasm, this diversity is not used in breeding programs, sufficiently.However, for exploration of diversity in pomegranate germpelasm, it is essential evaluation of morphological and agronomic traits. During the last two decades, DNA-based molecular markers have been extensively used for a variety of purposes in many animals and plant systems [7]. Among various DNA markers, simple sequence repeats (SSR) because of their co-dominant nature [8], their high degree of polymorphism[9] and their distribution in the whole genome [10] are considered to be the most important molecular markers, which have been used in various study

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[11-14]. Microsatellite markershave been successfully used in many genetic diversity [15, 16], fingerprinting [17, 18] and constructing linkage map and QTL analysis in various crop species such as: cotton [19, 20], wheat [21], maize [22] and etc.Thought map-based QTL analysis is efficient in detecting QTL, it is time consuming and laborious [23]. In order to overcome these limitations and as an alternative to planned populations, molecular marker-trait association identifications have been conducted through the combination between the present germpelasm and the regression technique [24-29] and increasingly adopted in many plants [30]. Multiple regression analysis (MAR), based on association of a marker with the phenotype gives estimates and test of significance of the parameters of multiple linear regression equations. It also provides the coefficient to determination (R^2) which indicates the proportion of variability of a dependent variable that can be explained by a linear function of independent variables [31].

Here, we report association of SSR markers with morphologic and pomologic traits in pomegranate following multiple regression analysis. A total of 30 *Punica* SSR developed by Pirseyedi *et al* [32] were used. But only seven markers showed polymorphism among the markers used.

MATERIAL AND METHODS

Plant Materials: Plant material in this study consisted of 202 pomegranate accessions representing 22 Province of Iran, belonging to Iranian pomegranate collection held at Markazi Province. Genotype names and accession codes of material is shown in Table 1. Accessions are labeled according to their Province number, material number and taste.

Morphologic Evaluation: The pomegranate used for this study have been planted in a α- Latis design in two replication at the pomegranate research station of Saveh. Saveh is a town in the Province of Markazi. Traits studied inthis researchwere evaluated based on a descriptor list for pomegranate developed by the CIHEAM Collaborative Working Group on Underutilized Fruit Crops in the Mediterranean Region [33]. These traits were: Fruit height, Fruit diameter, Fruit height/Diameter ratio, Calyx height, Calyx diameter, Calyx height/Diameter ratio, Fruit taste, Fruit shape, Calyx shape, Calyx type, Fruit size, Skin color, Sunburn sensivity, Hull cracking, Flower length, flower width and style length. 10 mature fruits [34] and 25 flowers [35] were taken at random from each genotype for morphological analysis.

DNA Extraction and Microsatellite Analysis: High-quality genomic DNA was isolated from young leaves usingGMO DNA Extraction Kit (BioNEER) following the instructions of the manufacturer. Quality and quantity of DNA in the extracted sample solutions were measured with NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware) and electrophoretic separation through a 0.8% (w/v) agarose gel. Extracted DNA was subjected to SSR analysis. Amplification was carried out using iCycler™ BioRAD in a total volume of 15ml containing 1.5 ml of 10X of PCR Buffer, 1.0 mMof $mgcl_2$, 1 ml of 1 mMdNTPs, 0.2 U of Taq DNA Polymerase, 20-30 ng of template DNA and 6.1 ml of ddH_2o. The amplification profile consisted of an initial denaturation at 95°C for 4 min (step 1), followed by 10 cycles (step 2) of 94°C for 30 s,65°C for 30 s and 72°C for 60 s with touch down by 1°C in each cycle from 65°C to 55°C followed by 25 cycles (step 3) of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. final extension cycle was carried out at 72°C for 5 min (step 4). Amplification reaction products were separated on a 5% denaturing polyacrilamide gel using a Sequi-Gen GT Sequencing Cell 50 cm gel apparatus (Bio-Rad Laboratories Inc.).

Data Analysis: The bands of DNA fragments on SSR analysis were scored in a 0-1 binary format (0 for absence, 1 for presence). The polymorphism information content (PIC)for each primer was calculated according to the formula: $PIC= 1-\Sigma p_i^2$, where K is the total number of alleles detected for a locus of a marker and P_i the frequency of the *i*thallele in the specified locus. The effective number of alleles was calculated using the POPGEN software program version 3.1 [36]. Association analysis between molecular date (as independent variables) and mean of two replication of morphological data (as dependent variables) was performed using multiple regression analysis to identify informative markers associated with the studied traits.Multiple regression analysis was conducted using "stepwise" method of "linear regression analysis" option of SPSS version 17. Markers showing significant regression values were considered as associated with the trait under consideration.

RESULTS

Among thirty microsatellite markers that were used in this study, seven primers showed polymorphism.A total of 23 alleles were detected and the number of alleles per locus ranged from two for mp07, mp12, mp30 and mp39

Table 1: Pomegranate samples studied including genotype names and accession cods

Genotypes	Code	Genotypes	Code
Poost-Nazok-Ardal	1-1-N	Shahvar-Kashmar	12-102-W
Poost-Ghermez-Dareh-Hourand	2-2-S	Ghand-Kashmar	12-103-W
Dane rize-Dare hourand	2-3-W	Bi daneh-Kashmar	12-104-W
Nar shirin-Dareh-Hourand	2-4-W	Garche-Shahvar	12-105-N
Poost-Nazok-Dareh-Hourand	2-5-WS	Ghandi-Poost-Sefid-Bejeston	12-106-W
Meikhosh-Dareh-Hourand	2-6-W	Torsh-Shooshtar	13-107-S
Binam-Kouhestan-DarehHourand	2-7-N	Meikhosh-Behbahan	13-108- WS
Poost-Ghermez-Dareh- Hourand	2-8-W	Malas-Behbahan	13-109-WS
shirinriz-Dareh-Hourand	2-9-W	Danehghermez-A lot-Baneh	14-110-S
Shirin-Sourati-DarehHourand	2-10-W	Abbasi-Kordestan	14-111-N
Poost-Sefid-DarehHourand	2-11-W	Dane Ghermez-Lorestan	15-112-W
Dane Dorosht-Dareh-Hourand	2-12-S	Khoramabad-Lorestan	15-113-N
Zoodres-Dareh-Hourand	2-13-W	Jafari-Shei-Nesha-Lorestan	15-114-WS
Shekarnar-Tasuj-Shabestar	2-14-W	Ghermez-Poost-Koloft-Tang seab	15-115-W
Dane Sefid-Mehran	3-15-W	Soz-Poost-Koloft-Lorestan	15-116-W
Dane-Ghermez-Mehran-Elam	3-16-S	Poost-sorkh- tang seab	15-117-W
Sabz-Shirin-Kalam-Elam	3-17-W	Soz-Lori-Shi-Nesha-Lorestan	15-118-S
Malas-Charmak-Elam	3-18-W	Bavasi-Poost-Sefid-Lorestan	15-119-WS
Binam-Salehabad-Mehran	3-19-W	Ghermez-Shirin-Kouhdasht-Lorestan	15-120-W
sefid-Elam	3-20-W	Zard-Mahaligerab-Lorestan	15-121-W
Sabz-Charmak-Elam	3-21-S	Gol-Khoramabad	15-122-N
Kadro-Poost-Koloft-Kazeron-Fars	4-22-W	Shirin-Nami-Khoramabad	15-123-W
Abdorahomkhani	4-23-N	Poost-Sefid-Khoramabad	15-124-S
Torbat-Sefid-Shiraz	4-24-W	Dane Sefid-Lorestan	15-125-W
Atabaki-Shiraz	4-25-WS	Meikhosh-Poost-Koloft-Lorestan	15-126-WS
Shirin-Shahbar-Shiraz	4-26-W	Binam-Lori-Khoramabad-Lorestan	15-127-W
Shirin-Sabz-Shiraz	4-27-W	Meikhosh-Bavasi-Shei-Nesha-Lorestan	15-128-WS
Khoramrize-Shiraz	4-28-W	Shirin-Lori-Khoramabad-Lorestan	15-129-W
Berit-Mamoli-Kazeron	4-29-S	Gavdamagh-Kouhdasht	15-130-S
Berit-Mamoli-Kazeron	4-30-W	Abbasi-Khoramabad	15-131-W
Ghojagh-Ghom	5-31-WS	Bi daneh-Saveh	16-132-WS
Jangali-Talesh-Rasht	6-32-S	Meikhosh-Saveh	16-133-W
Dareh-Loushan	6-33-S	Malas-Saveh	16-134-WS
Hajiabad-**B**andar abbas	7-34-W	Shirinseah-Saveh	16-135-W
Minab-Bandar abbas	7-35-W	Alak-Parand-Saveh	16-136-W
Meikhosh-PishRas-Kouhpayeh	8-36-WS	Malas-Torsh-Saveh	16-137-W
Poost-Nazok-Natanz	8-37-W	Alak-Shirin-Saveh	16-138-N
Bi name-Dastjerd	8-38-S	Tabestani-Saveh	16-139-W
Poost-Ghermez-Kouhpayeh	8-39-W	Dane dorosht-Shahsavar	17-140-W
Bihasteh-Najafabad	8-40-W	Shirin-Behshahr	17-141-W
Malas-Mortazavi	8-41-WS	Ardestani-Daneh-Sorkh-Semnan	18-142-S
Zaghi-Kouhpayeh	8-42-S	Torsh-Zabol	19-143-S
Damagh baste-Kouhpayeh	8-43-N	Meikhosh-Zahedan	19-144-WS
Khatooni-Poost-Sefid-Natanz	8-44-W	Poost-Sabz-Shirin-Zahedan	19-145-W
Pishras-Najafabad	8-45-W	Shirin-Zabol	19-146-W
Daneh-Ghermez-Natanz	8-46-WS	Torsh-Poost-Sabz-Zahedan	19-147-S
Malas-Isfahan	8-47-WS	Vahshi-Tamin-Khash	19-148-W
Dane-Sefid-Kouhpayeh	8-48-W	Bi daneh-Pishva	20-149-W
Sabz-Dane-Ghermez-Zavare-Ardestan	8-49-W	Marsel-Shouravi-Varamin	20-150-WS
Sarbarik-Kouhpayeh	8-50-WS	Rabab-Ghermez-Pishva	20-151-WS
Anbari-Poost-Koloft-Kashan	8-51-S	Torki-Pishva	20-152-WS
Poost-Sefid-Yaran	8-52-S	Gouzal-Shouravi-Varamin	20-153-W
Narak-Kouhpaye-Isfahan	8-53-W	Ghojagh-Pishva	20-154-WS
Malas-Shirin-Dastjerd	8-54-W	Togh-Pishva	20-155-WS
Khodroo-Vahshi-Najafabad	8-55-W	Piyazi-Ghermez-Pishva	20-156-N
Khatooni-Natanz-Isfahan	8-56-S	Ghahvedan-Kan	20-157-WS

Table 1: Countinue

Genotypes	Code	Genotypes	Code
Shomareyek-Kashan	8-57-S	Ghiyasin-Shirin-Kan	20-158-W
Poost-Ghermez-Natanz	8-58-W	Ghiyasin-Zati-kan	20-159-WS
Abanmahi-Isfahan	8-59-N	talghid-kan	20-160-W
Torsh-Mar mar	8-60-S	Poost-Keremi-Pishva	20-161-W
Poost-Sefid-Najafabad	8-61-W	Tokhm-Save dar-Kan	20-162-N
Dane seah-Isfahan	8-62-N	Maroof be ghomi-Kan	20-163-WS
Shirin Gar-Najafabad-Isfahan	8-63-W	Malas-Kan	20-164-WS
Mamuli-Kouhpayeh-Isfahan	8-64-WS	Ghaojagh-Shahpar-Varamin	20-165-WS
Bihaste-Isfahan	8-65-N	Shahpar-Pishva-Varami	20-166-WS
Bezi-Isfahan	8-66-W	Poost-Nazok-Saghand	21-167-W
Torsh-Isfahan	8-67-S	Poost-Koloft-Saghand	21-168-W
Haste riz-Najafabad	8-68-W	Daneh-Ghermez-Saghand-Yazd	21-169-WS
Bi haste-Shirin-Khabar	9-69-W	Bafti-Poost-Koloft-Saghand	21-170-N
Dandedar-Khabar-Baft	9-70-W	Tafti-Marvest-Yazd	21-171-S
Haste dar-Khabar-Baft	9-71-W	Bafti-Poost-Nazok-Saghand	21-172-W
Kam bar-Khabar-Baft	9-72-N	Karche-Tafti-Torsh	21-173-S
Poost-Sefid-Khabar	9-73-W	Se-anbeli-Taft-Yazd	21-174-S
Shahvar-Poost-Nazok-Baft	9-74-WS	Zagh-Poost-Ghermez-Saghand	21-175-W
Khodroo-Vahshi-Baft	9-75-N	Togh-Gardan-Torsh-Yazd	21-176-N
Daneh-Ghermez-Ravar	9-76-S	Meikhosh-Ardekan	21-177-W
Vahshi-Narak-Shahdad	9-77-W	Mamulii-Saghand-Yazd	21-178-W
Togh-Ravari-Malas	9-78-WS	Poost-Sefid-Chakchak-Ardekan	21-179-W
Dopayeh-Rize-Ravar	9-79-WS	Malas-Torsh-Yazd	21-180-WS
Meikhosh-Haste-Rize-Shahdad	9-80-WS	Teloz-Shirin-Yazd	21-181-W
Meikhosh-Soorati-Rafsanjan	9-81-W	Abanmahi-Torsh-Yazd	21-182-S
Daneh-Ghermez-Sirjan	9-82-WS	Zagh-Karche-Torsh-Yazd	21-183-S
Golabi-Poost-Ghermez-Ravar-Torsh	9-83-W	Zoodras-Yazd	21-184-N
Haste-Rize-Baft	9-84-W	Garche-Shabar-Shirin-Yazd	21-185-W
Golnar-Farsi-Shahdad	9-85-N	Gabri-Yazd	21-186-W
Bihasteh-Chenje-Rijab	10-86-W	Malas-Ardekan	21-187-WS
Poost-Nazok-Rijab	10-87-WS	Poost-Seah-Yazd	21-188-W
Ghomi-Poost-Nazok-Rijab	10-88-N	Torsh-Yazd	21-189-S
Maroof be sheryan-Ghasreshirin	10-89-W	Garche-Dadashi-Poost-Nazok-Ashkzar	21-190-W
Poost-Sfid-Ghasreshirin	10-90-N	Shour-Poost-Koloft-Saghand	21-191-W
Shahvar-Ghasreshirin	10-91-N	Zagh-Ardekan	21-192-W
Ghomi-Poost-Ghermez	10-92-W	Shahvar-Dadashi-DarajeYek-Ashkzar	21-193-W
Shahrbani-Torsh-Rijab-Bakhtaran	10-93-S	Zagh-poost-Sefid-Ashkzar	21-194-W
Poost-Sefid-Rijab	10-94-S	Koohi-Siri-Tabas-Torsh	21-195-S
Poost-Koloft-Rijab	10-95-W	Koohi-Siri-Tabas	21-196-WS
Poost-Koloft-Rijab-Bakhtaran	10-96-S	Nabati-Poost-Sefid-Ashkzar	21-197-W
Razhnar-Ravansar-Paveh	10-97-S	Ratki-Daneh-Sefid-Bafgh	21-198-W
ShirinPaveh	10-98-W	Dadash-Peivandi-Ashkzar	21-199-WS
Shirin-Nar-Paveh	10-99-W	Kartchi-Por Bar-Bafgh	21-200-WS
Mamoli-Birjand	11-100-N	Poost-Nazok-Zanjan	22-201-WS
Malas-Sabzevar	12-101-WS	Shahvar-Miveh-Dorosht-Zanjan	22-202-W

*Province code: (1) Chahar-Mahall-va-Bakhtiari, (2) East-Azarbayejan, (3) Elam, (4) Fars, (5), Ghom, (6) Gilan, (7) Hormozgan, (8) Isfahan, (9) Kerman, (10) Kermanshah, (11) Khorasan-Gonubi, (12) Khorasan-Razavi, (13) Khuzestan, (14) Kordestan, (15) Lorestan, (16) Markazi, (17) Mazandaran, (18) Semnan, (19) Sistan-baluchestan, (20) Tehran, (21) Yazd, (22) Zanjan

**Taste: (S) Sour, (W) Sweet (WS) Sweet-Sour, (N) Unknown

Tabe 2: Number of amplified alleles, number of effective alleles, polymorphism information content (PIC) and Major allele frequency of tested simple sequence repeat (SSR) primers

Primer	No. of alleles	No. of effective alleles	PIC	Major allele frequency
ABRII-MP26	9	2.102	0.48	0.48
ABRII- MP30	2	1.934	0.48	0.48
ABRII-MP51	3	1.913	0.42	0.42
ABRII-MP28	3	1.983	0.44	0.44
ABRII-MP12	2	1.025	0.024	0.024
ABRII-MP07	2	1.010	0.01	0.01
ABRII-MP39	2	1.999	0.56	0.56
mean	3.28	1.709	0.3487	0.3487

Table 3: Marker-trait association detected in pomegranate genotypes through multiple regression analysis

Plant characteristics	Locus	Adjusted R^2	P-value
Fruit height	MP26-2,MP26-8,MP39-22	14	000.0
Fruit diameter	MP26-2	4.0	0.012
Calyx height	MP26-2,MP30-11,MP51-13,MP51-16,MP07-21	29	0.000
Calyx diameter	MP30-10	3.0	0.028
Fruit height/Diameter ratio	MP26-2	5.0	0.005
Calyx height/Diameter ratio	MP30-11, MP28-16,MP07-21	27	0.000
Fruit shape	-	-	-
Calyx shape	MP26-2	2.0	0.036
Fruit size	MP26-2	6	0.002
Fruit taste	MP28-16	2.0	0.041
Sunburn sensitivity	MP26-3	4.0	0.013
Hull cracking sensitivity	-	-	-
Skin color	-	-	-
Calyx type	-	-	-
Flower height	MP26-2, MP26-5, MP28-3	11	0.000
Flower diameter	MP12-2	6.0	0.000
Style height	MP12-2	8.0	0.000
Flower height/Diameter ratio	MP26-5, MP26-6, MP28-2	11	0.000

to nine for mp26 with an average number of 3.28 alleles per locus. Major allele frequency ranged from 0.4 to 0.99 with a mean of 0.7. The polymorphic information content (PIC) of the markers varied from 0.01 to 0.5 with an average of 0.34. Marker mp39 revealed the highest PIC 0.56, while marker mp07 had the lowest PIC of 0.01 (Table 2).

Regression analyses showed significant regression of each of the 14 traits (excluding fruit shape, calyx type, Hull cracking sensitivity and skin color) on a total of 14 of the 23 polymorphic SSR bands. Details of regression analysis are available in table 3. Significant association was observed for 14 of 23 polymorphic markers with at least one of the 18 traits. The number of marker associated with individual traits ranging from 1-5 markers (Table 3). Among these 18 traits, Fruit diameter, Calyx diameter, Fruit height/Diameter ratio, Calyx shape, Fruit size, Fruit taste, Sunburn sensitivity, flower diameter and style height showed significant regression on only one SSR marker, while calyx height showed significant regression on 5 SSR markers. The association markers explained2% to29% of the variation for individual traits. The most variation of calyx height (0.29) was accounted by MP30-11, MP51-13, MP51-16 and MP07-21. MP26 marker was linked with most traits such as: Fruit height, Fruit diameter, Calyx height, Fruit height/Diameter ratio, Fruit size, Calyx shape and Sunburn sensitivity in this research. SSR loci associated with fruit diameter, calyx shape and fruit size was the same. Besides, our results showed that there is a significant and positive correlation among these traits (data not shown).MP12 marker associated with style height, in this study.

DISCUSSION

The mean number of alleles for each microsatellite locus (3.28) in this study is higher than some previous research in pomegranate. For example, an average of 2.77 [37] and 2.44 [38] alleles per locus has been reported for pomegranate genotypes. The high number of amplified alleles and relatively high PIC value in this experiment show that the microsatellite markers used in this study is a useful tool for differentiation of genotypes in pomegranate.

In this study, some of the markers were found to be associated with more than one trait in multiple regression analysis. Such an association may arise due to pleiotropic effect of the linked QTLon different traits [39-41]. However, for better understanding of these relationships, preparation of segregating population and linkage mapping can be useful [42]. Closely linked QTLs affecting different traits may also lead to a single marker showing association with multiple traits which would be reflected in correlations between such traits. This study has identified one informative SSR marker ABRII-MP26linked with majority of the traits studied in this research which can be used in breeding program of pomegranate.

Style height in pomegranate flower is one of the important traits in this species because flowers with long style in pomegranate are known as hermaphrodite flower (fertile= perfect) and are developed to fruit but the male flowers produce well-developed mal parts, but rather have degenerated female parts. Male flowers typically drop and fail to set fruit [5, 43]. On the other hand, there is a positive correlation between the bearing capacity and the percentage of perfect flowers [44, 45]. Association between MP12 and style height will be introduced this marker as highly reliable marker in breeding program for providing high performance genotypes in pomegranate.In the present study also we found significant correlation between fruit diameter, calyx shape and fruit size (data not shown). This correlation was also evident in shared associated markers for these traits. For example, in multiple regression analysis, ABRII-MP26 marker was found to associate with these traits.

Many possibilities for future research can be suggested from this study, according to the results all SSR markers used in this study, particularly ABRII-MP26 marker, showed significant association with at least one of the 18 traits; therefore, it is possible to use these markers along with morphological traits in pomegranate breeding programs for identification of suitable parents to produce mapping populations and hybrid varieties. Also, since the marker-trait association identification will play an important role in plant MAS/QTL breeding programs, especially in plants that genetic information such as linkage map and Quantitative Trait Loci is not available about them, it would be interesting to identification the new SSR markers for an effective MAS and to achieve the optimal results in pomegranate.

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