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Evaluation of Genetic Diversity among South Tunisian Pomegranate (*Punica granatum* L.) Accessions Using Fruit Traits and RAPD Markers

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ABSTRACT

Morphological and RAPD markers were used to investigate the genetic diversity among 21 accessions of pomegranate originating from South Eastern Tunisia. Thirteen morphological traits were studied and results showed significant differences for all morphological characters (P< 0.001). Clustering based on fruit traits, using Ward's method, divided the accessions into three main groups. In RAPD analysis, 6 out of 15 employed random primers showed good amplification and polymorphism on pomegranate samples with a total of 63 bands, of which 56 were polymorphic. The lowest percentage of polymorphism (50%) was observed with TIBMBA-03 while the highest (50%) was observed with primer TIBMBB-03. According to Jaccard coefficient, the lowest (0.29) and highest (0.94) similarities were detected between genotypes. UPGMA clustering based on data from polymorphic RAPD bands resulted in three clusters at a similarity of 0.46. The Stress value for the nonmetric multidimensional scaling plot was 0.071, showing an excellent representation of the data. The comparison between groupings based on the fruit traits and RAPD data did not produce a significant correlation (r= -0.09). Using a stepwise linear regression, significant regressions were found between 13 morphological traits and 63 molecular markers revealing association between RAPD molecular markers and some traits.

Keywords: Morphology, Random primers, Fruit traits, RAPD markers, Regression association.

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the *Punicaceae* family and is a suitable crop for cultivation and land validation in arid and semi-arid regions with high salinity in soils and water resources. It is an important commercial fruit crop that is extensively cultivated in parts of Asia, North Africa, the Mediterranean, and the Middle East (Sarkhosh *et al*., 2006). Tunisia is one of the most important pomegranate producers and exporters in the world; with the main production centers in Gabes, Gafsa, Cap bon, the region of Bizerte, and Sousse in the Sahel.

The production and consumption of pomegranate have increased worldwide due to its usage in various fields. For breeding and commercialization of promising pomegranate cultivars, a precise determination and discrimination of the genotypes is needed. Selection is the most important activity in all tree breeding programs. Study on fruit characters was the prevalent method for genotype identification in fruit trees such as pomegranate, however, these traits are mostly affected by environmental and cultivation conditions and often do not result in a clear discrimination (Struss *et al*., 2001). Application of DNA based on molecular markers is the solution for the need of a clear discrimination between genotypes and

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cultivars. Various approaches are available for DNA fingerprinting such as: Amplified Fragment Length Polymorphism (AFLP) (Ipek *et al.*, 2003), Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeat (SSR) (Hasnaoui *et al*., 2012), and Inter Simple Sequence Repeat (ISSR) markers (Zahuang *et al.*, 2004). During recent years, the use of Random Amplified Polymorphic DNA (RAPD) for resolving the genetic diversity has been remarkably increased in fruit trees (Sesli and Yegenoglu, 2009; Sarabi *et al*., 2010). This technique is characterized by its simplicity, which allows detection of differences at the DNA level using small amounts of genomic DNA. RAPD does not require any information on DNA sequences and has been previously used by several authors in the development of genetic maps of the pomegranate (Ercisli *et al*., 2007) and other species including *Arabidopsis*, lucerne and barley (Beigi *et al*., 2013).

The variety Gabsi is one of the well-known pomegranate cultivars with very appreciable sensory quality originating from South East of Tunisia. The main objective of the present study was to evaluate the genetic diversity of 21 pomegranate accessions by morphological traits and RAPD molecular markers, and also to find any associations between morphological traits and RAPD data.

MATERIALS AND METHODS

Plant Material

A total of 21 pomegranate accessions (Table 1) were used in this study. Leaf and fruit samples were collected from mature trees originating from six oases in the region of Gabes in the South-East of Tunisia, which is characterized by an arid bioclimate typical for the Mediterranean with a mild winter. The leaf samples were washed three times in sterile distilled water, frozen in liquid nitrogen, and kept at -60°C until use.

Fruit Characterization

A sample of 10 fruits per plant were harvested in full maturity to determine the variables presented in Table 1 that were previously reported to be important in pomegranate evaluation (Sarkhosh *et al*., 2006; Vinson *et al*., 2001). Qualitative traits were coded as the following: peel color (1: Yellow; 2: Green; 3: Pink, 4: Red), aril color (1: White; 2: Pink; 3: Red, 4: Red-purple), seed hardness: (1: Soft; 2: Semi-soft, 3: Hard).

Molecular Characterization

Total genomic DNA was extracted using CTAB based method of Doyle and Doyle (1990). DNA quantity and quality were determined using spectrophotometer (PerkinElmer, Lambda EZ201, USA) at 260 and 2% agarose gel electrophoresis, respectively.

Fifteen 10-mer primers (TIBMOLBIOL Co., Germany) were used for screening of the accessions in this study.

The reaction mixture of 20 µL contained 10 ng of genomic DNA, 2.5 µL 10X Taq DNA polymerase buffer, 10 µM of a single primers, 0.6 µL of each dNTP (2 mM) and 0.2 µL of Taq DNA polymerase (5 U μL^{-1}). Amplifications were carried out using a Cleaver Scientific Thermocycler. The PCR programm included an initial denaturation step at 94°C for 5 min, followed by 45 cycles of 1 minute at 94°C, 1 minute at 43.5°C, 1 minute at 72°C, and final extension at 72°C for 7 minutes.

After amplification, the DNA fragments were separated by electrophoresis for about 2h under constant voltage of 60V in 3% agarose gel submersed in 1X TBE buffer. The gels were stained with ethidium bromide solution and observed under ultraviolet light. Each gel was photo documented using the image capturing system bio print.

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Table 1. The studied pomegranate accessions, their origin and the mean of measured morphological traits.

Accessions	Code	Origin	Fruit weight (g)	Peel thickness (mm)	Percentage of peel (%)	100 Aril weight (g)	Percentage of aril (%)	Volume of juice (%)	PH	TA[a] (%)	TSS[b] (%)	Seed hardness	Aril color	Peel color
P[c]			***	***	***	***	***	***	***	***	***			
GME1	1	Metouia	283.67[d]	5.12	34.45	18.49	62.77	71.00	3.73	0.20	14.60	2	2	4
GME3	3	Metouia	326.17	3.23	29.77	17.25	67.04	76.13	3.51	0.35	15.00	3	3	2
GME5	2	Metouia	222.50	3.23	38.30	17.31	59.04	77.00	3.45	0.43	13.80	3	3	3
GO1	4	Ouedhref	377.00	4.44	41.27	13.60	57.73	77.47	3.46	0.42	15.20	1	1	4
GO2	5	Ouedhref	442.67	3.35	35.03	11.30	62.97	80.27	3.34	0.42	12.20	2	3	4
GO3	6	Ouedhref	499.67	3.83	37.22	13.90	61.45	76.50	3.45	0.20	17.47	2	2	4
GG1	7	Gabes	363.33	3.69	29.04	14.70	68.96	74.93	3.23	0.50	14.20	1	2	3
GG4	8	Gabes	479.17	4.23	26.67	21.87	71.33	82.77	3.23	0.51	14.83	2	2	2
GG5	9	Gabes	245.00	5.07	21.14	21.71	77.19	80.00	3.03	1.14	13.77	3	1	2
GC1	10	Chenini	348.83	3.13	27.10	26.93	70.57	82.73	3.27	0.44	14.93	3	3	1
GC2	11	Chenini	472.83	2.93	26.29	27.53	71.38	85.50	4.34	0.20	13.80	2	4	1
GC3	12	Chenini	429.00	3.55	24.16	22.93	73.84	83.13	4.44	0.19	16.10	1	2	3
GC5	14	Chenini	419.17	4.07	30.08	27.10	67.92	85.33	4.22	0.19	16.80	1	2	3
GC6	13	Chenini	537.83	3.35	24.02	26.67	73.98	77.03	4.49	0.18	17.70	1	1	3
GM1	15	Mareth	428.33	3.26	27.22	23.93	71.11	70.00	4.60	0.17	16.57	1	1	3
GM2	16	Mareth	401.17	3.79	23.26	24.83	75.07	71.03	3.63	0.22	15.27	2	2	3
GM3	17	Mareth	410.50	3.85	19.10	18.47	78.90	65.00	3.56	0.21	13.73	2	1	2
GM4	18	Mareth	349.00	4.00	22.26	17.15	75.74	80.33	3.26	0.44	13.60	2	2	3
GK1	21	Kettana	328.83	3.75	22.73	22.27	75.61	76.43	3.41	0.42	14.03	2	2	4
GK2	19	Kettana	350.67	3.24	31.04	20.75	66.63	64.67	3.47	0.42	14.53	3	4	2
GK4	20	Kettana	418.83	4.55	27.62	18.03	70.38	77.80	3.53	0.34	13.97	1	2	4

[a] Titrable Acidity; [b] Total Soluble Sugar; [c] *P* values were determined by Fisher's exact test, *** significant at 1%, [d] Values are average of three individual samples.

Statistical Analysis

For morphological analysis, data processing was performed using SPSS software (version 18.0). A variance analysis (ANOVA) was done for the quantitative morphological characters. Results were significant when $P< 0.05$. Mean values recorded for each parameter were used to classify the accessions into similarity groups using cluster analysis with Ward's method.

For molecular analysis, NTSYS software, version 2.1 was used. Amplified fragments were classified as present (represented with 1) or absent (represented with 0) (Du *et al*., 2001). The binary data so generated was used to estimate the levels of polymorphism, by dividing the polymorphic bands by the total number of scored bands, and the pairwise genetic distances between genotypes using Jaccard coefficient in the SIMQUAL (Staub *et al*., 2000).

The polymorphism information content (PIC) was calculated by the formula: $PIC = 1 - \sum_{j=1}^{n} Pij^2$ (Smith *et al*., 2000), where, *Pij* is the frequency of the J^{th} RAPD pattern for marker *I* and the summation covers *n* patterns. The ability of the primers to discriminate among accessions was assessed using the resolving power (Akbarpour *et al*., 2010) coefficient (Prevost and Wilkinson, 1999) that has been described to correlate strongly with the ability of loci to distinguish between accessions. The formula is according to Gilbert *et al*. (1999): $Rp= \sum I_b$ where $I_b= 1-(2x|0.5-p|)$, where, *p* is the proportion of genotypes having present (1) band.

To estimate the relationships among the genotypes, a dendrogram was constructed using the similarity matrix data applying the UPGMA method with the SHAN module of NTSYS-pc (Rohlf, 1994) and the MDSCALE to identify the number of groups based on the original binary data matrix. The lack of agreement was measured by a statistic called Stress value (Krzanowski, 1988). The original genetic distance matrix was compared to the corresponding cophenetic value matrix using the MXCOMP algorithm of the NTSYS in order to compute the cophenetic correlation, i.e. to test the goodness of fit of the cluster analysis to the similarity matrix. The stepwise regression analysis was performed by SPSS software to evaluate the association between 13 morphological traits and RAPD data of 6 polymorphic primers.

Similarity between matrices, based on different marker system (morphological and RAPD), was calculated using the standardized Mantel coefficient (Mantel, 1967).

RESULTS AND DISCUSSION

Fruit Traits

Mean values of the morphological and chemical characteristics studied are shown in Table 1. The analysis of variance (ANOVA) showed highly significant differences between accessions for all studied variables ($P< 0.001$). Significant positive and negative correlation coefficients were observed among characteristics (data not shown).

Grouping of accessions based on fruit traits at distance of 10 allows grouping them into three main clusters (Figure 1). The first cluster (A) holds 5 accessions (GG1, GO1, GC3, GC5 and GM1) all having soft seeds. The second group (B) was formed by only one accession (GG5) characterized by the smaller fruit, the thickest peel, the highest acidity and the lowest TSS. It has white arils and hard seeds. The third cluster (C) included 3 sub-groups. The first composed of 8 individuals (GME3, GME5, GO2, GG4, GC1, GC2, GM4 and GK1) which have the thinner peel and the highest volume of juice and high acidity. Their seeds are hard or semi hard with red or red-purple arils. The second was formed by only two accessions (GO3 and GC6) characterized by the highest fruit height and TSS and the lowest acidity. The third contained 5 genotypes (GM3,

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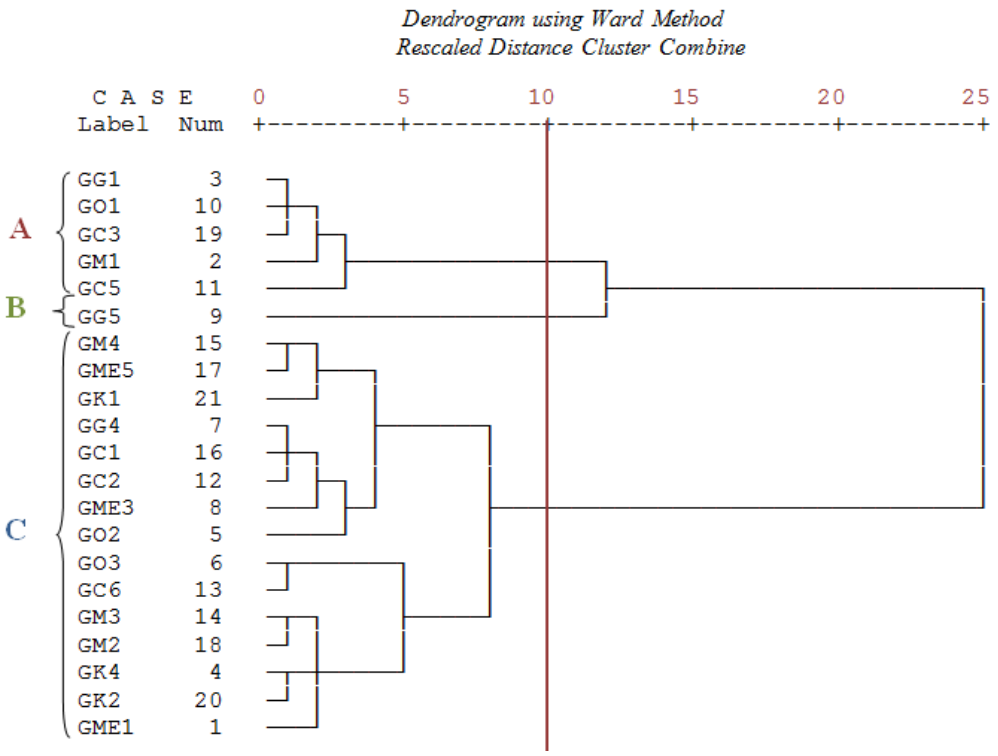

Figure 1. Dendrogram of genetic distance for 21 accessions based on morphological traits using Ward's method by SPSS software. Codes are in accordance with Table 1.

GM2, GK4, GK2 and GME1) characterized by the lowest volume of juice.

Morphological polymorphism plays an important role in the conduct of breeding programs and genetic mapping (Huguet *et al*., 2004). Many characteristics vary between pomegranate genotypes and they are the key to identify the consumer preference and the way for valorization. The most important traits are fruit size, peel color (ranging from yellow to purple, with pink and red as the most common colors), aril color (ranging from white to red-purple), hardness of the seed, juice content, acidity, sweetness, and astringency. Large fruit, red aril, red colored skin, and soft seed are among the desirable traits that could be considered in pomegranate breeding programs for selection of superior cultivars (Zamani *et al*., 2010). The peel thickness is also among the main selection criteria; fruits with thin peel are intended for processing, while those with thick peel that provides resistance to transport and storage are selected for fresh consumption. High juice content is a desirable attribute in pomegranate production, and other fruits, and it is the most important parameter from an industrial point of view (Cassano *et al*., 2004; Maestre *et al*., 2000). Referring to the parameters studied in this work, outlines of valorization can be advanced. The accessions GG1, GO1, GC3, GC5, GM1, GO3 and GC6 seem to be the most promising for fresh consumption since they are characterized by a medium size fruit with a thick red colored peel. They have also soft seed with low acidity and high soluble solids contents.

The accessions GME3, GME5, GO2, GG4, GC1, GC2, GM4, GK1, and GG5 have a smaller fruit with a thinner peel, their seeds are hard, the arils are red or dark red giving an abundant juice relatively rich in

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sugar with high acidity. Those properties make these accessions desirable for transformation.

RAPD Molecular Markers

Among 15 random 10-mer primers only 6 primers yielded reproducible and clear polymorphic banding profiles on these accessions (Table 2). These primers generated a total of 63 RAPD bands (Table 2), among which 56 (88.88%) were polymorphic and only 7 fragments were monomorphic, indicating a high degree of polymorphism among these pomegranate accessions. The number of bands generated per primer varied from 6 to 25.

The resolving power coefficient varied between 1.33 and 9.62. TIBMBA-08 was the primer with the most resolving power screened. This value was higher than that obtained by Zamani *et al*. (2010) with the primer TIBMBB-14. The lowest percentage of polymorphism (50%) was observed with TIBMBA-03 primer while the highest (100%) was observed with primer TIBMBB-03 with an average of 84.35%. Zamani *et al*. (2010) and Sarkhosh *et al*. (2009) found that the averages of genetic diversity, based on RAPD analyses of 36 population and 21 accessions from Iran, were 21.18 and 29.45%, respectively. The degree of RAPD polymorphism detected was relatively higher than that reported in melon (18%; Garcia-mas *et al*., 2000), watermelon (21%; Lee *et al*., 1996), pumpkin (23%; Gwanama *et al*., 2000), and ash gourd (28%; Sureja *et al*., 2006). The size of amplified fragments ranged between 180 to 1800 bp for all primers.

The polymorphism information content (PIC) allows us to measure the level of polymorphism of each marker. *PIC* values fluctuated from 0.80 for TIBMBA-03 to 0.93 for the most polymorphic locus TIBMBA-08 with an average of 0.85 indicating a high level of polymorphism for all loci. This high value reflects the efficiency of the markers used to study the genetic diversity of 21 pomegranate accessions (Table 2).

Jaccard's genetic similarity coefficient varied from 0.29 to 0.94. Sarkhosh *et al*. (2006) also found similar results. The lowest similarity of 0.26 was measured between GM2 (N°16) and GK2 (N°20). These two accessions can be considered genetically distant. The highest Jaccard's genetic similarity coefficient (0.94) was observed between the accessions GME5 (N°3) and GO2 (N°5). They are considered genetically very close or similar.

The UPGMA cluster based on RAPD data divided the accessions into three main groups at 0.46 similarity coefficient (Figure 2). The first cluster consisted of eleven

Table 2. Characteristics of RAPD banding profiles obtained among the 21 studied pomegranate accessions.

Primer	Number of amplified bands	Number of polymorphic bands	% of polymorphism	MW [b] min-max (pb)	Resolving Power (RP)	PIC [c]
TIBMBA-03 [a]	6	3	50	180-800	1.33	0.80
TIBMBA-06	7	6	85.71	380-800	3.14	0.82
TIBMBA-07	8	7	87.5	290-1200	3.9	0.84
TIBMBA-08	25	23	92	230-1800	9.62	0.93
TIBMBB-03	6	6	100	410-750	3.43	0.81
TIBMBB-09	11	10	90.90	180-1750	5.05	0.89
Total	63	56	88.88	180-1800	26.47	5.09
Mean	10.5	9.16	84.35		4.41	0.85

[a] TIB Molbiol, [b] Molecular Weight, [c] Polymorphism Information Content

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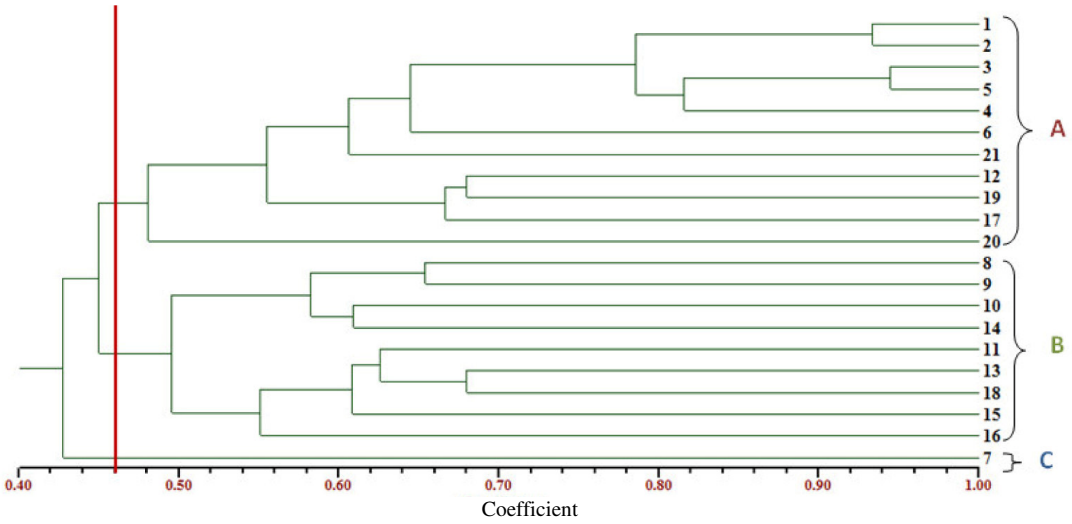

Figure 2. Dendrogram derived from UPGMA clustering analysis using Jaccard's coefficient of similarity of RAPD marker. Codes of accessions are in accordance with Table 1.

individuals, which were the accessions of Metouia, Ouedhref, Kettana, one accession from Chennini, and one from Mareth. They were characterized by a number of bands between 14 and 24 with a molecular weight between 180 to 1,800 pb. The second group contained nine accessions with a higher number of bands (18 to 25 and a molecular weight from 180 to 1,800) and characterized by the total absence of the band of size 1,050 bp. The third group included the accession GG1, which had 14 bands with molecular weight varying from 200 to 1,750 bp. Cophenetic correlation coefficient indicated a correlation of r= 0.80 between the similarity matrix and cophenetic matrix measured from the dendrogram data, indicating a good fit of the cluster analysis to the similarity matrix among the studied genotypes. Similar results were found by Sarkhosh *et al*. (2006; 2009) and Zamani *et al*. (2010). Other methods were used for the calculation of pairwise genetic distances between genotypes as DICE and Simple Matching (SM) coefficients (results not shown). Correlation between similarity matrix obtained by Jaccards and the other two methods (DICE and SM) were computed showing significant correlations r= 0.99 and r= 0.92, respectively.

MDSCALE analysis reflected the confidence of the diversity of the accessions that were divided into three distinct groups (Figure 3). The first group comprised 4 accessions all having soft seeds and red peel. The second group contained the majority of the accessions (7 accessions) all having semi hard seeds. The third group had five accessions which were geographically close. Other accessions were classified individually. The Stress value for the nonmetric multidimensional scaling plot was 0.071, reflecting the large number of data points. Indeed, Kruskal (1964) considered that the values less than 0.1 indicated an excellent representation of the data.

The correlation between derived similarity matrix from morphological and molecular data was not significant (r= -0.09). In group 1, there were 11 pomegranate accessions with different morphological characteristics; most of them had red peel, pink aril and semi-soft seed, but there were also genotypes with pink, green, and yellow peel, white, red, and red-purple arils and soft and hard seeds. The accessions of group 2 revealed large variation for the morphological parameters (Table 1). This result indicated that the cultivars within cluster 1 were genetically closer to each

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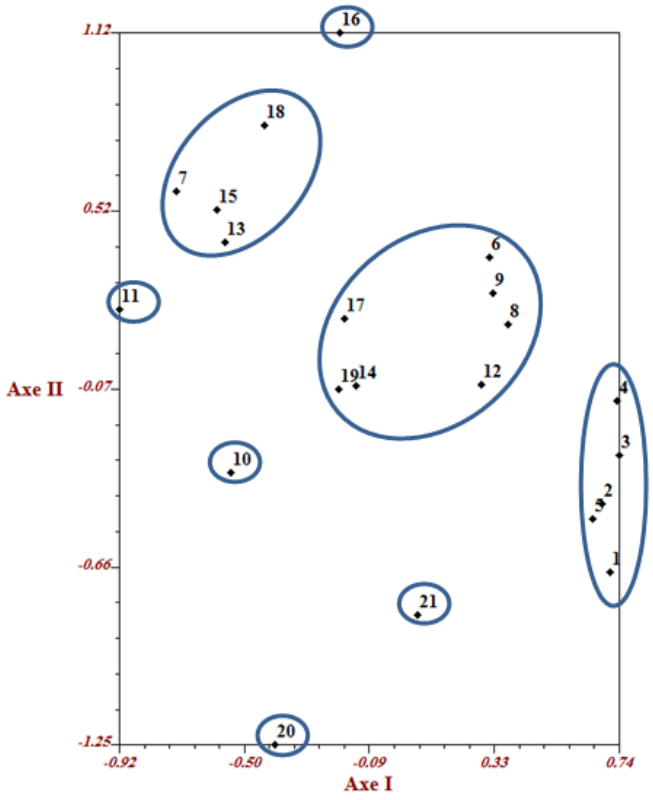

Figure 3. Nonmetric multidimensional scaling analysis of Jaccard coefficient between 21 accessions of pomegranate (The Stress value is 0.071).

other than to the other cultivars. GG1 (N°7), which belonged to the second group according to fruit traits, was grouped individually. Differences between morphological and RAPD clustering have been reported for Iranian pomegranate (Zamani *et al*., 2010) and other fruits such as olive (Hagidimitriou *et al*., 2005) and banana (Uma *et al*., 2004). These findings support the view that morphopomological characteristics are not reliable in estimating genetic relationships among large and diverse groups of cultivars and should be used mainly for discrimination. This can be related to many reasons; one of which is the effects of different climatic conditions on morphological traits, which do not influence RAPD markers (Kumar, 1999; Gupta and Rustgi, 2004).

Associations between morphological traits and RAPD markers are shown in Table 3. Significant regressions were observed for each 13 measured traits and 63 molecular markers, by using stepwise linear regression. TSS, seed hardness, peel and aril colors traits showed significant regression with only one molecular marker, while pH and the Titratable Acidity (TA) showed high significant regression with eleven and nine markers, respectively. The highest R^2 was related to 4 markers associated with aril weight (55.6%) and, among them, TIBMBA-06_{550} had the maximum R^2. The percentage of peel and the titratable acidity traits contained also a relatively high adjusted R^2 with, respectively, 41.3 and 41.1%. It is possible that the location of these markers in the genome may coding to the genes related to these traits and could be used for future breeding programs.

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Table 3. Regression analysis for 13 fruit traits and RAPD markers in pomegranate accessions.

No.	Trait	RAPD			
		Number of markers	Main marker	R^2 (%) max	R^2 (%) total
1	Fruit weight	4	TIBMBB-09_{290}	21.8	54.5
2	Peel thickness	2	TIBMBA-08_{670}	18.2	35.8
3	Percentage of peel	4	TIBMBB-09_{180}	41.3	72.2
4	Aril weight	4	TIBMBA-06_{550}	55.6	79.2
5	Percentage of aril	7	TIBMBB-09_{1750}	28.3	90.5
6	Volume of juice	5	TIBMBB-09_{790}	19.6	80.6
7	pH	11	TIBMBA-07_{1000}	31.1	99.3
8	Titrable acidity	9	TIBMBB-09_{550}	41.1	95.4
9	TSS	1	TIBMBA-08_{462}	36.3	36.3
10	TSS/TA	3	TIBMBA-08_{1200}	27.4	54.9
11	Seed hardness	1	TIBMBA-08_{660}	23.8	23.8
12	Aril color	1	TIBMBA-07_{575}	27.6	27.6
13	Peel color	1	TIBMBA-08_{690}	40.8	40.8

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ارزیابی تنوع ژنتیکی بین انارهای نمونه جنوب تونس با استفاده از صفات میوه و نشانگر هایRAPD

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چکیده

برای بررسی تنوع ژنتیکی بین ۲۱ نمونه ثبت شده انار که مبداء آنها جنوب شرقی تونس بود از نشانگر های شکلی و RAPD استفاده شد. سیزده صفت شکلی بررسی شدند و نتایج تفاوت های معنی داری ($p<0.001$)برای همه ویژگی های شکلی نشان داد . خوشه بندی بر مبنای صفات میوه با استفاده از روش وارد(Ward) نمونه ها را به سه گروه دسته بندی کرد. در تجزیه RAPD، ۶ مورد از ۱۵ پرایمر اتفاقی، تکثیر و چند شکلی خوبی در نمونه های انار نشان دادند با تعداد کل ۶۳ باند که ۵۶ تای آنها چند شکلی بودند. کمترین درصد چند شکلی (۵۰٪) با پرایمر TIBMBB-03 مشاهده شد.بر اساس آزمون ژاکارد، کمترین (۰/۲۹) و بیشترین (۰/۹۴) مشابهت ها بین ژنوتیپ ها یافت شد. خوشه بندی UPGMA بر مبنای باند های چند شکلی RAPD منجر به سه خوشه با مشابهت۰/۴۶ شد. مقدار استرس(Stress) برای مقیاس چند بعدی غیر متریک ۰/۰۷۱ بود که نمایندگی بسیار خوبی از داده ها نشان می داد. مقایسه بین گروهبندی ها بر مبنای صفات میوه و داده هایRAPD همبستگی معنی داری به دست نداد ($r = -0.09$). با استفاده از رگرسیون گام به گام، رگرسیون معنی داری بین ۱۳ صفت شکلی و ۶۳ نشانگر مولکولی به دست آمد که همراهی بین بعضی نشانگرهای مولکولی و بعضی صفت ها را آشکار کرد.

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