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Full Length Article

Evaluation of Genetic Diversity among Jordanian Pomegranate Landraces by Fruit Characteristics and Molecular Markers

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Abstract

The level of variation in various fruit traits was described among 14 Jordanian pomegranate landraces and the genetic relatedness was investigated using RAPD and SSR markers. Euclidean distances among studied landraces ranged from 3.33 to 12.01, with a mean of 7.65. Fruit and aril traits explained the variation in the first component (28.92%), while other traits were present in the second (17.615) and third (12.81%) component, and therefore contributed less to the variability. Genetic distances based on RAPD scores ranged from 0.24 to 0.66, indicating that considerable level of divergence exists among studied pomegranate landraces. The set of SSR markers used in this study was monomorphic, which might be due to the fact that available SSR markers are too few to identify polymorphic SSR markers to differentiate between landraces present within a small geographic area. Multivariate analysis showed that dendrograms constructed based on fruit related traits as compared with that based on RAPD scores were not consistent. Pomegranate landraces displayed high variability in fruit and aril related traits, which could be considered a valuable source of genes for commercial uses. Results revealed the presence of small seeded variety that has large arils. High morphological and RAPD variation exist among Jordanian pomegranate landraces could be exploited in pomegranate breeding. © 2016 Friends Science Publishers

Keywords: Diversity; Fruit traits; Pomegranate; RAPD; SSR

Introduction

Pomegranate (*Punica granatum* L.) is considered one of the ancient and sacred fruit tree in the Mediterranean zone (Stover and Mercure, 2007), it is believed that the center of origin of pomegranate is the region extended from Iran to the Himalayas in northern India, and then it spread to other parts of the world (Levin, 1994). Pomegranate tree is well known in the Mediterranean countries where high diverse genetic resources are still available in Syria, Lebanon, Jordan and Arab Peninsula (Barone *et al*., 2001; Sarkhosh *et al*., 2009). Pomegranate were used as antimicrobial and antimutagenic agent and has an antioxidant activity (Negi *et al*., 2003; Seeram *et al*., 2005), and has been used in commercial cosmetics preparation and could be utilized as therapeutic agent (Kim *et al*., 2002).

Pomegranate landraces in the world display high diversity level in fruit traits such as fruit size and external fruit colour, juice taste and colour, seed hardiness, pest resistance and times to maturity (Barone *et al*., 2001; Sarkhosh *et al*., 2009). Aril is the edible part of the fruits and constitutes about one half of total fruit mass (Sarkhosh *et al*., 2006). Aril is very variable in taste, in arils colour, and in seed hardness (Zamani, 1990; Sarkhosh *et al*., 2009).

Varietal identification in pomegranate is based mainly on fruits external and internal features (Barone *et al*., 2001; Sarkhosh *et al*., 2009) however, it is not an easy task for many reasons. Firstly, the duration required from seedling until the tree bears fruit is too long (4-5 years in average) and quantitative traits used for varietal identification vary according to the prevailing environmental conditions (Melgarejo *et al*., 2000). DNA-based markers are not affected by environment and molecular genetic tools may help to quickly and precisely characterize plant varieties. Restriction Fragment Length Polymorphisms (RFLPs) of the total genome (Melgarejo *et al*., 2009) and Random Amplified Polymorphic DNA (RAPD) were successfully used in characterizing pomegranate germplasm (Talebi Baddaf *et al*., 2003; Sarkhosh *et al*., 2006). Awamleh *et al*. (2009) used Amplified Fragment Length Polymorphism (AFLP) technique to evaluate the level of genetic diversity among pomegranate landraces. SSR markers in pomegranate were developed and characterized by several groups (Currò *et al*., 2010; Hasnaoui *et al*., 2010; Pirseyedi *et al*., 2010).

In Jordan, pomegranate was subjected to genetic erosion during the last decades, because of the replacement of pomegranate with olive plantations and due to unavailability of water for supplementary irrigation due to successive seasons of drought. Many old pomegranate plantations have been removed and a few local varieties are propagated in commercial nurseries and used in the new

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plantations. Therefore, the objectives of this study were to: (i) estimate the level of polymorphism in economically important traits in Jordanian pomegranate fruit trees landrace collections, (ii) identify pomegranate germplasm with desirable fruit traits for pomegranate improvement, and (iii) studying the extent of DNA variation in pomegranate landraces using RAPD and SSR markers.

Materials and Methods

Localization of Pomegranate Landraces

Field trips were organized to collect stem cuttings from pomegranate landraces according to their local names from Ajloun district, Jordan. In total, 14 pomegranate landraces were localized in farmers' field, namely; 'Khashaby', 'Khdaree Hello', 'Malesse', 'Bradee Sharabee Asfar', 'Hasmasi', 'Hmaree Hmadee', 'Bradee Sharabee Ahmar', 'Zeklabee', 'Zokom Albagel', 'Khratee', 'Ahmar Hello', 'Khdaree Hmadee', 'Lfani Sharabi', 'Zarori and Esari'. The fourteen Jordanian pomegranate landraces were propagated by stem cuttings according to the conditions proposed by Owais (2010). Thereafter, rooted cuttings were planted in plastic bags to be conserved at Mu'tah University Agricultural Station *ex situ*. From each landrace, stem cuttings were collected from four trees to have 56 accessions in total.

Morphopomological Characterization

Fruits samples of pomegranate landraces were collected from farmers' field from mature trees at Ajloun, Jordan area for two successive seasons 2010 and 2011. Four trees per landrace were evaluated. Each tree was considered as a replicate. Five fruits per replication were harvested at maturity; *i.e.* 20 mature fruits in total for each landrace were evaluated. Four qualitative traits were recorded with diverse phenotypic classes including peel colour (green red, yellow red, red), aril colour (white, light pink, pink and red), seed hardiness (soft, semi-hard, hard) and juice taste (sour, sweet-sour and sweet). Twenty seven quantitative fruit traits were recorded and analyzed (Table 1). Fruit, crown and neck dimensions were initially recorded. Thereafter, fruits were weighed and carefully opened to separate arils from pericarp and internal membrane surrounding arils. Afterwards, the total number of arils and their weight per fruit were recorded. Peel total weight (pericarp) was calculated by subtracting aril weight from fruit weight. Peel percentage was calculated by dividing peel fresh weight by fruit fresh weight. Aril fresh weight percentage was calculated by dividing total aril weight by the total number of arils. Bulbs containing fresh arils were manually squeezed and then the seeds separated from other residues, washed with fresh water and dried with paper towel. Thereafter, seeds number and seed total fresh weight were recorded. Juice percentage was recorded by dividing juice weight by the total weight of fresh arils. One hundred seeds were oven-dried for 48 h at 70°C to record seed dry weights.

Amplification of DNA Fragments and PCR Conditions

Genomic DNA was isolated from individual trees (replicate) from fresh leaves using CTAB based protocol described by Murray and Thompson (1980). DNA was diluted to 5 µg/µL for PCR amplification. As an initial step, sixty two primers from Operon kits were screened to identify the primers with polymorphic amplifications as shown in Table 2 (Williams *et al*., 1990). PCR reaction was performed in 20 µL volumes under the following conditions: 20 ng genomic DNA, 250 n*M* of 10-mer primer, 200 n*M* dNTPs, 1 U Taq polymerase and 1.5 m*M* $MgCl_2$. DNA amplifications were performed using the following program: an initial denaturation step of 94ºC for 4 min, followed by 35 cycles of 92ºC for 1 min, 37ºC for 1 min, 72ºC for 2 min and a final extension at 72ºC for 10 min. Agarose gel with a concentration of 1.5% (w/v) was used to separate the PCR products using horizontal gel electrophoresis in TBE buffer (Tris–boric acid–EDTA). Amplified products were visualized under UV light using ethidium bromide staining.

SSR markers were selected from previous studies (Currò *et al*., 2010; Hasnaoui *et al*., 2010; Pirseyedi *et al*., 2010) as shown in Table 3. PCR was carried out in 20 µl volumes under the following conditions: 50 ng template DNA, 250 nM of each primer, 200 nM dNTPs, 1 U Taq polymerase and 1.5 mM MgCl2. All PCR reactions were performed as recommended in previous studies (Currò *et al*., 2010; Hasnaoui *et al*., 2010; Pirseyedi *et al*., 2010). Amplified fragments were separated using 3.5% (w/v) MetaPhor agarose gel.

Statistical Analysis

Quantitative fruit data were subjected to analysis of variance and some descriptive statistics were calculated. The level of significance was recorded for each trait. Pearson's correlation coefficients were used to determine the strength and direction of interrelationship among pomegranate fruit characteristics. All quantitative data were converted to Z-scores to avoid any differences due to scaling before multivariate analyses. Thereafter, euclidean distance matrix was constructed for pairs of varieties. Euclidean distance matrix was used to construct dendograms based on unweighted pair-group method of arithmetic average (UPGMA), and to perform principle component analysis (PCA). RAPD DNA markers were scored for the presence (1) and absence (0) of homologous amplified products. For RAPD data, Nei's genetic distance (D) matrix was calculated (Nei, 1972), which in consequence used to construct dendrograms and to perform PCA for genotypic discrimination of pomegranate collection. All data were analyzed using NTSYSY-pc (Numerical Taxonomy and Multivariate Analysis for personal computer) software program version 2.00 (Rohlf, 1998).

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Table 1: Fruit characteristics measured for pomegranate landraces characterization

No.	Trait	Unit	Description
1	Peel colour	score	1. Yellow red, 2. Green red and 3. Red
2	Aril colour	score	1. white, 2.light pink, 3. pink, 4. dark pink and 5. red
3	Taste	score	1. sweet, 2.sour, and 3. sweet sour
4	Seed hardiness	score	1. hard, 2. semi-hard and 3. soft
5	100 seed dry weight	g	The weight of 100 dry seeds, drying will be performed at 68 ºC for 48 hours
6	Aril dry weight percent	%	Total aril dry weight per fruit/total aril fresh weight per fruit, drying will be performed at 68ºC for 48 hours
7	Aril total weight per fruit	g	Weighing total fresh arils present in each fruit
8	Aril percent per fruit	%	Total aril fresh weight/total fruit fresh weight
9	Peel total weight	g	Total fresh weight of external peel
10	Peel percent	%	Peel fresh weight/fruit fresh weight
11	100 aril fresh weight	g	The weight of 100 fresh arils
12	100 aril dry weight	g	The weight of 100 dry arils, drying will be performed at 68 ºC for 48 hours
13	Seed dry weight percent	%	Weight of dry seeds/weight of fresh seeds
14	Peel thickness	mm	Thickness of external peel
15	Aril length	mm	The average length of 10 arils
16	Aril diameter	mm	The average diameter of 10 arils
17	Aril length/aril diameter	ratio	The ratio between aril length and aril diameter
18	Seed length	mm	Average length of 10 seeds
19	Seed diameter	mm	Average width of 10 seeds
20	Seed length/seed diameter	ratio	The ratio between seed length and seed diameter
21	100 seed fresh weight	g	Weighing of 100 fresh seeds
22	Fruit weight	g	Average of 5 fruits per tree
23	Fruit length	mm	The distance from crown base to the fruit eye
24	Fruit diameter	mm	The wider width of fruit
25	Fruit length/fruit diameter	ratio	The ratio between fruit length and fruit width
26	Fruit crown length	mm	The length from the top to the base of crown area
27	Fruit crown diameter	mm	The widest part of the crown area
28	Fruit crown length/Fruit crown diameter	ratio	The ratio between crown length and crown diameter
29	Fruit neck diameter	mm	The widest part of the neck
30	Juice % in Ariles	%	Juice weight×100/total fresh weight of the sample
31	Total soluble solids (TSS)	%	The percentage of soluble solids present in the juice

Table 2: List of the selected informative RAPD primers and the degree of polymorphism obtained among the 14 studied pomegranate landraces

No.	Primer	Sequence 5' →3'	Total No. of bands	No. of polymorphic bands	Polymorphism%	Bands range (Kb)
4	OPD-02	GGACCCAACC	9	9	100	0.42-1.9
8	OPO-03	CTGTTGCTAC	6	5	83.3	0.44-1.41
9	OPO-12	CAGTGCTGTG	10	9	90	1.25-1.50
27	OPA-05	AGGGGTCTTG	8	5	62.5	0.42-1.42
29	OPA-10	GTGATCGCAG	4	2	50	1.03-1.92
31	OPA-13	CAGCACCCAC	5	3	60	0.42-1.35
38	OPB-05	TGCGCCCTTC	5	4	80	0.42-1.43
54	OPZ-01	TCTGTGCCAC	7	6	85.7	0.36-1.42
56	OPZ-03	CAGCACCGCA	10	6	60	0.76-2.46
57	OPZ-04	AGGCTGTGCT	7	1	14.2	0.39-1.51
59	OPZ-06	GTGCCGTTCA	8	6	75	0.35-1.91
62	OPZ-18	AGGGTCTGTG	7	5	71.4	0.52-1.92
	Total		86	61	70.9	

Results

Range of Variation in Morphological Traits

The peel colour was highly variable among pomegranate landraces. 'Hmaree Hmadee' was red, while four landraces ('Khdaree Hello', 'Khdaree Hmadee', 'Lfani Sharabi' and 'Zarori') were green red, and the rest were yellow red. Aril colour was highly polymorphic ranging from white to red. One landrace (Malesse) was white, three landraces were light pink ('Hmaree Hmadee' and 'Esari') and three ('Khdaree Hello', 'Zokom Albagel' and 'Zarori') had red arils, while the rest was with pink arils. Differences were also found in seed texture based on panel test, pomegranate landraces were divided into four classes: soft, semi-soft, semi-hard and hard seeded (Table 4). Two landraces ('Malesse' and 'Bradee Sharabee Asfar') were recorded as real soft-seed; three landraces ('Khdaree Hello', 'Zeklabee and 'Zokom Albagel') as semi-soft or semi-hard-seeded, and the remaining landraces were classified as hard seeded (Table 4). The panel test also revealed that six landraces were sweet in taste ('Khashaby, Khdaree Hello, 'Malesse', Hasmasi', 'Zeklabee' and 'Khratee Ahmar Hello'), four landraces ('Hmaree Hmadee', 'Zokom Albagel', 'Khdaree Hmadee' and 'Esari') were with sour taste and the rest had sweet-sour taste.

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Table 3: Sequences of the 17 SSR pairs of *Punica granatum* used in the current study

No.	Name	Sequence 5'→3'	
1	POM_AAC1	F: GGGTCTTCCTAATTCTCTGG;	R: TACAACTTCGGACTCACTTGC
2	POM_AAC14	F: CGAGAACCGTTAGTCATGC;	R: AGTGACGGCAGGACAAGAAC
3	POM_AGC5	F: TTCGATATTGTTTATTGTGTCG;	R: CAACGAACTAGACGACACAC
4	POM_AGC11	F: CGTCATCCCTTATGTTCTTC;	R: CTGGGGAAGTCGACGAAG
5	ABRII-MP12	F:TTGAGTCCCGATCATATCTC;	R:TCAATCTGTCAGGAACAACA
6	ABRII-MP26	F:TTTCTCGAAGAATTGGGTAA;	R:CTGAGTAAGCTGAGGCTGAT
7	ABRII-MP28	F:ATCCTCTGTCTTTGTGTTCG;	R:TGAGTAATTCCGGTCAGAAG
8	BRII-MP30	F:CCCAGTTTGTAGCAAGGTA;	R:AAGCTGACATTCTTTGAAGC
9	ABRII-MP42	F:GAGCAGAGCAATTCAATCTC;	R:AACAATTTCCCATGTTTGAC
10	Pom006	F-TACTAGGTGGAACCGAACTT;	R-CCTTGACAACCTCATCTCAT
11	Pom021	F-GACTGGAAGAAGCAGAGACT;	R-GAAAAGGAAGTAGCAGAGCA
12	Pom024	F-GGAGATTTGAATTGGGAAGT;	R-GTGGACTAACTCAAGCAAGG
13	ASSR17	R:CCGACTATAACATCCAGAAGG;	F:GGACTGGACTGTGGATTGTTTTTG
14	ASSR46	R:GCGCCCCAAACACCAGAATA;	F:AATGGTCTATGAACACCTCTC
15	ASSR54	R:TCGAGGAGTTGCAGAGTATGAA;	F:CTTGGCTGGCTTCACTGC
16	Pchcmsl	F:GGGTAAATATGCCCATTGTGCAATC;	R:GGATCATTGAACTACGTCAATCCTC
17	Ps9f8	F:GGTTCTTGGTTATTATGA;	R:ACATTTCTATGCAGAGTA

Table 4: The studied pomegranate landraces and their peel and aril colour, taste and seed hardeners

No.	Genotype*	Abbrev.	Peel colour	Aril colour	Seed hardiness	Taste
1	Khashaby	Kha	Yellow-Red	Pink	Hard	Sweet
2	Khdaree Hello	KH	Green –Red	Red	Semi-hard	Sweet
3	Malesse	Mal	Yellow-Red	White	Soft	Sweet
4	Bradee Sharabee Asfar	BSAs	Yellow-Red	Pink	Soft	Sweet-Sour
5	Hasmasi	Has	Yellow-Red	Pink	Hard	Sweet
6	Hmaree Hmadee	HH	Red	Light pink	Hard	Sour
7	Bradee Sharabee Ahmar	BSAh	Yellow-Red	Pink	Hard	Sweet-Sour
8	Zeklabee	Zek	Yellow-Red	Pink	Semi-soft	Sweet
9	Zokom Albagel	ZA	Yellow-Red	Red	Semi-hard	Sour
10	Khratee Ahmar Hello	KAhH	Yellow-Red	Pink	Hard	Sweet
11	Khdaree Hmadee	KHm	Green –Red	Pink	Hard	Sour
12	Lfani Sharabi	LS	Green –Red	Pink	Hard	Sweet-Sour
13	Zarori	Zar	Green –Red	Red	Hard	Sour
14	Esari	Esa	Yellow-Red	Light pink	Hard	Sour

*According to the names at the original growing places

Significant ($P < 0.01$) differences among the 14 pomegranate varieties were observed for all quantitative traits recorded in this study (Table 5). Across the 14 pomegranate studied, fruit weight, fruit crown diameter, fruit crown length/fruit crown diameter, aril total weight per fruit and peel total weight were with high phenotypic coefficient of variation (CV > 32%), while aril dry weight percent, seed dry weight percent, seed length, fruit length/fruit diameter, juice % in arils showed comparatively low values (CV < 10%). The other four traits exhibited intermediate CV values (range = 11.12-17.65%). Pomegranate fruits for landraces under study ranged from 110.50 to 353.00 g/fruit (Table 6). 'Zarori ' and 'Bradee Sharabee' had the heaviest fruits (377.25 and 376.00 g/fruit respectively), followed by 'Zokom Albagel', 'Esari', 'Khratee Ahmer Hello', and 'Khashaby' with 353.25, 341.00, 328.50 and 319.00 g, respectively. The lightest fruits were observed in 'Malesse', 'Hasmasi' and 'Hmaree Hmadee' with average fruit weight of 110.50, 114.25 and 170.25 g/fruit, respectively. Other fruit, seed and aril traits were highly variable (Tables 6 and7). Fruit weight was strongly and positively correlated with fruit dimensions (length and diameter with r= 0.96** and 0.98** respectively), aril total weight (0.89**), hundred aril dry weight (0.56**) and juice percentage (0.623**).

Multivariant Analysis for Morphological Traits

Pomegranate landraces displayed wide euclidean distance coefficients ranging from 3.33 to 12.01, with a mean of 7.65 (Fig. 1A). The most similar landraces (Distance = 3.33) were 'Khashaby' and 'Khratee Ahmar Hello', while the most divergent landraces (Distance = 12.07) were 'Malesse' and 'Bradee Sharabee Asfar'. The dendrogram divided pomegranate genotypes into two main clusters (Fig. 1B). The first main cluster contained all pomegranate varieties except 'Bradee Sharabee Asfar' with unique traits such as soft seeds and large fruit, was placed isolated from other landraces. The first sub-cluster within the first main cluster included 'Hmaree Hmadee', 'Hasmasi' and 'Malesse' with relatively small fruits and fruit dimensions, whereas the second sub-cluster included all other landrace varieties with intermediate to large fruit size and fruit dimensions.

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Table 5: Measured fruit characteristics, range of variability, means and coefficient of variability among studied pomegranate landraces

No.	Trait	Unit	Min	Mean	Max	CV%	F-value
1	100 seed dry weight	g	1.79	2.52	3.10	17.54	**
2	Aril dry weight percent	%	18.20	21.30	26.67	9.90	**
3	Aril total weight per fruit	g	231.5	160.78	68.00	33.89	**
4	Aril percent per fruit	%	41.28	58.92	68.99	13.33	**
5	Peel total weight	g	32.75	101.22	165.25	35.47	**
6	Peel percent	%	27.65	36.96	49.10	16.61	**
7	100 Aril fresh weight	g	20.25	30.08	38.55	20.17	**
8	100 aril dry weight	g	4.17	6.32	8.01	17.65	**
9	Seed dry weight percent		36.92	45.50	52.23	9.50	**
10	Peel thickness	mm	2.45	3.60	4.58	15.79	**
11	Aril length	mm	6.00	8.79	10.00	14.34	**
12	Aril diameter	mm	5.25	6.46	8.00	12.50	**
13	Aril length/aril diameter	ratio	0.76	1.43	1.75	17.26	**
14	Seed length	mm	5.25**	6.79	7.99	9.41	**
15	Seed diameter	mm	2.35	2.85	4.00	13.71	**
16	Seed length/seed diameter	ratio	2.07	2.44	2.95	11.12	**
17	Fruit weight	g	110.50	278.46	377.25	32.77	**
18	Fruit length	mm	46.55	71.16	81.78	15.89	**
19	Fruit diameter	mm	59.78	80.13	91.03	12.73	**
20	Fruit length/fruit diameter	ratio	0.78	0.89	0.97	5.49	**
21	Fruit crown length	mm	7.55	13.79	20.78	25.85	**
22	Fruit crown diameter	mm	7.16	16.27	28.13	34.53	**
23	Fruit crown length/Fruit crown diameter	ratio	0.53	1.11	2.04	48.18	**
24	Fruit neck diameter	mm	11.68	17.69	23.58	17.16	**
25	Juice % in Ariles	%	65.42	74.14	79.61	5.08	**
26	Total soluble solids	%	10.50	15.13	17.88	12.15	**

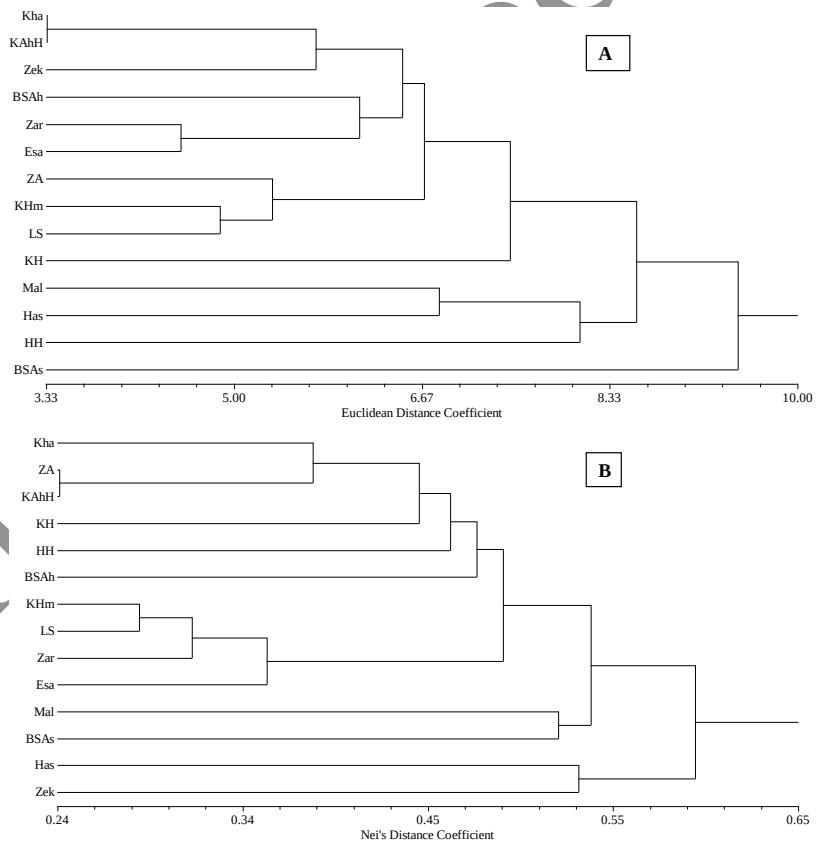

Fig. 1: Dendrogram based on the cluster analysis of the 14 pomegranate landraces from Jordan: (a) Distance matrix based on morphological traits; (b) Distance matrix based on RAPD marker data. For landraces' name abbreviations see Table 4

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PCA was performed to identify traits that that contributed most to the phenotypic total variation. The results of the principle component analysis are shown in table 8. The first seven PC explained 88.64% of the morphological variation among the landraces tested. The first function accounted for 28.92% with high load on fruit (fruit weight and dimensions, peel total weight and thickness and fruit neck diameter) and aril (aril weight and dimensions, and aril fresh and dry weight) related traits. The second and third functions accounted for 17.61% and 12.81 of total variation which were explained by seed characteristics mainly 100 seed and aril weights, seed length and seed hardiness. Other traits were consistently present in the other components and therefore contributed less to the variability. Plots of the first three eigenvectors calculated for the 14 pomegranate landraces gave clustering pattern similar to that obtained by UPGMA analysis (Figs. are not shown).

DNA Variation

The preliminary RAPD primers screening was done with 62 RAPD primers. The twelve primers which gave reproducible and polymorphic scorable bands were used in DNA characterization of pomegranate landraces (Table 2). A total of 86 RAPD bands were scored, of which 61 (70.9%) were polymorphic. The number of polymorphic bands varied from 1 in OPZ-04 primer to 9 in OPD-02, with band sizes ranged from 0.36 to 2.46 Kb and with a mean of 5.08 bands per primer. Bands with the same band sizes were considered as identical. All SSR markers used in this study were monomophic, therefore no statistical analyses were performed.

Multivariant Analysis for Molecular Data

Nei's genetic distances (Nei 1972) were calculated for paired comparison of the 14 pomegranate landraces from RAPD scores (Fig. 1B). The mean Nei's genetic distance based on RAPD scores was 0.51, ranging from 0.29 to 0.66. The highest genetic distances (range = 0.65-0.66) was detected between 'Hasmasi' and both 'Khashaby' and 'Malesse' and that between 'Zeklabee' and both 'Khashaby' and 'Khdaree Hello', while the most similar landraces (distance = 0.24) were 'Zokom Albagel' and 'Khratee Hmadee'. The UPGMA tree (Fig. 1B) based on Nei's genetic distance showed that the 14 pomegranate landraces

Table 6: Means of fruit traits for the 14 Jordanian pomegranate landraces

No.	Genotype	Fruit weight (g)	Fruit length (mm)	fruit diameter (mm)	Fruit length/fruit diameter	Fruit crown length (mm)	Fruit crown diameter (mm)	Fruit crown length/Fruit crown diameter	Fruit neck diameter (mm)	Aril percent per fruit	Peel total weight (g)	Peel percent	Peel thickness (mm)	Juice percent age	Total soluble solids (g/l)
1	Khashaby	319.00	76.88	85.68	0.90	14.00	8.25	1.69	19.35	59.65	113.40	35.64	3.53	74.27	13.50
2	Khdaree Hello	256.00	76.33	78.90	0.97	14.25	13.13	1.73	17.78	61.58	93.50	36.49	3.48	67.55	15.13
3	Malesse	110.50	46.55	59.93	0.78	7.55	14.25	0.85	11.68	68.99	32.75	29.26	3.45	72.87	10.50
4	Bradee Sharabee Asfar	376.00	81.78	88.35	0.93	15.75	23.88	0.70	21.43	57.81	165.25	44.61	4.36	75.31	17.88
5	Hasmasi	114.25	51.43	59.78	0.86	12.00	21.50	0.59	14.63	59.71	44.50	38.95	2.45	72.72	15.00
6	Hmaree Hmadee	170.75	58.90	70.50	0.83	11.50	17.35	0.66	17.63	56.17	80.35	49.10	3.20	65.42	16.38
7	Bradee Sharabee Ahmar	231.00	65.05	76.88	0.85	14.50	28.13	0.53	20.00	65.85	92.00	39.88	4.30	73.81	15.13
8	Zeklabee	319.00	76.13	84.80	0.90	20.78	13.63	1.96	23.58	48.12	131.59	44.17	4.58	76.82	15.88
9	Zokom Albagel	353.25	78.13	85.85	0.91	13.00	17.25	0.82	16.13	66.69	123.41	35.72	3.20	77.55	17.44
10	Khratee Ahmar Hello	328.50	80.25	86.40	0.93	12.30	7.16	2.04	18.23	61.53	98.75	30.38	4.08	73.97	16.00
11	Khdaree Hmadee	261.50	70.28	78.75	0.89	17.25	17.25	1.01	15.93	60.88	72.03	27.65	3.33	75.76	13.44
12	Lfani Sharabi	340.50	79.68	86.30	0.92	8.00	13.63	0.64	17.90	66.55	110.00	31.95	3.80	75.57	16.00
13	Zarori	377.25	77.08	88.75	0.87	14.50	18.00	1.01	14.53	50.05	134.33	35.92	3.20	79.61	15.06
14	Esari	341.00	77.80	91.03	0.86	17.75	14.41	1.27	18.98	41.28	125.19	37.64	3.50	76.69	14.44
	$LSD_{0.05}$	72.76	7.71	8.30	0.06	4.37	8.33	0.95	4.73	13.47	27.18	11.57	0.70	7.80	1.57

Table 7: Means for seed and aril traits for the 14 Jordanian pomegranate landraces

No.	Genotype	100 seed dry weight (g)	Aril dry weight percent (g)	Aril total weight per fruit (g)	100 Aril fresh weight (g)	100 Aril dry weight (g)	Seed dry weight percent (g)	Aril length (mm)	Aril diameter (mm)	Aril length/aril diameter	Seed length (mm)	Seed diameter	Seed length/seed diameter
1	Khashaby	2.96	20.24	189.49	33.25	6.74	47.41	9.00	6.00	1.71	7.00	2.92	2.40
2	Khdaree Hello	1.79	18.20	157.50	29.50	5.34	36.92	7.50	6.00	1.29	6.41	2.35	2.74
3	Malesse	2.47	19.97	75.75	32.00	6.39	44.34	7.25	6.50	1.23	6.74	2.53	2.69
4	Bradee Sharabee Asfar	1.83	21.13	231.50	31.00	6.55	45.49	6.00	8.00	0.76	5.25	2.49	2.11
5	Hasmasi	1.96	20.74	68.00	20.25	4.17	38.89	8.50	5.25	1.63	6.64	2.72	2.47
6	Hmaree Hmadee	2.72	26.67	91.62	21.11	5.56	52.23	8.33	5.75	1.47	7.03	2.93	2.45
7	Bradee Sharabee Ahmar	2.67	20.56	151.88	34.00	6.99	47.57	10.00	7.00	1.43	6.10	2.85	2.14
8	Zeklabee	2.79	20.48	144.81	37.54	7.69	39.55	10.00	7.00	1.47	7.99	2.72	2.95
9	Zokom Albagel	2.09	24.80	229.07	24.04	5.89	46.45	9.50	5.50	1.75	6.82	2.68	2.56
10	Khratee Ahmar Hello	3.10	21.39	200.50	35.75	7.65	48.22	10.00	6.75	1.49	7.56	2.77	2.74
11	Khdaree Hmadee	2.36	20.53	159.88	23.75	4.88	49.25	9.00	6.00	1.52	6.91	2.82	2.47
12	Lfani Sharabi	2.76	22.24	226.00	26.00	5.79	47.30	8.00	6.00	1.62	6.88	3.10	2.22
13	Zarori	2.93	20.97	187.96	38.55	8.01	48.59	10.00	7.50	1.34	6.59	3.00	2.20
14	Esari	2.83	20.26	136.99	34.38	6.84	44.74	10.00	7.25	1.38	7.09	4.00	2.07
	$LSD_{0.05}$	0.30	2.95	41.56	5.39	1.06	5.35	1.51	1.19	0.31	0.54	0.83	0.42

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were separated into two subclusters inconsistent with clustering based on euclidean distance matrix (Fig. 1B): the first cluster contained two landraces ('Hasmasi' and 'Zeklabee'), while the second cluster included other pomegranate landraces. Two subclusters existed within the second main cluster: two landraces were ('Bradee Sharabee Asfar' and 'Malesse') clustered together, while the other ten landraces were placed together in another subcluster. Even though dendrograms based on phenotypic traits compared with that based on RAPD scores were not consistent, however, some common groupings were observed in both dendograms. Seven landraces ('Khashaby', 'Kharatee Ahmar Hello', 'Bradee Sharabee Ahmer', 'Zarori', 'Esari', 'Zokom Albagel', 'Lafani Sharabi' and 'Khdaree Hello') tended to cluster together, and 'Malesse', 'Bradee Sharabee Asfar' and 'Hasmasi' were always very close to each other. The other three landraces ('Hmaree Hmadee', 'Khdaree Hmadee' and 'Zeklabee') were inconsistently distributed in the both dendograms.

The distances based on morphological and RAPD markers were significantly correlated (r = 0.46**). Plots of the first three eigenvectors calculated for the 14 landraces produced a separation similar to that obtained by UPGMA dendrogram based on Nei's genetic distance matrix. The first three functions account for 28.92, 17.61 and 12.81% of the total genetic variation, respectively while the PC4, PC5, PC6 and PC7 together explained. 29.3% of the total genetic variation (Table 9).

Discussion

Morphological Variation

Most traits used to characterize pomegranate landraces are with economic interest for pomegranate breeding programs and pomegranate consumers. Jordanian pomegranate landraces are a valuable source of genes for commercial uses. ANOVA revealed high significant differences between pomegranate landraces for fruit traits with considerable CV values for most studied traits. Qualitative traits displayed two or more phenotypic classes. Pomegranate landraces characterized in this study were localized in the same geographic site with very minor microclimate effect. Therefore, phenotypic variation is mostly representing genetic difference rather than weather condition effects on studied traits. The results obtained in this study are comparable with the report of other authors (Drogoudi *et al*., 2005; Sarkhosh *et al*., 2009) who found that some traits such as juiciness, fruit shape, and aril and seed related traits had highest loading in the first components. In accordance, fruit size, colour and juice characteristics in Tunisian pomegranate displayed high discriminating power as compared to other traits (Mars and Marrakchi, 1999).

Fruit weight is the most pertinent criteria used during the sorting process. Pomegranate fruit destined for the export markets are usually 275-325 gms (Najan, 2014).

Table 8: Loading values of morphological variables on the first three principle components for the 14 Jordanian pomegranate landraces

Trait	PC1	PC2	PC3
100 seed dry weight	0.38	0.70	0.43
Aril dry weight percent	-0.13	-0.25	0.64
Aril total weight per fruit	0.69	-0.03	0.06
Aril percent per fruit	-0.53	-0.19	-0.20
Peel total weight	0.90	-0.36	0.07
Peel percent	0.16	-0.41	0.05
100 Aril fresh weight	0.69	0.39	-0.24
100 aril dry weight	0.74	0.33	0.00
Seed dry weight percent	0.09	-0.04	0.76
Peel thickness	0.68	-0.06	-0.34
Aril length	0.37	0.61	0.39
Aril diameter	0.68	-0.16	-0.09
Aril length/aril diameter	-0.19	0.52	0.35
Seed length	0.12	0.83	0.08
Seed diameter	0.31	0.32	0.66
Seed length/seed diameter	-0.18	0.44	-0.59
100 seed fresh weight	0.41	0.84	0.02
Fruit weight	0.90	-0.16	0.14
Fruit length	0.87	-0.15	0.02
Fruit diameter	0.93	-0.08	0.18
Fruit length/fruit diameter	0.49	-0.26	-0.33
Fruit crown length	0.62	0.00	-0.13
Fruit crown diameter	-0.17	-0.67	0.16
Fruit crown length/Fruit crown diameter	0.48	0.58	-0.48
Fruit neck diameter	0.73	-0.16	-0.21
Juice % in Ariles	0.57	0.09	0.17
Total soluble solids	0.46	-0.59	0.13
Peel colour	-0.26	-0.12	0.44
Aril colour	-0.34	0.07	-0.01
Tast	0.13	-0.32	0.84
Seed hardiness	0.43	-0.66	-0.30
% variation	28.92	17.61	12.81
Cumulative	28.92	46.53	59.34

Table 9: Eigen values and cumulative variances resulted from factor analysis for the first 8 principal components among the 14 pomegranate landraces from Jordan based on RAPD markers

	Components							
	1	2	3	4	5	6	7	8
Eigen value	13.69	8.86	7.94	5.99	5.41	4.77	4.20	3.37
% of variance	22.44	14.53	13.01	9.81	8.87	7.81	6.88	5.52
Cumulative% of total variance	22.44	36.97	49.98	59.79	68.66	76.47	83.35	88.88

Most landraces under study had an average fruit weight higher than 250 g, which are suitable for marketing, packaging and shipment. In general, fruit weight of Jordanian pomegranate landraces is low to medium in size as compared with other pomegranate collections in the Mediterranean basin (Mars and Marrakchi, 1999; Polat *et al*., 1999; Al-Maiman and Ahmad, 2002; Yildiz *et al*., 2003; Ozkan, 2005; Grundogdu, 2006) with a fruit sizes ranging from 192.00 to 806.00 g/fruit. The shape index obtained from the ratio between fruit length and fruit width indicate that flattened fruit shape is prevailing among pomegranate landraces from Jordan as compared with pomegranate collections from Iran (Sarkhosh *et al*., 2009) and Tunisia

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(Mansour *et al*., 2011) and Turkey (Durgaç *et al*., 2008), but they are very similar in shape as compared with Sicilian pomegranate varieties (Barone *et al*., 2001). Results showed strong correlation between fruit weight and both fruit dimensions (length and diameter) and fruit components (peel total weight, aril total weight, hundred aril dry weight and juice percentage) indicating that selection for larger fruits will lead to juicy fruits with greater total aril weight and vice versa. Fruits of thick peels might have the ability to resist peel cracks. A thick skin (peel) enclosing the edible arils protects the fruits from pest and pathogens that enters the fruits via these cracks (Jalikop *et al*., 2006, 2005). Landraces with thick pericarp might be used to breed for varieties that resist cracking.

Improvement of juice quality, seed mellowness, and aril and fruit appearance are major recent breeding objectives for pomegranate breeding programs (Crites *et al*., 2014). Varieties showing pink or red arils with small and soft seeds are targets for pomegranate breeders (Crites *et al*., 2014; Fawole and Opara, 2013). Three landraces were classified as sour-sweet and six landraces were sweet, while the remaining studied landraces were sour. Sweet and sour-sweet varieties are acceptable for pomegranate fresh consumption. Soft seeds and red fruit peel is the most attractive trait to pomegranate consumers and important quality attribute in pomegranate marketing (Janick and Moore, 1975; Drogoudi *et al*., 2005; Ashton *et al*., 2006). ’Malesse’ and ’Bradee Sharabee Asfar’ were the only genotypes presenting soft seeds, Fruit peel colour varied among landraces. One landraces "Hmaree Hmadee" was with red peel, while the rest with yellow red and green red colour. The results of these studies revealed that pink- or red-arils are the most common traits among pomegranate landraces, and one of the most desirable traits for commercial cultivars of pomegranates used for fresh fruit consumption and juice making. Varieties with soft seeds, big arils, high juice content, thin peel and no sourness are suitable for the extraction of arils (Zavala and Cozza, 2012). For example, ’Bradee Sharabee Asfar’ landrace shows a combination of desirable traits with attractable yellow-purple skin colour at maturity with pink-soft arils that is high in juice with a slightly acid taste.

From industrial point of view, high juice content might be more desirable than fruit size (Holland *et al*., 2009). Jordanian pomegranate is considered as a rich source of genes for juicing with high percentages of juice to fruit weight, the juice makes up 65.42-79.6% percent of the pulp weight. ’Zarori’ showed high juice percentage with red arils and sour taste, while ’Hmaree Hmadee’ had the lowest fruit juice (65.42%). In general, juicing in pomegranate is genotype dependent and juice makes up 45-70% percent of the fruit weight (Ashton *et al*., 2006). The percentage of fruit juice is relatively higher than those reported for Spanish varieties (range = 50.26% to 64.17%) (Martinez *et al*., 2006) and Indian varieties (44.96% to 68.55%) (Viswanath *et al*., 1999).

TSS contents significantly differed among the pomegranate landraces, ranging from 10.50 to 17.88% for “Malesse” and “Bradee Sharabee Asfar”, respectively. “Bradee Sharabee Asfar” displayed also other preferable quality traits such pink aril juice, soft seeds and sweet-sour taste. High TSS content is highly desirable industrial traits in pomegranate fruit juice making as it associates with sweetness and flavor especially if it combined low juice acidity and tannin concentration (Shwartz *et al*., 2009; Zarei *et al*., 2011). TSS values of pomegranate aril juice are within the range of TSS values (10-20%) reported in Iranian collections (Akbarpour *et al*., 2009; Sarkhosh *et al*., 2011; Nemati *et al*., 2012) and those reported for pomegranate selections from Tunisia (Mansour *et al*., 2011).

RAPD Variation

The results indicate that there is wide genetic polymorphism among Jordanian pomegranate landraces. The mean Nei's genetic distance among the studied genotypes was 0.51 (range = 0.24 to 0.66) indicating a high level of divergence among pomegranate landraces. Previous works on pomegranate comparably wider in ranges for Nei's genetic distances (range = 0.10 -0.83) (Zamani *et al*., 2007; Durgaç *et al*., 2008; Sarkhosh *et al*., 2009; Ercisli *et al*., 2011). Even though the area cultivated with pomegranate is limited in Jordan, such range of genetic distances clearly indicate the considerable variation present among studied landraces. Such variation could be exploited in pomegranate breeding programs. When the euclidean distances based on morphological traits and that based on RAPD scores were compared, a significant correlation was obtained between the two marker systems. This indicates that RAPD markers might be closely linked with genes controlling such traits. The set of SSR markers used in this study was monomorphic which might be due to the narrow gene pool or, due to the fact that 17 SSR markers were too few to identify polymorphism among pomegranate varieties within a small geographic area.

Conclusion

Broad phenotypic diversity was existed among pomegranate landraces from Jordan. It is strongly recommended using both morphological and molecular assays as complementary methods to describe diversity in Jordanian pomegranate landraces. Both morphological and RAPD markers revealed considerable variation among pomegranate landraces. The considerable phenotypic variation in fruit traits reported in this study indicates that such collection is valuable genetic resources for pomegranate improvement.

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