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Morphological and molecular evaluation of some Egyptian pomegranate cultivars

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Six Egyptian pomegranate (*Punica granatum* L.) cultivars were characterized by fruit characteristics (physical and chemical) and two molecular markers; Inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP). Genetic diversity of the pomegranate genotypes was evaluated. Physical fruit traits were determined (weight, volume and diameter), calyx [diameter, length (mm) and Carpels number], fruit firmness (Newton), peel as (weight and thickness), arils weight (g), volume of juice (ml), seeds [fresh and dry weight (g)], and color parameter of (fruit skin, internal peel, arils, juice and seeds). The chemical traits such as soluble solids contents (SSC), vitamin C content, anthocyanin content, pH, and titratable acidity (TA) were assessed and wide variations were observed in each of these traits among the studied cultivars. The genetic variability and relationships among six Egyptian pomegranate cultivars were tested using ISSR and AFLP analyses. The level of polymorphism across cultivars was 53 and 90.7% as revealed by ISSR and AFLP, respectively. ISSR and AFLP revealed different genetic similarities among the six pomegranate cultivars. Each analysis differs not only in its underlying principle, but also in their in-formativeness with regard to the type and amount of polymorphism detected. Genetic similarity matrices estimated from ISSR and AFLP data, showed similarity coefficients to range from 0.77 to 0.94 and 0.33 to 0.73, respectively. ISSR and AFLP characterized the six pomegranate cultivars by a large number of unique markers being 23 and 46 unique markers, respectively. The fruit weight ranged between 479.4 to 185 g of 'Nab El Gamal' and 'Assuity', the firmness was 79.98 of 'Nab El Gamal' and 71.84 Newton of 'Manfalouty' cv. The fruit peel thickness varied from 0.6 mm 'Araby', 'Hegazy' and 'Wardi' to 0.3 mm 'Assuity'. The arils weight ranged from 87.5 to 275 g of 'Assuity' and 'Nab El Gamal' cvs. The percentage net of arils weight/ fruit weight was the highest (59.34% of 'Manfalouty' cv). The juice volume ranged from 62.41 to 71.81 ml/100 g arils for ' Wardi' and 'Nab El Gamal' cvs. The SSC content ranged between 16.01 'Hegazy' and 12.55% 'Assuity'. V.C. content ranged from 3.21 to 14 mg. vitamin/100 ml juice of 'Nab El Gamal' and 'Assuity'. The anthocyanin content ranged from 1.47 to 10.03 for 'Araby' and 'Hegazy'. The pH values varied from 3.3 (Wardi) to 2.9 (Araby). The Egyptain cultivars of pomegranate have a wide variation in the morphological and chemical characteristics for many uses of fresh fruit and of industry purpose.

Key words: Morphological and chemical fruits characterization, pomegranate, inter simple sequence repeat (ISSR), DNA markers, amplified fragment length polymorphism (AFLP).

INTRODUCTION

The pomegranate *Punica granatum* L. (Punicaceae) is one of the oldest known edible fruits. Its history dates back to very ancient times. This fruit tree is one of the species mentioned in the Bible and the Koran and is

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often associated to fertility. It is native to Persia and perhaps some surrounding areas. It was cultivated in ancient Egypt. Pomegranate is considered an excellent tree for growing in arid zones for its resistance to drought conditions. It is now widely cultivated in the Mediterranean, tropical and subtropical areas. Botanically, the pomegranate (*P*. *granatum* L.) is included in the family of Punicaceae with 2n = 16 or 18. The genus punica is known to include two species *P. protopunica* and *P. granatum* (Mars, 2000). Genetic studies are lacking entirely because pomegranate has not been subject of much scientific investigation. Some studies based on morphometric and phytochemical criteria have recently been performed to determine the degree of polymorphism within local material. A high genetic diversity is expected to occur in pomegranate genotypes cultivated in Egypt due to long historical cultivation and different environmental conditions in which, these cultivars are growing. Therefore, our study is concerned with identification of genetic diversity (by using different molecular markers) in pomegranate cultivars as these data may be of further use in hybridization and selection programs.

Molecular markers have been overcoming limitations of morphological and biochemical markers due to the influence of environment on the performance of genotypes. A wide range of molecular markers have been used to assess genetic diversity of pomegranate cultivars as well as wild genotypes from different parts of the world. Inter-Simple Sequence Repeats (ISSR) analysis is considered as an efficient molecular marker, showing genetic variation in the wild pomegranate populations (Zahra et al., 2012). Amplified fragments length polymerphism (AFLPs) are another source of markers which has been used to evaluate genetic diversity within and among Chinese pomegranate populations (Yuan et al., 2007) and Tunisian cultivars (Jbir et al., 2008). Up to now, more than 137 microsatellite loci in pomegranate genome (Curro et al., 2010; Hasnaoui et al., 2010; Soriano et al., 2011) show different ranges of genetic polymorphism in the studied genotypes. In recent years, there has been an increasing interest in determining antioxidant properties of red fruits, because they are rich as dietary sources of antioxidant phenolics and anthocyanins (Velioglu et al., 1998; Kaur and Kapoor, 2001; Moyer et al., 2002; Ozgen et al., 2007; Arjmand, 2011). Epidemiological studies have suggested that consumption of red fruit juices such as grape, berry juices and pomegranate, correlates with reduced risk of coronary heart disease, stroke, certain types of cancers and aging (Prior, 2003; Malik and Mukhtar, 2006). It has been reported that pomegranate juice is one of the important sources of anthocyanins (cyanidin, delphinidin, and pelargonidin), which give the fruit and aril its red colour (punicalagin and ellagic acid) (Kulkarni and Aradhya, 2005). Thus, pomegranate has become more popular because of its health-promoting phytonutritional content (Malik et al., 2005; Adams et al., 2006; Faria et al., 2007; Khan and Mukhtar, 2007).

Statistics provided by the statistic of Agriculture Directorates of Governorates in 2011 indicate that a total of 5471.78 hectare are planted with pomegranate trees, with a total production of 64574 ton in Egypt new area mainly planted by “Wonderful” cv. The local cultivars were grown at Assuit governorate (the most concentrated area of pomegranate in Egypt). In this investigation, we aimed to study fruit characterization and genetic diversity among pomegranate Egyptian local cultivars to assess the appropriate uses of these cultivars (fresh fruit or industry), to elucidate their biodiversity by PCR-based markers in breeding programs to improve their characteristics and expansion of cultivation.

MATERIALS AND METHODS

Plant material and cultivation

The adult trees of the six cultivars (Araby, Assuity, Hegazy, Manfalouty, Nab El Gamal and Wardi) were grown in loamy soil at Faculty of Agriculture Farm, Assuit University, Egypt. Samples were collected from four different trees.

Fruit physical characteristics

Samples of 16 random mature fruits were collected, the maturity of the fruit was judged by the development of shiny reddish yellow peel colour, full opening of the calyx and deep red coloured juicy arils. Diseased, bruised and injured fruit were rejected, and sound fruit of uniform size and appearance randomly distributed into different lots (Nanda et al., 2001) were used for the determination of fruit parameters such as weight, volume and diameter, calyx diameter, length (mm) (measured by precision vernier caliper, Steco, Germany), Carpels number, fruit firmness (Newton) measured by using pressure tester (Effegi), peel (weight and thickness), arils weight (g), juice of 100 g, arils volume (ml), seeds [fresh and dry weight (g)], weights measured by weighting device (RADWAG , Model: WTB200) with an accuracy of 0.001 g and color parameter of (fruit peel, internal peel, arils, juice and seeds). The external peel color were (three different measurements at three equidistant points on the equatorial region of each individual fruit), color of arils and the juice of 100 g arils by using a Minolta CR-400/410 Chroma-meter (Minolta, Japan). Hunter scale (L*, a*, b*) system was used.

The Chroma meter was calibrated with a white standard tile with illuminant D65 (Y = 93.3, x = 0.3161 and y = 0.3328) equivalent to HL system: HL = 97.10, a = -0.17 and b = 1.80. Calibration was made at each using. The parameter L*
represents the brightness of the color, a* the hue range of the colors red (+), green (-), b* hue range of colors yellow (+) and blue (-).

$$\text{Chroma} = (a_2 + b_2)^{½}$$

$$\text{Hue angle} = \tan^{-1}(b/a)$$

Fruit chemical characteristics

Chemical characteristics as soluble solids contents (SSC) were measured using digital refractometer (ATAGO Pr-32). pH were determined by pH meter. The titratable acidity (TA) was expressed

Table 1. ISSR primer names, sequence and annealing temperature.

Serial number	Primer code	Sequence	Ta (°C)
1	890	ACG $(GT)_7$	50
2	17898[b]	$(CA)_6$ GT	40
3	835	$(AG)_8$ CC	55
4	851	$(GT)_8$ CG	55
5	809	$(AG)_8$ G	53
6	HB10	$(GA)_6$ CC	40
7	17898[a]	$(CA)_6$ AC	40
8	Bec	$(CA)_7$ TC	42
9	Had2	CT$(CCT)_3$ CAC	42
10	HB15	$(GTG)_3$ GC	38

as percent citric acid according to A.O.A.C (2005a). The anthocyanin content were measured at 535 nm, using spectrophotometer JENWAY Ltd. model 4600 according to A.O.A.C (1990). The vitamin C (V.C.) ascorbic acid content was determined as A.O.A.C (2005b).

Genomic DNA extraction

Three leaves were collected from each plant and three plants per cultivar were subjected to molecular analysis. Leaves (200 mg) were ground to a powder using liquid nitrogen in microphage tubes and then DNA were isolated using AxyPrep multisource Genomic DNA Mini-Prep Kit (Axygen Bioscience, USA, cat. No. Ap-MN-MS-GDNA-50) according to manufacturer's manual. DNA samples of each cultivar were analyzed individually to determine intra-cultivar variations and bulked to assess inter-cultivar variations.

Inter simple sequence repeat (ISSR)

An initial screening of 20 ISSR primers [successfully utilized in other plant species (Nagaoka and Ogihara, 1997; Awasthi et al., 2004)] was performed in order to test their readability and amplification profiles for polymorphism. After this screening procedure, 10 ISSR primers were selected (Table 1).

PCR for analysis was performed in 25 µl volume containing 2.5 mM $MgCl^2$ 0.2 mM dNTPs, 20 µM primer, 50 ng genomic DNA and 1 U Taq DNA polymerase (Bioron, Germany). All reactions were performed in a Perkin Elmer 2400 thermal cycler. The ISSR program was performed as 1 cycle of 94°C for 4 min and 35 cycles of 94°C for 1 min, 44° for 45 s, 72°C for 1.5 min, and, a final extension step of 72°C for 8 min.

Amplified fragment length polymorphism (AFLP)

AFLP analysis was performed using the AFLP analysis system1-Promega according to the manufacturer's protocol. Genomic DNA samples were digested with EcoR1 and Mse1 restriction enzymes in which the EcoR1 and Mse1 adapters were ligated to the digested DNA fragments. Pre-amplification was carried out using EcoR1 primer plus one extension base at the third position (A) and Mse1 primer plus one extension base at the third position (C) to amplify complementary sequences. Two primer combinations between EcoR1 and Mse1 primer extension bases (E-ACC/M-CAA and E-ACT/M-CTT) were used to selectively amplify the DNA fragment matching the primer-extension sequence.

Detection of PCR products

The products of ISSR based PCR were detected by electrophoresis on agarose gel (1.2% in 1X TBE buffer), then stained with ethidium bromide (0.3 ug/ml). AFLP products were detected by electrophoresis in polyacrylamide denaturing sequencing gel. DNA silver staining system (Promega, CA, USA) was used for band detection then gel was photographed. Amplicon size were estimated using 1 kb DNA standard (Bioron, Germany) with ISSR while 100 bp DNA standard (Fermentes) with AFLP.

Data analysis

The data were subjected to ANOVA and were evaluated by MSTATC program. The differences between means were compared using LSD test at 5% level. Reproducible bands visualized on the gels were scored using a binary system (1/0) for their presence or absence for both ISSR and AFLP based on the UVP gel documentation system (Gel Works ID advanced software, UVP). Bands of the same mobility were scored as identical. UPGMA was used to measure the genetic similarity resulted from the analysis software of Non-Linear Dynamics Corporation (UK).

RESULTS

Fruit physical characteristics

Tables 2, 3 and 4 show the fruit physical characteristics of the six pomegranate cultivars as fruit (weight, volume, diameter, length and firmness), calyx (diameter, length and the number of carpels), peel (thickness, fresh weight and dry weight), arils weight, juice volume, seeds (fresh and dry weight) and the color parameters of the (fruit skin, internal peel, arils juice and seeds); Figures 1 and 2 demonstrate cross sections and arils of the six cultivars. The fruit weight ranged between 479.4 to 185 g for 'Nab El Gamal' and 'Assuity'; fruit volume was highest (416.6 cm^3) and lowest 214.8 cm^3 for 'Nab El Gamal' and 'Wardi', respectively; there was a close relationship between the fruit weight and volume. Calyx diameter was ranged between 2.3 to 1.6 cm for 'Araby' and Assiuty, and similarly calyx length was 1.8 to 0.8 cm for 'Araby'

Table 2. The fruit and calyx physical characteristics of the pomegranate cultivars.

Cultivar	Fruit					Calyx		
	Weight (g)	Volume (cm^3)	Diameter (cm)	Length (cm)	Firmness (Newton)	diameter (cm)	Length (cm)	Number of carpels
Araby	233.9^{C}	230.0^{C}	7.731^{C}	7.325^{C}	75.30^{CD}	2.300^{A}	1.844^{A}	5.875^{A}
Assuity	185.0^{D}	222.5	7.500^{C}	6.600^{D}	77.08^{B}	1.600^{D}	0.850^{D}	6.000^{A}
Hegazy	334.7^{B}	281.3^{B}	8.544^{B}	8.300^{B}	76.33^{BC}	1.619^{CD}	1.013^{C}	5.000^{C}
Manfalouty	353.9^{B}	302.2^{B}	8.631^{B}	8.144^{B}	71.84^{E}	1.788^{BC}	1.131^{B}	5.438^{B}
Nab El Gamal	479.4^{A}	416.6^{A}	9.538^{A}	8.887^{A}	79.98^{A}	1.944^{B}	1.150^{B}	5.125^{BC}
Wardi	226.5^{C}	214.8^{C}	7.559^{C}	7.019^{C}	74.42^{D}	1.663^{CD}	1.200^{B}	5.250^{BC}
LSD at 0.05	37.26	43.32	0.3235	0.3407	1.76	0.1725	0.1179	0.4319

Means in the same column for each characteristics followed by the same letter are not significantly different at 5% level.

Table 3. The fruit and calyx physical characteristics of the pomegranate cultivars.

Cultivar	Peel			Aril	Juice	Seed	
	Thickness (mm)	Fresh weight (g)	Dry weight (g)	Weight (g)	Volume (ml)	Fresh weight (g)	Dry weight (g)
Araby	0.6125^{A}	96.98^{C}	25.57^{A}	132.4^{B}	66.56^{B}	23.93^{B}	8.216^{A}
Assuity	0.3000^{C}	97.50^{C}	21.13^{BC}	87.50^{E}	67.00^{B}	27.17^{A}	6.653^{B}
Hegazy	0.6313^{A}	140.6^{B}	24.94^{A}	181.3^{C}	68.19^{B}	20.12^{C}	5.230^{C}
Manfalouty	0.4938^{B}	141.3^{B}	23.70^{AB}	210.0^{D}	67.63^{B}	21.09^{C}	6.072^{B}
Nab El Gamal	0.5437^{AB}	186.6^{A}	26.12^{A}	275.0^{A}	71.81^{A}	17.39^{D}	4.431^{D}
Wardi	0.6313^{A}	84.46^{C}	18.13^{C}	130.8^{D}	62.41^{C}	24.32^{B}	8.018^{A}
LSD at 0.05	0.09708	15.26	3.108	25.35	2.511	2.61	0.6972

Means in the same column for each characteristics followed by the same letter are not significantly different at 5% level.

and Assiuty; generally the number of carpels was five except ‘Assuity’ which was six carpels. The firmness was 79.98 for ‘Nab El Gamal’ and 71.84 Newton for ‘Manfalouty’ cv. The fruit peel thickness varied was 0.6 mm for ‘Araby’, ‘Hegazy’ and ‘Wardi’ and 0.3 mm for ‘Assuity’, also the peel moisture percentage ranged from 73.63 to 86.0% for ‘Araby’ and ‘Nab El Gamal’, respectively. The arils weight was highest (275 g) for ‘Nab El Gamal’. The seeds moisture percentage varied from 65.67 to 75.51% for ‘Araby’ and ‘Assuity’ (Table 3). The aril percentage (arils weight/ fruit weight) was highest (59.34%) for ‘Manfalouty’ cv.

‘Nab El Gamal’ had the highest juice volume (71.81 ml/100 g arils); these results agree with those of Hassan et al. (2012) and similar values were obtained from Iranian pomegranates cultivars (Tehranifar et al., 2010).

Fruit chemical characteristics

Table 5 shows the chemical characteristics of the six pomegranate cultivars (as SSC, V.C. content, anthocyanin, TA and pH). The SSC content ranged between 16.01 for ‘Hegazy’ and 12.55°Brix for ‘Assuity’; whereas ‘Assuity’ had the highest value for TA and V.C. content. ‘Hegazy’, ‘Wardi’ and ‘Manfalouty’ had the highest anthocyanin content were insignificant. The pH values varied from 3.3 (Wardi) to 2.9 (Araby). These values were near the values reported by Hassan et al. (2012). Similar values were obtained for pomegranate cultivars grown in Turkey, and some Spanish varieties evaluated by Martinez et al. (2006).

Identification of ISSR markers

ISSR is a class of molecular markers based on inter-tandem repeats of short DNA sequence. These inter repeats are highly polymorphic, even among closely related genotypes, due to the lack of functional constraints in these non-functioning region. A total number of 90 ISSR bands were obtained. Forty eight bands were polymorphic (53%) and 42 were monomorphic (46%). The highest number of amplicons were generated from Assuity (71 amplicons), while Araby cultivar generated the lowest number of bands (59 amplicons). The highest number of amplicons was generated from primers 851, bec and HB15 (14 amplicons), while the lowest was generated from primer 17898a (3 amplicons) (Table 6 and Figure 3 as a model). A number of 23 amplicons were a specific markers in which 14 of them were scored for the presence of a

Table 4. The color characteristics (skin, internal peel, arils, juice and seeds) of the pomegranate cultivars.

Cultivar	Color parameter														
	Fruit skin			Internal peel			Arils			Juice			Seeds		
	L	a	b	L	a	b	L	a	b	L	a	b	L	a	b
Araby	55.43^{A}	20.15^{C}	35.12^{A}	80.17^{A}	-3.282^{C}	27.02^{A}	46.54^{A}	2.075^{C}	13.37^{A}	26.98^{A}	2.736^{D}	-2.942^{A}	56.38^{BC}	4.599^{D}	19.63^{A}
Assuity	50.22^{BC}	23.65^{C}	12.84^{E}	77.07^{A}	-2.365^{C}	25.25^{AB}	33.59^{C}	16.92^{A}	10.99^{A}	22.50^{B}	12.03^{B}	-5.165^{BC}	60.31^{A}	9.185^{C}	19.73^{A}
Hegazy	49.67^{BC}	29.51^{B}	17.21^{D}	76.82^{A}	0.7600^{AB}	17.82^{E}	32.44^{C}	16.00^{A}	2.065^{C}	19.95^{C}	16.03^{A}	-4.655^{B}	53.78^{C}	17.00^{A}	13.31^{C}
Manfalouty	47.22^{C}	34.84^{A}	21.58^{C}	81.57^{A}	-1.881^{BC}	21.34^{CD}	32.04^{C}	17.66^{A}	4.562^{BC}	19.56^{C}	15.50^{A}	-5.190^{BC}	55.37^{BC}	14.92^{A}	13.77^{C}
Nab El Gamal	51.39^{B}	24.69^{BC}	25.44^{B}	79.35^{A}	-0.8906^{ABC}	18.81^{DE}	38.61^{B}	11.36^{B}	6.114^{B}	20.68^{BC}	12.02^{B}	-5.615^{C}	57.18^{B}	11.86^{B}	16.51^{B}
Wardi	52.11^{B}	35.53^{A}	26.54^{B}	70.74^{B}	1.773^{A}	23.83^{BC}	43.18^{A}	16.43^{A}	12.61^{A}	14.97^{D}	8.222^{C}	-3.022^{A}	53.45^{C}	11.36^{BC}	16.68^{B}
LSD at 0.05	3.032	5.206	2.743	5.798	2.797	2.636	4.02	3.388	2.686	2.106	1.862	0.704	2.971	2.607	2.134

Means in the same column for each characteristics followed by the same letter are not significantly different at 5% level.

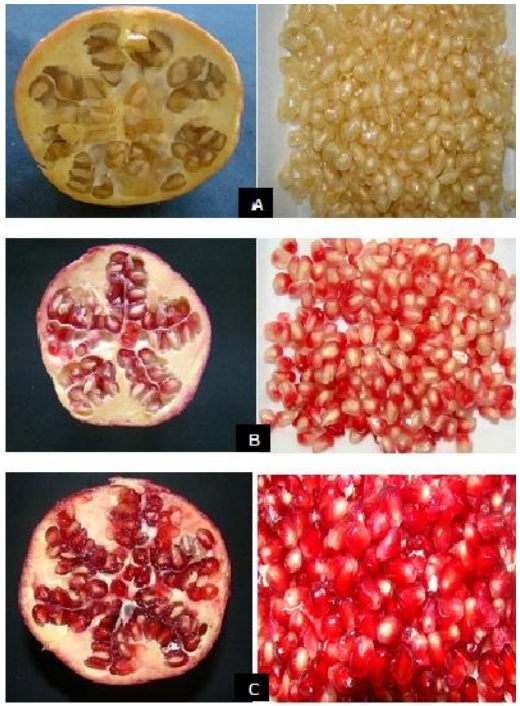

Figure 1. Pomegranate fruit cross section and arils of 'Araby', 'Assuity' and 'Hegazy' cvs (A, B and C) respectively.

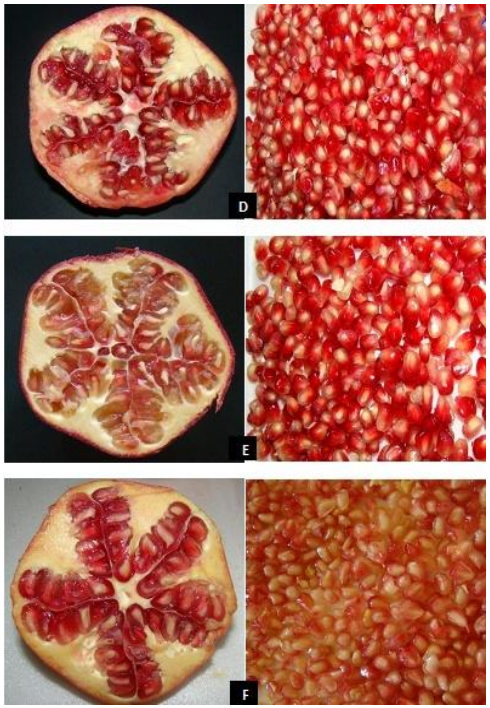

Figure 2. Pomegranate fruit cross section and arils of 'Manfalouty', 'Nab El Gamal' and 'Wardi' cvs. (D, E and F) respectively.

unique band for a six given cultivars (positive markers), while 9 were scored for the absence of a common band (negative marker). The highest number of cultivar-specific markers (9) was scored for Wardi cultivar, while Manfalouty cultivar did not scored any specific markers, as shown in Table 7.

Zahra et al. (2012) used three molecular markers including RAPD, ISSR and SSR to evaluate genetic diversity of thirty six Iranian pomegranate genotypes.

Genetic relationships and cluster analysis as revealed by ISSR data

The genetic similarity between the six cultivars indicated that the highest genetic similarity was scored between Assuity and Hegazy cultivars (94%), while the lowest genetic similarity was scored between Wardi and both Araby and Nab El gamal (77%) (Table 8). The dendrogram grouped the six pomegranate cultivars into two clusters, the first contained Wardi cultivar, while the second contained the rest of pomegranate cultivars. The later was further divided into two

Table 5. The fruit chemical characteristics of the pomegranate cultivars.

Cultivar	SSC (°Brix)	V.C (mg vitamin/100 ml juice)	Anthocyanin (mg/100 g fresh arils)	TA (%)	pH
Araby	12.84^{C}	3.785^{D}	1.470^{D}	0.700^{D}	2.956^{BC}
Assuity	12.55^{C}	14.00^{A}	3.870^{C}	2.600^{A}	3.000^{BC}
Hegazy	16.01^{A}	11.40^{C}	10.03^{A}	1.694^{C}	3.006^{BC}
Manfalouty	15.47^{A}	12.90^{B}	9.451^{A}	2.094^{B}	2.950^{C}
Nab El Gamal	14.41^{B}	3.215^{D}	6.143^{B}	2.294^{B}	3.056^{B}
Wardi	14.36^{B}	4.000^{D}	9.528^{A}	0.868^{D}	3.338^{A}
LSD at 0.05	0.5956	0.9848	1.855	0.2325	0.1045

Means in the same column for each characteristics followed by the same letter are not significantly different at 5% level.

Table 6. Number of amplified fragments markers of six pomegranate cultivars based on ISSR analysis.

Cultivar		ISSR primer										
		890	17898b	835	851	809	HB10	17898a	Bec	Had	HB15	Total
Wardi	AF	6	5	8	9	5	5	3	8	4	10	63
	SM	1	1	0	1	0	0	0	4	2	0	9
Araby	AF	7	5	8	7	5	5	3	4	6	9	59
	SM	2	1	0	0	2	0	0	1	0	1	7
Manfalouty	AF	7	4	8	7	5	5	3	8	7	10	64
	SM	0	0	0	0	0	0	0	0	0	0	0
Assuity	AF	7	4	11	8	5	5	3	8	7	13	71
	SM	0	0	1	2	0	0	0	0	0	0	3
Hegazy	AF	7	3	11	5	5	5	3	9	6	13	67
	SM	0	0	1	0	0	0	0	0	0	0	1
Nab-El Gamal	AF	7	4	9	8	5	5	3	4	6	10	61
	SM	0	1	0	2	0	0	0	0	0	0	3
TSM		3	3	2	5	2	0	0	5	2	1	23
TAF		8	7	12	14	6	5	3	14	7	14	90
PB		3	4	4	10	2	0	0	14	3	8	48
%polymorphism		37	57	33	71	33	0	0	100	42	57	53

TAF = Total number of amplified fragment; PB = polymorphic bands; AF = amplified fragment; SM = marker, including either the present or absence of a band in pomegranate cultivar; TSM = total number of specific markers across pomegranate cultivars.

subcultures, the first contained Araby while the second contained four cultivars, Manfalouty, Assuity, Hegazy and Nab El gamal (Figure 4).

Polymorphism detected by AFLP analysis

The two primer combinations used in the AFLP analysis revealed 141 amplicons including 128 polymorphic amplicons (90.7%) among the six pomegranate cultivars as shown in Table 9 and Figure 5 as a model. Zahra et al. (2012) evaluated the diversity of a number of Iranian pomegranate cultivars using fruit morphological characteristics and AFLP markers. Our results for the rate of polymorphism on pomegranate genome are in agreement with the results obtained by Sezai et al. (2011). The size of AFLP fragments in the present study ranged from 1700 to less than 100 bp and the polymorphic fragments were distributed across the entire size range. The primer combination E-ACC/M-CAA was

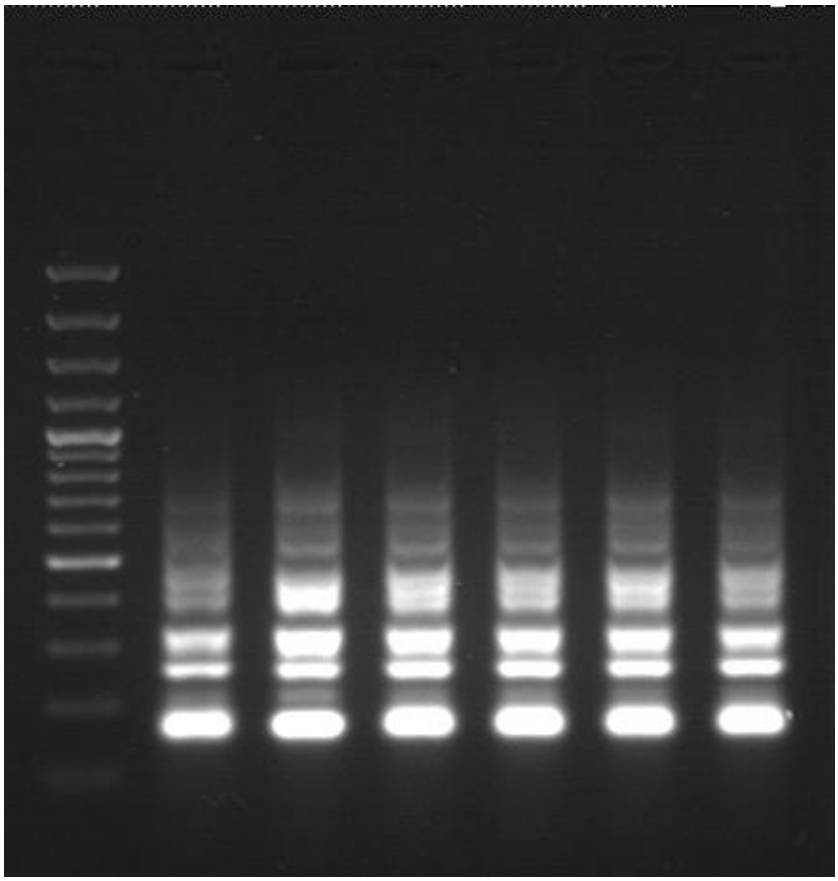

Figure 3. ISSR profile of the six pomegranate cultivars amplified with ISSR 890 primer. M: 1 kb ladder marker. Lanes 1 through 6 refer to pomegranate cultivars: Wardi, Araby, Manfalouty, Assuity, Hegazy and Nab El gamal.

Table 7. Cultivar-specific markers resulting from ISSR analysis.

Pomegranate cultivar	Positive marker	Negative marker	Total
Wardi	5	4	9
Araby	3	4	7
Manfalouty	0	0	0
Assuity	3	0	3
Hegazy	1	0	1
Nab El gamal	2	1	3
Total	14	9	23

Table 8. Similarity matrix resulting from ISSR data for the six pomegranate cultivars.

Population	Wardi	Araby	Manfalouty	Assuity	Hegazy	Nab El gamal
Wardi	1.00					
Araby	0.77	1.00				
Manfalouty	0.83	0.80	1.00			
Assuity	0.79	0.80	0.90	1.00		
Hegazy	0.82	0.81	0.90	0.94	1.00	
Nab El gamal	0.77	0.83	0.85	0.82	0.84	1.00

Table 9. Polymorphism detected by AFLP marker for the six pomegranate cultivars.

Primer combination	Total number of amplicons	Polymorphism number	% polymorphism
E-ACC/M-CAA	72	65	90.2
E-ACT/M-CTT	69	63	91.3
Total	141	128	90.7

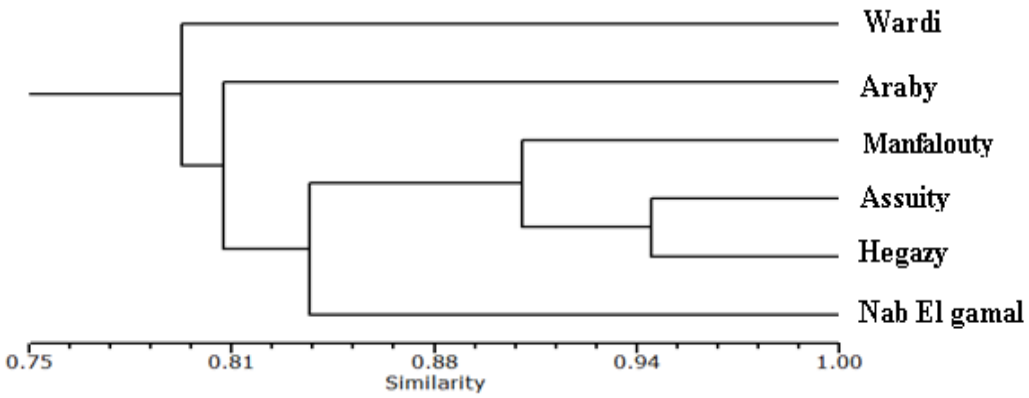

Figure 4. Cluster analysis as revealed by ISSR data number 1 through 6 refers to pomegranate cultivars: Wardi, Araby, Manfalouty, Assuity, Hegazy and Nab El gamal, respectively.

found to be the most informative one, which confirmed the high multiplex ratio expected with this type of markers. This high level of in-formativeness represents one of the most important advantages of AFLP; hence, provide wide range coverage of the genome (Krauss, 1999). The number of polymorphic amplicons produced by the different primer combinations ranged from 65 (E-ACC/M-CAA) to 63 (E-ACT/M-CTT) and the average number of scorable bands per gel was 128 (Table 9). The level of polymorphism ranged from 90.2 to 91.3% in primer combinations E-ACC/M-CAA and E-ACT/M-CTT, respectively. The two AFLP primer combinations characterized the six pomegranate cultivars by a total of 45 unique AFLP markers (43 positive and 2 negative). Manfalouty cultivar was characterized by the highest number of unique AFLP markers (15 unique positive markers), while Hegazy was characterized by 13 unique AFLP markers; Wardi was characterized by the lowest number of unique markers, only two positive band (Table 10).

Sezai et al. (2011) reported that the molecular characterization is necessary to get reliable assessment of the relationships among pomegranate genotypes and AFLP markers can be used effectively in this case.

Genetic relationships and cluster analysis as revealed by AFLP data

The similarity level among the six pomegranate cultivars according to Nei (1972) coefficient ranged from 73 to 33% between Wardi and Araby, and Assuity and Hegazy, respectively (Table 11 and Figure 6). The dendrogram grouped the six pomegranate cultivars into two clusters where Hegazy cultivar formed a separate cluster. The second cluster was divided into two sub clusters, one containing cultivar Assuity and the other subcluster grouped contains Wardi, Araby, Manfalouty and Nab El gamal.

The combined similarity matrix of the six pomegranate cultivars based on ISSR and AFLP markers

The similarity matrix according to the combined dendrogram based on ISSR and AFLP markers for the six pomegranate cultivars is shown in Table 12. The ISSR and AFLP similarity matrices data showed that the range between the lowest and highest similarity coefficient was wider for AFLP (0.33 to 0.73) than ISSR (0.77 to 0.94) which indicates that the AFLP system was able to detect a higher level of polymorphism between pairwise combinations. However, the combined similarity matrix of ISSR and AFLP data showed that the similarity coefficient ranged from 0.62 to 0.75. The lowest genetic similarity coefficient (0.62) was between cultivars (Hegazy and Wardi); whereas, the highest similarity coefficient (0.75) was between cultivars (Wardi and Araby). The resultant dendrogram divided the cultivars in

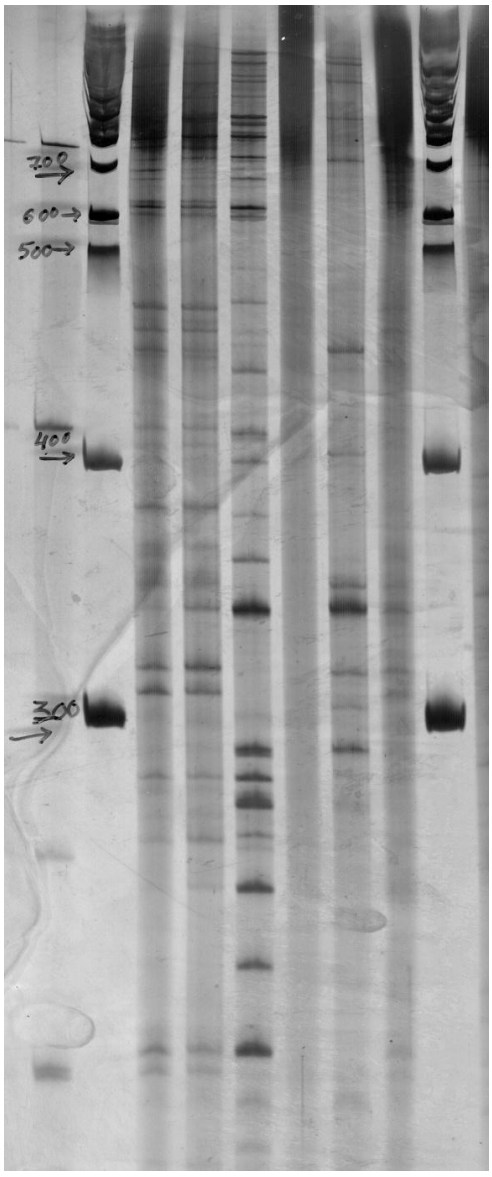

Figure 5. AFLP profile of the six pomegranate cultivars amplified with E-ACC/M-CAA primer. M: 100 bp ladder marker. Lanes 1 through 6 refer to pomegranate cultivars: Wardi, Araby, Manfalouty, Assuity, Hegazy and Nab El gamal, respectively.

Table 10. Cultivar-specific markers resulting from AFLP analysis.

Pomegranate cultivar	Positive marker	Negative marker	Total
Wardi	2	0	2
Araby	4	0	4
Manfalouty	15	0	15
Assuity	7	1	8
Hegazy	12	1	13
Nab El gamal	3	1	4
Total	43	3	46

Table 11. Similarity matrix resulting from AFLP data for the six pomegranate cultivars.

Pomegranate cultivar	Wardi	Araby	Manfalouty	Assuity	Hegazy	Nab El gamal
Wardi	1					
Araby	0.73	1				
Manfalouty	0.5	0.62	1			
Assuity	0.46	0.48	0.39	1		
Hegazy	0.44	0.44	0.50	0.33	1	
Nab El gamal	0.60	0.61	0.46	0.54	0.38	1

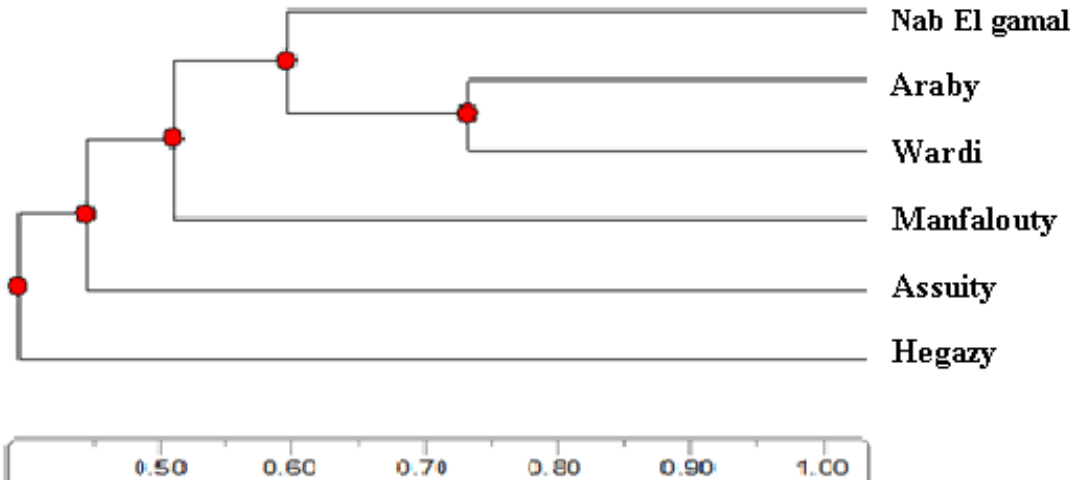

Figure 6. Cluster analysis as revealed by AFLP data number 1 through 6 respectively refers to pomegranate cultivars: Wardi, Araby, Manfalouty, Assuity, Hegazy and Nab El gamal.

Table 12. The combined similarity matrix for the six pomegranate cultivars based on ISSR and AFLP markers.

Pomegranate cultivar	Wardi	Araby	Manfalouty	Assuity	Hegazy	Nab El gamal
Wardi	1					
Araby	0.75	1				
Manfalouty	0.66	0.70	1			
Assuity	0.65	0.65	0.65	1		
Hegazy	0.64	0.62	0.69	0.68	1	
Nab El gamal	0.69	0.72	0.65	0.70	0.64	1

two main clusters. Three cultivars (Wardi, Araby and Nab El gamal) fell in one cluster while the second was comprised of the other cultivars (Manfalouty, Assuity and Hegazy) (Figure 7). It was observed that there was a correspondence with the separate dendrogram of either ISSR or AFLP that the cultivars clustered together.

DISCUSSION

This study aimed to detect the fruit characteristics of local pomegranate cultivars for the appropriate use for fresh fruit or processing for the production of juices, jam, molasses, vinegar and extract dyes and natural colors. It could be noted that 'Nab El Gamal' and 'Manfalouty' were the biggest fruit size and arils weight. 'Hegazy' and 'Manfalouty' cvs revealed the highest value of SSC, the anthocyanin and the red color of juice; these may be more suitable for fresh cut and the juice industry whereas 'Assuity' recorded the highest values for TA and V.C. content. The present study calls for the development of molecular methods suitable for the assessment of pomegranate genetic polymorphisms; thus, using the designed method in the investigation of a large number

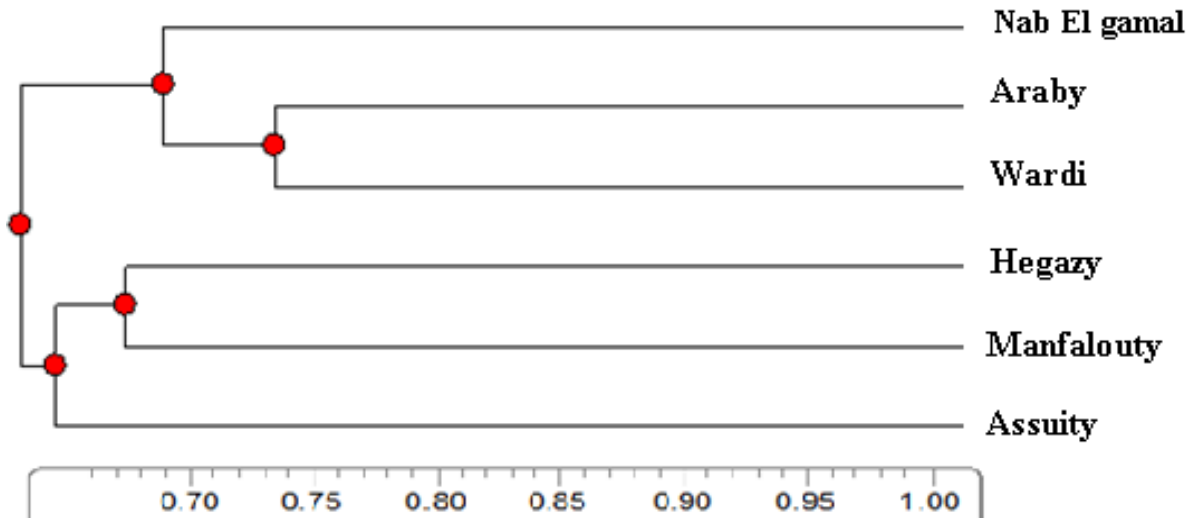

Figure 7. The combined dendrogram for the six pomegranate cultivars based on ISSR and AFLP markers.

either of ecotypes and / or primers would provide inferences about the genetic diversity structure of Egyptian pomegranate cultivars. Work is currently in progress in order to either gain a deeper insight into the genetic diversity and to molecularly characterize the Egyptian pomegranate germplasm or to enhance its cultivation throughout the establishment of selection programs. All ISSR primers used in the present study allowed for enough distinction among the six pomegranate cultivars as used earlier in previous work (Zahra et al., 2012).

AFLP was able to differentiate among all pomegranate cultivars with a higher number of unique markers compared to ISSR. Furthermore, the combined analysis (ISSR and AFLP) provided higher resolution for the distinction between the studied cultivars.

Conclusion

'Manfalouty' cv. has good appearance and taste for fresh fruit marketing and can be considered as a strong competitor to the wonderful pomegranate cv. if the growers apply the good agricultural practices. Keeping post-harvest good quality and resistance to fruit handling are desirable traits. The other cultivars can be used for different industrial purposes. ISSR and AFLP are dominant markers, but AFLP is more complicated than ISSR. Different markers differ in their ability to differentiate between individuals, the mechanism of detecting polymorphism, genome coverage and the ease of application. Therefore, they could be complementary to each other depending on technical availability. Selection assisted by these molecular markers may also be helpful to produce new cultivars with improved productivity, abiotic tolerance and pest resistance. Furthermore, marker-assisted selection is important in the assessment of the genotypes used in the local pomegranate cultivars improvement programs in Egypt.

In conclusion, additional pomegranate germplasm needs to be collected from other locations and characterized to ensure the representation of most of the genetic diversity is conserved *in situ*.

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