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Morphological and molecular characterization of ancient pomegranate (*Punica granatum* L.) accessions in Northern Italy

D. Beghè [1 (*)], A. Fabbri [1], R. Petruccelli [2], M. Marieschi [3], A. Torelli [3], T. Ganino [1, 2]

[1] *Department of Food and Drug Science, University of Parma. Parco Area delle Scienze, 27/a, 43124 Parma, Italy.*

[2] *Institute for the Bioeconomy (IBE), Italian National Research Council (CNR), 50019 Sesto Fiorentino, Italy.*

[3] *Department of Chemistry, Life Sciences and Envinronmental Sustainability, University of Parma. Parco Area delle Scienze, 11/a, 43124 Parma, Italy.*

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(*) Corresponding author:
deborah.beghe@unipr.it

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Abstract: The Italian research on *P. granatum* L. is still limited, although the study of local germplasm is extremely important in order to preserve the existing biodiversity and to identify potential useful characters for a renewed industry. The study aimed at characterizing for the first time ancient pomegranates, grown in Emilia Romagna (Italy), through 38 quantitative morphometric descriptors related to leaf, flower, fruit and seed, 42 RAPD and 12 SSR markers. Morphological analyses showed large variation of traits among accessions and the descriptors related to fruit and seed had the highest power of discrimination. The considerable variation found was consistent with ANOVA and PCA results. Among all RAPDs tested, 7 were selected for their polymorphism; whereas among selected SSRs, 8 presented differences in the genetic profiles allowing a good discrimination of the local pomegranate accessions. The genetic relationships among pomegranates were studied by UPGMA cluster analysis and the accessions were clearly regrouped in four different genotypes. The study has highlighted significant differences and interesting pomological characteristics in the local pomegranates, which confirmed the good potential of this germplasm for the pomegranate industry.

1. Introduction

Pomegranate *(Punica granatum* L.) is one of the world most ancient domesticated fruit crops and it is believed to have been first grown in the region between the Caspian Sea and the Caucasus (Zohary and Spiegel-Roy, 1975). Its diffusion occurred across the millennia due to man's activities or gene flow in quite varied environments as concerns climatic conditions, has produced a rich and diversified germplasm. *P. granatum* L. has

a large genetic pool, represented by over 500 described cultivars throughout the world, and by a wide amount of wild plants, and its germplasm is so far only partially explored (Beghè *et al.*, 2016). In spite of the wide genetic diversity, only 50 cultivars were widely cultivated in the main growing areas at the time of the last official survey made by international institutions (IPGRI, 2001). Consequently, the risk of a drastic loss of the existing biodiversity is high.

In the last decade the interest on pomegranate has grown and the world production (estimated to be around 1.5 million tonnes, of which 90% provided by the main producers: India, China, Iran) has rapidly increased (da Silva *et al.,* 2013). The renewed interest in this crop is to be ascribed to socio-economical and cultural factors, which led to a change in food habits in the West, with a growing attention to the nutritional quality of foods (Negri, 2003). Pomegranate, in this respect, is considered one the fruits with the most valuable nutritional properties (Calani *et al.,* 2013).

Pomegranate cultivation is also growing in Italy, and in a ten-year period (since 2008) the surface covered, from a mere 7 ha (2 ha in Calabria and 5 in Sicily), passed to 1142 ha (mainly in Southern Italy, Sicily (364 ha) and Apulia (363 ha) and, with minor productions, also in Venetia (152 ha), Latium (101 ha), Emilia Romagna (44 ha), Tuscany (21 ha) and Lombardy (20 ha). Productions rose from only 69 tons to over 12531 tons (AGRISTAT, 2017).

One of the trends of recent years has been to import from Israel pomegranate cultivars created and patented by Israel breeders, to be utilised in Italian orchards. Although this strategy has led to an increase in production, it hinders any attempt at exploiting Italian cultivars, which have been known for centuries but are not utilised in commercial cultivation.

In Italy, there are several local cultivars which are little known and scarcely diffused in the country. Scarce research, mostly confined to Southern Italy, has been devoted to the characterization of *P. granatum* L. Italian diversity, by means of molecular, morphological and biochemical markers (Adiletta *et al.,* 2018). A research on the whole national territory is unavoidable to select autochthonous genotypes suited to the different environments, and to promote a pomegranate industry able to satisfy the internal demand and to compete with the international product.

The best suited Italian areas for pomegranate growing are the central and southern regions, characterized by a Mediterranean climate, unlike the northern regions, which have a continental climate, with cold and snowy winters. However, in some zones of Emilia-Romagna (44°-45° N and 11°-12° E, Northern Italy) microclimate conditions are such as to allow the cultivation of this species and of other Mediterranean species like olive (Lona *et al.,* 1981; Calani *et al.,* 2013). Ancient pomegranate trees, survived for hundreds of years and adapted to local conditions have been retrieved in these areas. This local germplasm is of particular interest, being the result of a selection process occurred during many centuries in the unfavorable conditions of this territory.

The present research is part of a multidisciplinary project aiming at valorising ancient pomegranate cultivars of Northern Italy. We utilized morphological and molecular markers (Random Amplified Polymorphic DNA (RAPDs) and Simple Sequence Repeat (SSRs)) to characterize ancient pomegranate accessions present in Emilia-Romagna Region. The pomological comparisons also had the purpose of determining the peculiar features and the potential of these plants, for a possible introduction in commercial plantings or for their use in breeding programs.

2. Materials and Methods

Plant materials

The pomegranate germplasm subject of this study was represented by very ancient trees located in a small hill area of Parma province (Emilia-Romagna) (44°69 N, 10°02 E), at an altitude from 150 to 250 m a.s.l. Eight accessions of *P. granatum* L., tagged with an alpha-numerical code (ID): ME1, ME2, ME3, ME4, ME5, ME6, ME7 and ME8 (Table 1), were studied during two seasons, 2014 and 2015. The selected plant material was maintained at pomegranate germplasm collection field in the low hills of the Emilia Appennins, where each accession was replicated 4 times. The pomegranate trees were planted at a spacing of 5 x 5 m and trained to form a bush.

Morphological characterization

The accessions were characterized according to the guidelines proposed by the project EC Project GENRES 29 "Conservation, evaluation, exploitation and collection of minor fruit tree species" (Bellini and Giordani, 1998), integrated by the list of characters proposed by Bellini *et al.* (2007) and by the International Union for the Protection of New Varieties of Plants (UPOV, 2012).

Plant material was randomly sampled from

around the canopy of four plants per each accession, by collecting 40 flowers (20 hermaphrodite also called "long-styled" and 20 male also called "short-styled") at full bloom, on June 1st, 40 adult leaves, from the middle part of the shoot in summer and 12 fruits, at ripening, in the first decade of October. All seeds were extracted from each fruits and 25 of them were randomly selected; arils (the seed fleshy coats, containing edible juice, that represent the seed outer integument or testa) were hand removed to analyze also the tegmen (seed lignified inner integument). The morphological characters evaluated included 38 quantitative traits (Table 2). The linear dimensions were determined with a caliper, and the weight was measured using a semi-analytic electronic scale. From these values other indices have been calculated as indicated in Table 2. Furthermore, some qualitative characters were observed; these traits are reported in Table 1.

DNA extraction and molecular characterization

Total cellular DNA was extracted from young leaves following the CTAB (cetyl trimethylammonium bromide) as reported in Ganino *et al.* (2008).

Forty-two decamer oligonucleotide primers belonging to the AI, AH, OPA, OPC, OPX and OPK series (Table S1 of supplementary data) and twelve couples of SSR primers belonging to the PgAER (Çalişkan *et al.,* 2017), PGKVR (Ravishankar *et al.,* 2015), Pom (Hasnaoui *et al.,* 2012), POM-AGC (Currò *et al.,* 2010) and PG (Ebrahimi *et al.,* 2010) were used for polymorphism detection on the samples. RAPD amplifications were performed as reported in Marieschi *et al.* (2016).

The RAPD profiles obtained with each utilized primer were analyzed by comparison with Gene Ruler 100 pb DNA Ladder plus marker (M-Medical, Milano, IT), with the Kodak digital sciences 1 D Images Analysis Software, calculating the size in base pairs (bp) of each amplicone present in the electrophoretic run of each sample.

SSR amplification reaction was performed as reported in Ganino *et al.* (2008). The amplification condition, for the PGKVR, PgAER, POM-AGC and PG series, were: a first step at 95°C for 5 min followed by 35 cycles of 45 s at 94°C, 45 s at 57°C, 45 s at 72°C, for denaturation, annealing, and primer extension; the last step included 8 min of incubation at 72°C. For the "Pom" serie, the following thermal cycling protocol was used: a first step at 95°C for 3 min followed by 10 touchdown cycles of 30 s at 94°C, 40 s at 65°C (-1°C per cycle), 30 s at 72 and 25 cycles of 30 s at 94°C, 30 s at 55°C, 40 s at 72°C with final extension time of 8 min at 72°C. The amplification products were separated with a CEQ 2000 Genetic Analysis System (Beckman Coulter, Inc.) sequencer on acrylamide gel CEQ Separation Gel LPA-1 (Beckman Coulter, Inc.). A marker CEQ DNA Size Standard kit 400 (Beckman Coulter, Inc.) was used to estimate the molecular weight of the amplified products.

Data analysis

The quantitative morphological characters were evaluated: means, minimum and maximum, standard deviation. The coefficient of variation (CV) was calculated as indicator of variability. All data were subjected to one way analysis of variance (ANOVA) followed by Tukey test to determine the statistically significant differences ($p \leq 0.05$). Correlation analyses between descriptors to reveal possible relationships were car-

Table 1 - *Punica granatum* L. accessions studied, coded (ID) and main qualitative characteristics of their fruit, leaf and flower

ID	Fruit shape	Size	Epicarp colour	Calyx type	Leaf shape	Petiol colour	Mucro	Blade colour	Flower petal colour	Shape short-stiled	Shape long-stiled
ME1	oblate/rounded-spheroid	large/very large	reddish-yellow/red	semi-closed/closed	elliptic	yellow	no	yellow	red/orange	medium bell	sinuolate jug
ME2	oblate/rounded-spheroid	large/very large	reddish-yellow/red	semi-closed/closed	elliptic	red	no	yellow	red/orange	medium bell	sinuolate jug
ME3	oblate/rounded-spheroid	large	reddish-yellow/red	semi-closed/closed	elliptic	red	no	yellow	red/orange	medium bell	sinuolate jug
ME4	oblate/rounded-spheroid	large/very large	reddish-yellow/red	semi-closed/closed	elliptic	red	no	yellow	red/orange	medium bell	sinuolate jug
ME5	oblate/rounded-spheroid	large/very large	reddish-yellow/red	semi-closed/closed	elliptic	red	no	yellow	red/orange	medium bell	sinuolate jug
ME6	oblate/rounded-spheroid	large	reddish-yellow	semi- closed/open	elliptic	red	no	yellow	red/orange	broad bell	jug with base
ME7	oblate/rounded-spheroid	very small	reddish-yellow/red	open	elliptic	yellow	no	yellow	red/orange	narrow bell	sinuolate jug
ME8	oblate/rounded-spheroid	very small	reddish-yellow/red	open	elliptic	yellow	no	yellow	red/orange	narrow bell	sinuolate jug

Table 2 - Quantitative traits used for characterizing pomegranate accessions and their descriptive statistics analysis using the mean, minimum, maximum, standard deviation (SD) and coefficient of variation (CV)

Trait	Trait code	Mean	Minimum	Maximum	SD	CV (%)
Leaf						
Leaf fresh weight (g)	LFW	0.10	0.070	0.160	0.029	29.13
Leaf length (cm)	LL	5.44	4.450	6.920	0.828	15.21
Leaf width (cm)	LW	1.62	1.260	2.150	0.278	17.21
Leaf shape (length/diameter)	LS	3.50	2.970	3.910	0.341	9.76
Flower						
Flower diameter long-styled (cm)	FDL	1.51	1.220	1.810	0.221	14.57
Flower length long-styled (cm)	FLL	4.75	2.900	5.950	1.193	25.11
Petal number long-styled (cm)	PNL	6.52	5.670	7.500	0.713	10.93
Pistil length long-styled (cm)	PLL	1.75	1.500	2.030	0.231	13.20
Flower diameter short-styled (cm)	FDS	1.48	1.200	1.770	0.163	11.06
Flower length short-styled (cm)	FLS	3.73	2.840	4.490	0.564	15.10
Petal number short-styled	PNS	6.50	6.000	7.400	0.510	7.86
Pistil length short-styled (cm)	PLS	0.43	0.310	0.600	0.105	24.43
Fruit						
Fruit weight (g)	FW	274.02	87.010	388.730	120.303	43.90
Fruit diameter equatorial (cm)	FD	8.22	5.070	9.600	1.799	21.88
Calyx diameter equatorial (cm)	CD	2.14	1.430	2.840	0.545	25.50
Fruit height without calyx (cm)	FL1	6.64	4.400	8.120	1.380	20.80
Total fruit length (cm)	FL2	8.11	5.570	9.530	1.568	19.34
Calyx height (cm)	CL	1.56	1.170	2.220	0.323	20.67
Fruit skin thickness equatorial (mm)	FT	0.42	0.300	0.550	0.096	22.69
Fruit skin and carpellary membranes weight (g)	SCW	129.94	28.980	191.810	66.526	51.20
Number of carpel in equatorial section	NC	6.88	5.330	8.000	0.993	14.43
Fruit shape index (height/diameter)	FSI	0.81	0.730	0.890	0.059	7.21
Calyx shape index (height/diameter)	CSI	0.80	0.610	0.880	0.095	11.93
% Skin and carpellary membranes	SC (%)	45.10	33.300	53.400	7.499	16.63
% Seeds	S (%)	54.92	46.600	66.700	7.491	13.64
Total seeds weight (g)	STW	141.918	58.076	202.556	53.394	37.62
Seed weight (g)	SW	0.27	0.144	0.381	0.080	29.57
Seed length (cm)	SL	0.96	0.786	1.080	0.113	11.73
Seed diameter (cm)	SD	0.69	0.547	0.804	0.090	13.04
Tegmen weight (g)	TW	0.02	0.016	0.029	0.005	19.95
Tegmen length (cm)	TL	0.65	0.544	0.769	0.074	11.35
Tegmen diameter (cm)	TD	0.31	0.253	0.416	0.052	16.88
Woody portion index (tegment weight/aril weight)	WPI	0.09	0.071	0.121	0.021	22.12
Seed shape (length/diameter)	SL/SD	1.39	1.310	1.540	0.083	5.93
Tegment shape (length/diameter)	TL/TD	2.15	1.880	2.410	0.176	8.19
Aril weight (g) [z]	AW	0.25	0.120	0.350	0.080	32.49
Aril weight/tegmen weight	AW/TW	10.04	7.400	12.390	2.162	21.53
% Aril	A (%)	90.35	88.200	92.600	1.653	1.83

[z] Aril weight were calculated by subtracting tegmen fresh weight from whole seed fresh weight.

ried out using a bilateral Pearson correlation. The same characters were also submitted to a principal component analysis (PCA) to evaluate the relationship between pomegranate accessions. The analysis was performed using XLSTAT 2009 software (AddinsoftTM1995-2009).

RAPD bands were treated as binary characters (present = 1 or absent = 0), XLSTAT 2009 software was used to estimate genetic similarities/dissimilarities using Jaccard's similarity coefficient and cluster analysis by using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm.

The size of SSR fragments was determined using a conservative binning approach (Kirby, 1990) through the statistical R software. The information content of the SSR markers under study was evaluated according to number of alleles per locus (N_a), observed (H_o) and expected (H_e) heterozygosity, and polymorphic information content (PIC) (Botstein *et al.,* 1980) using the Cervus 3.0 software (Kalinowski *et al.,* 2007). The level

of similarity/dissimilarity among examined accessions was obtained through the genetic similarity matrix utilizing Manhattan distance and cluster analysis (UPGMA) algorithm, with XLSTAT 2009 software.

Finally, to test the correlations between genetic distance matrices and between the morphological and genetic distance matrices among accessions, Mantel tests were performed (Mantel, 1967). Each matrix distance was obtained by calculating Pearson's index. Mantel tests were performed with 100,000 permutations (p = 0.05). Pearson's r-value was used to measure linear correlation between two matrices.

3. Results and Discussion

Morphological characterization

The data resulting from the 2-year study were grouped and the average values were used for statistical analysis. The accessions showed significant variability in many of the characters analyzed. Descriptive values for each quantitative trait are recorded in Table 2. The coefficient of variation (CV) was used to determine the total variability present in each trait. The CV varied from 1.83% (A%) to 51.20% (SCW%), with seven traits having CV between 15 and 20% and fourteen traits with CV value higher than 20%. According to Audergon (1987), the descriptors with a high CV are more discriminating than the other ones, and can be reliable markers for the characterization of pomegranate accessions. The highest CV values were evident in traits involving fruits and the lowest were in flowers (except FLL and PLS), leaves (except LFW) and seeds (except STW, SW, WPI, AW, AW/TW). These results are consistent with previous studies (Zamani *et al.,* 2007, Mansour *et al.,* 2011).

The mean leaf quantitative values are reported in Table S2 and the traits values presented significant differences between the accessions. Moreover, leaf blade margin color and petiole color next to the shoot was red for accessions ME1, ME7 and ME8, and yellow for the others. All accessions have an elliptic shape and absence of mucro (Table 1).

The flower characteristics are reported in Table 1 and Table S3 and the observed values are comparable with those of Lebanese genotypes studied by Dandachi *et al.* (2017). The flowers of ME7 and ME8 accessions showed a much smaller size than that of the flowers of the other, but presented a similar style length, a feature that favors pistil pollination. Moreover, the long-styled flowers presented a "sinuate jug", except the flowers of ME6 accession that presented "jug with base". ME7 and ME8 presented a "narrow bell" shape in the short-styled flowers whereas ME6 presented "broad bell" shape and other accessions presented flowers with "medium bell" shape.

The mean values of quantitative fruit and seed traits are reported in Tables 3 and S4. Significant variability was observed in total fruit weight (FW), in maximum equatorial diameter (FD) and in fruit length, with calyx (FL2) and without calyx (FL1). In particular, these characters have lower values for ME7 and ME8. All accessions showed fruits with shape "oblate or rounded-spheroid" and "closed or semi-closed calyx", except ME7 and ME8 that have a majority of fruits with "open calyx". The number of locules (NC) was higher in fruits of higher total weight.

Table 3 - Mean values, standard deviation and ANOVA analysis for fruit characteristics

ID	FW [z]	FD	CD	FL1	FL2	CL	FT	SCW	NC	FSI	CSI	SC%	S%
ME1	373.48 ±49.78 A	9.60 ±0.62 A	2.68 ±0.51 A	7.00 ±1.14 A	9.22 ±1.28 A	2.22 ±0.62 A	0.55 ±0.10 A	191.81 ±19.64 A	8.00 ±1.58 A	0.73 ±0.12 A	0.84 ±0.27 A	53.4 ±0.03 A	46.6 ±0.03 D
ME2	303.53 ±93.46 A	8.83 ±1.17 A	2.40 ±0.62 A	7.83 ±0.72 A	9.53 ±1.06 A	1.70 ±0.43 A	0.53 ±0.11 AB	144.42 ±51.87 A	6.67 ±0.58 AB	0.89 ±0.07 A	0.70 ±0.03 A	47.2 ±0.03 AB	52.8 ±0.03 CD
ME3	335.73 ±13.03 A	9.10 ±0.52 A	2.43 ±0.55 A	7.10 ±0.17 A	8.53 ±0.61 AB	1.43 ±0.49 A	0.44 ±0.10 BC	163.25 ±11.59 A	7.67 ±0.58 A	0.78 ±0.06 A	0.61 ±0.23 A	48.6 ±0.02 AB	51.4 ±0.02 CD
ME4	388.73 ±89.49 A	9.40 ±0.84 A	2.15 ±0.78 A	8.12 ±1.03 A	9.00 ±1.27 AB	1.61 ±0.41 A	0.48 ±0.11 ABC	185.73 ±46.34 A	7.60 ±0.55 A	0.86 ±0.06 A	0.82 ±0.31 A	48.3 ±0.03 AB	51.7 ±0.02 CD
ME5	345.68 ±87.85 A	9.50 ±1.14 A	2.84 ±0. 94 A	7.47 ±0.67 A	9.24 ±0.81 A	1.63 ±0.55 A	0.41 ±0.09 BC	181.74 ±47.63 A	7.25 ±0.58 AB	0.80 ±0.05 A	0.85 ±0.27 A	52.5 ±0.01 A	47.5 ±0.01 D
ME6	266.81 ±23.78 AB	8.60 ±0.71 A	1.70 ±0.14 A	6.45 ±0.64 AB	7.95 ±0.78 AB	1.50 ±0.14 A	0.37 ±0.08 BCD	111.03 ±6.31 AB	7.00 ±0.45 AB	0.75 ±0.01 A	0.88 ±0.01 A	41.9 ±0.06 BC	58.2 ±0.06 BC
ME7	87.01 ±21.77 B	5.67 ±0.57 B	1.43 ±0.06 A	4.73 ±0.38 B	5.82 ±0.58 B	1.25 ±0.11 A	0.30 ±0.09 D	28.98 ±7.48 B	5.33 ±0.58 B	0.84 ±0.02 A	0.88 ±0.19 A	33.3 ±0.01 D	66.7 ±0.01 A
ME8	91.21 ±27.45 B	5.07 ±0.11B	1.47 ±0.06 A	4.40 ±0.10 B	5.57 ±0.23 B	1.17 ±0.15 A	0.30 ±0.79 D	32.54 ±10.48 B	5.50 ±0.55 B	0.87 ±0.03 A	0.80 ±0.13 A	35.5 ±0.02 CD	64.5 ±0.02 AB

The same letter show no statistically significant differences (P<0.05).

[z] For explanation of character symbols, see table 2.

According to the list of "pomegranate descriptors" of Bellini *et al.* (2007), the fruits were classified as "large or very large" (ME1, ME2, ME3, ME4, ME5, ME6) and "very small" (ME7, ME8). The first group had a total weight mean values of about 335 g, comparable to those of the fruits of many Italian and Spanish cultivars (Martinez *et al.,* 2006 and Ferrara *et al.,* 2014). Moreover, the epicarp (or "skin") of the local fruits has presented different colors, ranging from reddish-yellow to red. The size of the fruit and the color of the epicarp are two important parameters considered in the international market as concerns the quality of the fresh product (Mansour *et al.,* 2011). Another important parameter for fruit quality was the descriptor "skin thickness". ME7 and ME8 fruits have a thinner "skin thickness" than other accessions, and in field their fruits were more subjected to cracking at the first rainfall in October.

Significant variability was observed in seeds total weight (STW), skin and carpellary membranes weight (SCW) and seeds percentage (S%) (Tables S4 and 3). The mean S% found was of 54.91% and it was similar to that reported in another Italian study (Cristofori *et al.,* 2011). Furthermore, our mean values were lower than those reported on Italian and Iranian cultivars (Ferrara *et al.,* 2014). The seed descriptors showed, for the majority of traits, significant differences between two groups of accessions: ME1, ME2, ME3, ME4, ME5, ME6 and ME7, ME8; the former had SL, SD, SW, TL, TW greater than the latter (Table S4).

Tegmen index (WPI), aril percentage (A%), aril weight (AW) and aril/tegmen ratio (AW/TW) are very important parameters from a qualitative point of view. The WPI is a parameter that refers to the quantity of lignified tissue contained in the seed compared to total seed weight, and consumers greatly appreciate seeds with a limited amount of lignified tissue (Martinez *et al.,* 2006). Accessions ME1, ME2, ME3, ME4, ME5 had a higher quantity of aril (AW) and a lower percentage of the tegmen index (WPI) than ME6, ME7 and ME8. The WPI presented an average value of 7.7% in the first group of accessions, and an average value of 11.9% in the accessions ME6, ME7 and ME8. These values were in agreement with those of other Italian (5.4 to 10%), Spanish (7.4 to 9.7%), Moroccan (6.1 to 10.7%) and Iranian (5.4 to 7.5%) accessions (Martinez *et al.,* 2006; 2012; Sarkhosh *et al.,* 2009). The aril is a tissue valued for the high production of juice; AW and A%, with reference to the individual seed, were high in all accessions, and A% showed an average value of about 90%; a value comparable and higher than that of other Italian and Spanish genotypes (La Malfa *et al.,* 2009).

Correlation among morphometric traits

The correlations found between the quantitative variables, significant at $p < 0.05$, are reported in figure 1. The correlation coefficient can provide infor-

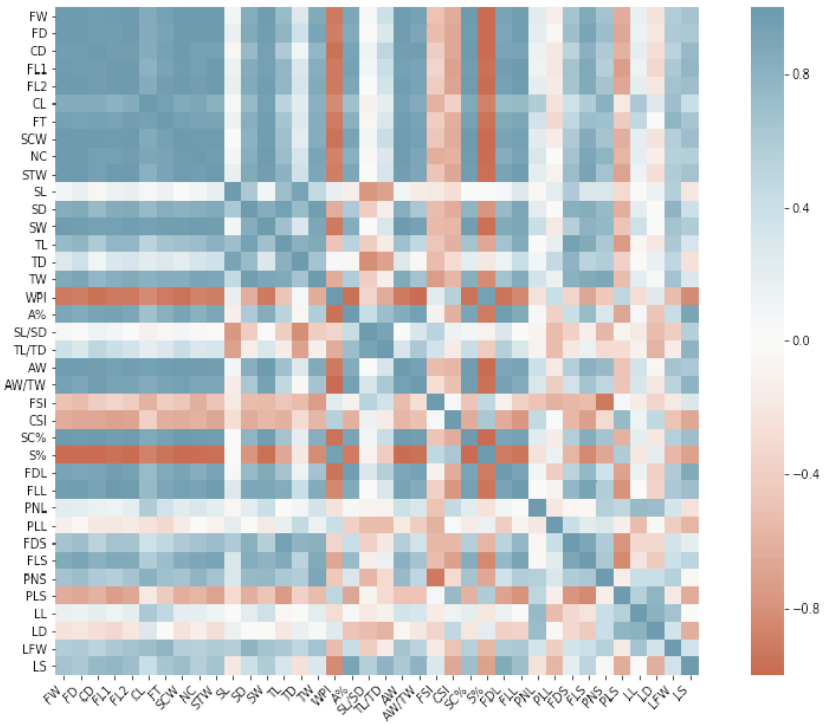

Fig. 1 - Pearson's correlation matrix of the quantitative traits in pomegranate accessions, visualized as a heat map plot. For explanation of character symbols, see table 2.

mation on the traits that are most important in assessing accessions (Norman *et al.,* 2011). Skinner *et al.* (1999) recommended analyzing correlation coefficients close to 0.7: in these conditions, the variance of one trait is strongly dependent on the others. According to this criterion, we estimated two hundred and fifteen valid correlations. Most of the significant correlations among traits coincide with those from the same plant organ, in particular the variables relative to fruits and seeds. A strong correlation value was found for fruit and seed descriptors, and these were also positively correlated with each other (*e.g.* FW was positively correlated with other fruit descriptors, FD, FL1, FL2, CL, SCW, NC, SC%, S% and seed descriptors, STW, SL, SD, SW, TW, A%, AW, AW/TW). The character WPI was negatively correlated with fruit traits relative to dimensions and with SW, SL, SD. The same results were observed in several former works that analyzed accessions from different countries (Martinez *et al.,* 2006; Zarei *et al.,* 2013). Moreover, the trait WPI could be an index of seed hardness, because this feature is strongly dependent on the trait WPI, as reported in Martinez *et al.* 2012 ($r = 0.63$ $p \leq 0.01$). In flowers, positive correlations were found among the variables relative to dimensions: between FLS and FDS and between FLL and FDL. In leaves, a positive correlation was between LD and LL, actually all accessions showed an elliptic leaf shape. Finally, some correlations were observed between seed (SW, A%, AW, TL, TW) and flower characteristics (FDL, FLL, FDS, FLS, PNS); we noted that accessions with small flowers (ME7-ME8) presented fruits and seeds of smaller dimensions. In fact, fruit growth potential is largely determined genetically through the ovary size in several species (Rosati *et al.,* 2009). These correlations between different traits could be due to genetic linkage or to a pleiotropic effect (i.e., when one gene influences two or more seemingly unrelated phenotypic traits) (Iezzoni and Pritts, 1991).

Principal component analysis

The results of PCA revealed the existence of large variability among accessions. The total variance explained by the first three principal components (PCs) in the model was 83.53%. A plot of the percentage of variance explained by seven PCs and eigenvalues associated with the first seven PCs for each quantitative trait are reported in supplementary material (Fig. S1 and Table S5), respectively. The PC1 explained the 54.60 % of total variance and the traits with the greatest weight on this component were related to fruit (FW, FD, CD, FL1, FL2, CL, FT, SCW, NC, SC%), seed (STW, SL, SD, SW, TW, WPI, A%, S%) and some flower traits (FDL, FLL, FLS). The PC2 explained 15.82% of the variability, and showed a strong negative load for TD, LD and PNS, whereas a strong positive load was present for SL/SD ,TL/TD, FSI, and LS. Finally, LD, LL relating to the leaf and PLS relating to the flower showed the highest contribution to PC3 (13.11% of the variability). The comparison of plot scores for PC1, PC2 and PC3 in figure 2 permits to obtain a view of accession dispersion and their clustering based on morphological traits. The accessions, for the first two PCs, were grouped into two main groups highly dissimilar: the first group consists of ME7 and ME8, the second group consists of three sub-groups (sub-group ME6; sub-group ME2, ME3, ME4, ME5 and sub-group ME1). The groups plotted for the PC1 and PC3 were very similar to those on PC1-PC2 plot, though accession ME6 showed less differences with the sub-group ME2, ME3, ME4, ME5. As already reported in the literature (Martinez-Nicolas *et al.,* 2016), fruit characteristics

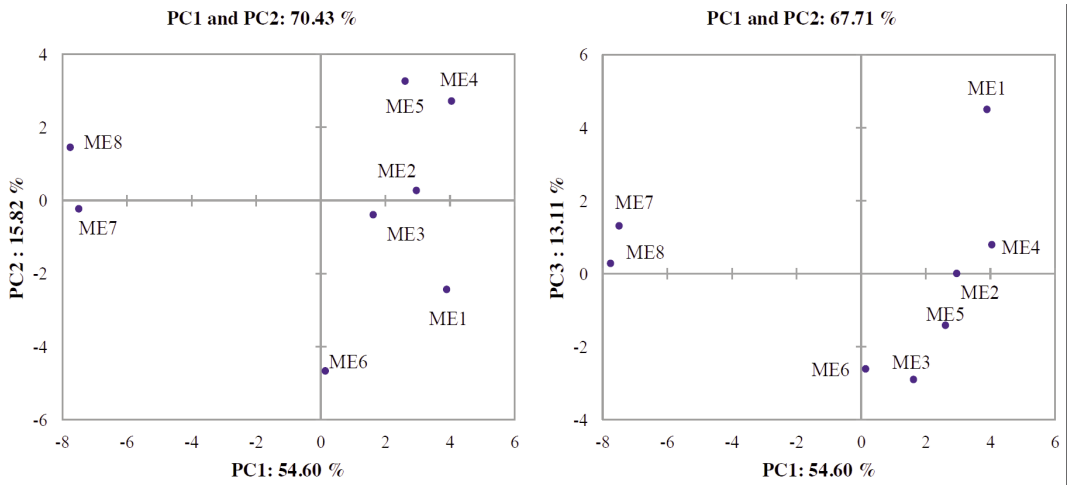

Fig. 2 - Loading plots of the first, second and third Principal Component showing the position of accessions.

had the highest loading values for the first component in principal component analysis. Our results confirmed that the traits related to fruit and seed had the highest power of discrimination, and were, therefore, the most useful for characterization of this local germplasm.

RAPDs and SSRs characterization and genetic relationships

Only 7 RAPD oligonucleotides (AI08, AI12, AI105, AH17, OPA19, OPB08, OPC16) out of 42 belonging to the AI, AH, OPA, OPC, OPX and OPK series, showed polymorphism in two or more accessions, by producing polymorphic and reproducible amplification patterns. The 7 oligonucleotides amplified a total of 84 RAPD fragments, 14 of which were polymorphic, making 16.67% polymorphism (Table 4). The number of polymorphic fragments found per primer was between 1 (OPB8, AI05 and AH17) and 3 (AI12, OPC16, AI08), with a mean of 2 (Table 4) and their size ranged from 250 to 3000 bp. The RAPD markers have been applied in many investigations aimed at the study of polymorphism in pomegranate, for their simplicity and low cost (Kathuria *et al.*, 2017), but these markers have often shown low polymorphism in this species. As reported in literature it is necessary an initial screening by a high number of RAPD primers to detect a discrete number of discriminating markers (Zamani *et al.,* 2010). The level of polymorphism detected in our study is lower than that reported in other works (Sarkhosh *et al.,* 2006; Zamani *et al.,* 2007). However, a percentage of polymorphism similar to ours was detected by Hasnaoui *et al.* (2010). In agreement with these authors we hypothesized that the slightly lower percentage of polymorphism detected could be due to the reduced dimension of the sample collection and to the limited variability in terms of geographical origin. Relationships among accessions were studied by cluster analysis (UPGMA) based on Jaccard's coefficient, and following statistical analysis a dendrogram was produced (Fig. 3A). The genetic distance among the accessions ranged from 0 to 0.8, showing genetic diversity among the pomegranate accessions. In the dendrogram, two main clusters could be identified: the first cluster included only two accessions (ME7 and ME8) at a 0 dissimilarity level (genetic identity). The second cluster comprised all other accessions which presented a level of dissimilarity that varied between 0 and 0.6. This last cluster presented three subgroups: ME1, ME6 and a subgroup that included accessions with genetic identity or with a very little (0.10) dissimilarity's distance (ME2, ME3, ME4, ME5).

Table 4 - Primer sequence of the most informative primers and level of polymorphism found by the RAPD analysis

RAPDs	Sequence	Total n° of bands	N° of polymorphic bands
OPA19	5'-d[CAAACGTCGG]-3'	13	2
OPB08	5'-d[GTCCACACGG]-3'	12	1
OPC16	5'-d[CACACTCCAG]-3'	13	3
AH17	5'-d[CAGTGGGGAG]-3'	13	1
AI08	5'-d[AAGCCCCCCA]-3'	15	3
AI12	5'-d[GACGCGAACC]-3'	15	3
AI105	5'-d[GTCGTAGCGG]-3'	3	1
TOT		84	14
mean		12	2

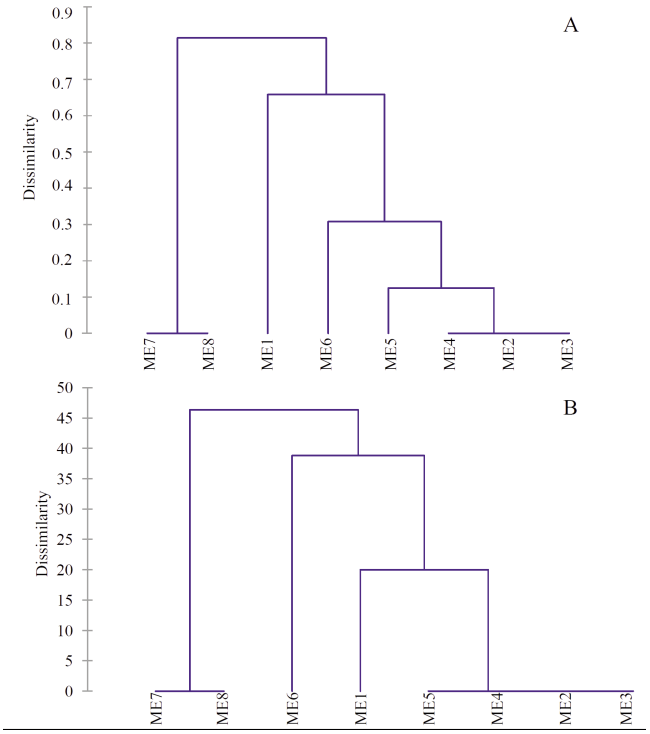

Fig. 3 - UPGMA clusters of 8 pomegranate accessions generated by RAPD markers using the Jaccard similarity coefficient (A) and by SSR markers using Manhattan distance (B).

The SSR molecular technique was utilized as a second molecular method to discriminate the pomegranates and to characterize their genetic profile. Although for *P. granatum* there is not yet a set of the best SSR primers with validity recognized at the international level, these markers have been successfully employed by several researchers to characterize the pomegranate germplasm (Beghè *et al.,* 2016). SSRs utilized in this study have been chosen between the primers which had shown a high discriminating capacity and yielded a total number of 31 reproducible fragments, which allowed a good discrimina-

tion of the local accessions (Table 5). Among 12 selected microsatellites, 10 showed polymorphism and 8 showed differences in the pomegranates genetic profiles. The alleles obtained by amplification of SSRs loci produced four different genetic profiles from eight ancient accessions analyzed (Table S6 of supplementary data). The number of alleles at each locus (N_a) varied between 1, for loci PGKUR114 and PGKUR127, and 5, for loci ssrOeUA-PG6 and Pom021, with an average value of 2.58 and their size ranged from 155 to 319 bp. The values of expected (H_e) and observed (H_o) heterozygosity were always above 0.500, except for the locus PGKVR027, where was not observed heterozigosity, and obviously for the two monomorphic loci (PGKVR114 and PGKVR127). It is important to underline that four primers (PG6, Pom021, Pom045, PgAER154) were highly polymorphic, showing a PIC> 0.5, as defined by Botstein *et al*. (1980) (Table 5). These last markers showed genetic parameters (H_o, H_e, PIC) higher (or similar) to those reported in previous studies where they have been developed (Ebrahimi *et al.,* 2010; Hasnaoui *et al.,* 2012; Caliskan *et al.*, 2017). Instead, the PGKVR-primers presented genetic parameters lower to these reported in literature (Ravishankar *et al.,* 2015); these primers had low polymorphism; of 6 primers 2 resulted monomorphic markers and only 3 (PGKVR027, PGKVR064 and PGKVR165) showed differences in the studied accessions. Similarly, the N_a, H_e, H_o and PIC values in other studies of pomegranate varieties also varied according to the primers tested and the geographic origin of population analyzed (Caliskan *et al.,* 2017). These results confirm the necessity to test different series of SSRs to obtain the best set of markers for each local germplasm.

Table 5 - Number of alleles (Na), Size (bp) expected (He) and observed (Ho) heterozigosity, polymorphic information content (PIC) at 12 loci in pomegranate accessions

SSRs	N_a	Size	H_e	H_o	PIC
PGKVR027	2	236-242	0.429	0	0.305
PGKVR064	2	239-241	0.536	0.750	0.359
PGKVR065	2	202-204	0.571	0	0.375
PGKVR114	1	258	-	-	-
PGKVR127	1	246	-	-	-
PGKVR165	2	307-319	0.571	1.000	0.375
POM-AGC11	2	183-185	0.536	0.250	0.359
PG4	2	198-244	0.571	1.000	0.375
PG6	5	191-199	0.786	0.750	0.653
Pom021	5	203-211	0.857	1.000	0.712
Pom045	3	155-163	0.750	0.750	0.581
PgAER154	4	262-300	0.786	1.000	0.630
TOT	31				
Mean	2.58		0.533	0.542	0.394

The UPGMA cluster based on SSR data divided the set of pomegranate accessions into two main cluster at a dissimilarity level of 45 (Fig. 3B). In the SSRs dendrogram, the first cluster included only two accessions (ME7 and ME8) with genetic identity. The second cluster instead presented all other accessions which a level of dissimilarity that varied between 0 and 38%. This last cluster comprised three genetic subgroups: ME1, ME6 and a subgroup that included accessions with genetic identity (ME2, ME3, ME4, ME5). As confirmed by Mantel's test ($r = 0.399$; $p \leq 0.033$), the SSRs clustering was very similar to that performed by RAPD markers. In fact, it showed the distinction of the same four genetic groups (Fig. 3A and B).

Comparison between morphological and molecular based clusters and potential use of pomegranate genetic resources

It is known that RAPD fragments derived from any region of the genome and that SSR fragments derived only from non-transcribed regions, therefore post-transcriptional modifications and non-nuclear inheritance of some characteristics can't be detected by these markers (Sarkhosh *et al.,* 2006). For these reasons, in literature there are contrasting results about the correlation between these molecular and morphological descriptors (Sarkhosh *et al.,* 2009; Zamani *et al.,* 2010; Basaki *et al.,* 2013). However, researchers agreed that the combination of morphological and molecular techniques are essential for a proper and complete characterization of the germplasm of this species (Beghè *et al.,* 2016).

In this study, the RAPD and SSR analysis reflected the main morphological differences observed among the local accessions studied; the molecular cluster analyses confirmed the same two main clusters detected with PCA analysis using quantitative morphological traits. Moreover, molecular analyses allowed to detect clearly four different genotypes. Analysis of correlation between distance matrices (morphological traits and molecular markers) by Mantel's test confirmed a high statistical significance ($r = 0.412$; $p \leq 0.034$ and $r = 0.583$; $p \leq 0.002$ for SSRs and RAPDs, respectively).

It is important to stress that in populations adapted to difficult ecological conditions, as the germoplasm in study, the polyphenolic content was high (Calani *et al.,* 2013). In this previous study the ME1, ME3, ME5 and ME8 accessions were subjected to phytochemical discrimination fingerprinting in pome-

granate juices. The Emilian pomegranates have presented interesting and peculiar phytochemical profiles. Moreover, the juices were rich in ellagitannins and had high total phenol content and total antioxidant capacity, especially ME8 pomegranate. For these reasons, the local germplasm studied could be considered a source of useful traits (*e.g.* resistance to diseases, frost tolerance, polyphenol synthesis) for genetic improvement of this species. According to pomological descriptors and phytochemical characteristics, we could appreciate peculiar features of these plants. Indeed, the ME7 and ME8 accessions showed some characteristics (*e.g.* small size of fruits, high woody portion in seeds, low pH) that make the fruits unlikely to be used for direct consumption, but have a juice with high antioxidant capacity, and could be successfully employed for the preparation of nutraceutical products or for industrial blending of juices. The other pomegranates (except ME6 accession that presented a high woody portion in seeds) presented good pomological characteristics for which a fresh use of the fruit could also be expected.

4. Conclusions

The present work is a first contribution to the genetic and morphological characterization of the pomegranate germplasm still present in Emilia Romagna region. The morphological traits, in particular those related to fruit and seed, seven RAPDs and eight SSRs have allowed to characterise the genetic diversity of ancient pomegranate accessions. The study, although preliminary and limited to a restricted area, highlighted significant differences and interesting pomological traits in local pomegranates. These results, presented in association with the study of Calani *et al.* (2013), clearly demonstrate a good potential of this germplasm for a commercial exploitation as fresh or processed fruits. The accessions could be used for new pomegranate plantings and could contribute to cross-breeding and the production of new genotypes suited to marginal environments.

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