Genetic and chemical characterization of pomegranate (*Punica granatum* L.) cultivars grown in southern Italy

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Abstract

The increasing interest in pomegranate as a functional fruit has recently favored a myriad of research projects aimed at studying cultivated germplasm by the world. In Apuglia, Southern Italy, the favorable climate conditions has allowed the diffusion of many unknown genotypes spread in different orchards and field collections. In this study, 19 SSR markers have been developed and characterized in *Punica granatum*. Eight selected loci were used to identify ten pomegranate accessions collected in different orchards in Apuglia region. Chemical-nutritional analyses (antioxidant activity, total phenolic, titrable acidity, pH and Brix) were also performed on a selected number of cultivars chosen on the basis of the results of DNA analysis. Results showed good levels of polymorphism and indicated a high antioxidant activity and total phenol content especially in the fruit of two accessions: Chionna and Cisternino.

Keywords: SSR, quality, fruit, local germplasm, *Punicaceae*

INTRODUCTION

The market trend, is facing a growing demand of high quality food intended as the whole organoleptic, nutritional and safe benefit food properties. These requirements have increased in the recent years, inducing fruit growers to consider new alternative crops deriving from typical species historically present in the country for many centuries. Fortunately, most of these interests have been focused on pomegranate due to its pleasant taste and providing diets rich in bioactive compounds highly recommended for human health (Lansky and Newman, 2007).

Southern Italy, in particular is the major area of pomegranate cultivation and where *Punica granatum* germplasm is collected. Besides the importance of the preservation of the genetic diversity, there are few information on the pomegranate biodiversity characterization and on the genetic structure of the populations. In this contest, there is a need for highly standardized methods of identification to evaluate the genetic resources and to tackle problems regarding confusion in plant material conservation due to the occurrence of cases of synonymy, homonymy and misnaming. Different methods are available to deal with germplasm identification up to now. Traditional genotype identification is based on morphological traits that cannot be used unambiguously as markers due to developmental and environmental influences. For these reasons, a wide number of genetic markers were developed to assess the genetic variability such as RFLP, RAPD, AFLP and SSR, but studies have demonstrated that the use of simple sequence repeat (SSR) markers was the method of choice for DNA typing. Thus, this research proposed to study and to evaluate 10 preliminary selected accessions using new microsatellite markers isolated by Akkak et al. (2012). Furthermore, chemical-nutritional parameters were also investigated.

MATERIALS AND METHODS

Plant material

This study was carried out on 10 accessions collected in different small orchards located in Apuglia region and they were: 'Masseria Rosa', 'Acido', 'Zio Sante', 'Pagliarulo', ‘Chionna’, ‘Melograno Acido’, ‘Melograno Dolce’, ‘Cisternino’, ‘Melograno Dente di Cavallo’ and ‘Dente di Cavallo 2’. Leaf for DNA extraction and fruit samples were collected at the end of September.

Genetic analysis

For microsatellite analysis, DNA was extracted from young leaves (0.2 g) following the procedure described by Thomas et al. (1993).

Samples were analyzed at 10 SSR loci isolated in *Punica granatum* (‘Dente di Cavallo’): UFPg1, UFPg5, UFPg6, UFPg14, UFPg16, UFPg18, UFPg20, UFPg24 (Akkak et al., 2012).

PCR amplification was carried out using a reaction mixture (20 µL) consisting of 2 µL 10x buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer, 0.5 U of Taq-DNA polymerase (AmpliTaq Gold polymerase, Applied Biosystems) and 50 ng of template DNA. Amplification cycles consisted of an initial step of 9 min at 95°C, followed by 28 cycles of 50 s at 94°C, 30 s at 56°C, 90 s at 72°C, with a final extension step of 7 min at 72°C.

Samples were then analyzed on a 3130 capillary sequencer (Applied Biosystems, Foster City, Calif., USA). Data were then processed by GeneScan Software 4.2 (Applied Biosystems) and alleles defined by their size (in bp) using a standard molecular size (GS500 LIZ, Applied Biosystems). DNA data obtained at six polymorphic loci were analyzed using identity 4.0 software; genetic distances between genotypes (computed as: 1-proportion shared alleles) were calculated using Microsat software. A phylogenetic tree was obtained by applying the UPGMA (unweighted pair group method with arithmetic mean) using Mega5 software (Tamura et al., 2011).

Chemical analysis

To determine the total phenols content, 1 g of juice was mixed for 30 min in distilled water. Total phenols were determined on the extract according to the method of Singleton and Rossi (1965). Each extract (100 µL) was mixed with 1.58 mL water, 100 µL of Folin-Ciocalteu reagent and 300 µL of sodium carbonate solution (200 g L^{-1}). After the solution stood for 2 h, the absorbance was read at 725 nm against a blank using a UV - 1700 Shimadzu spectrophotometer (Jiangsu, China). The content of total phenols was calculated on the basis of the calibration curve of gallic acid, and was expressed as mg of gallic acid per 100 g of fresh weight (mg GA 100 g^{-1}). Antioxidant assay was performed following the procedures described by Brand-Williams et al. (1995) with minor modifications. The diluted sample, 50 µL, was pipetted into 0.95 mL of DPPH solution to initiate the reaction. The absorbance was read at 515 nm after overnight incubation. Trolox was used as a standard and the antioxidant activity was reported in mg of Trolox equivalents per 100 g of fresh weight (mg TE 100 g^{-1}). For titratable acidity and pH, few drops of fruit juice were used to measure the total soluble solids content (TSS) with a digital hand refractometer (Atago, Japan). One g of pomegranate juice was then used to measure the pH value and the titratable acidity (TA), with an automatic titrator (T50 M Terminal, Mettler Toledo, Switzerland). TA was obtained measuring the volume of NaOH 0.1 N used to reach a final pH of 8.2, and results were expressed as per cent of citric acid (applying the acid milliequivalent factor 0.064) referred to the juice.

RESULTS AND DISCUSSION

All DNA samples were amplified at the 8 loci and analyzed on 3130 sequencer. All loci revealed clear SSR patterns and polymorphism within the expected allelic range. They showed at least 1 or 2 alleles per locus. We evaluated 8 loci and 5 of them were interesting (UFPg1, UFPg6, UFPg14, UFPg18 and UFPg20) considering their good polymorphism. The locus UFPg16 showed only two alleles and two genotypes. The remaining loci UFPg5 and UFPg16 were not able to detect any variation in this group of samples. The combination of all analyzed loci has determined 7 different genotypes and 2 cases of synonymy and 1 case of mislabelling.

Cluster analysis (Figure 1) produced a dendogram that grouped cultivars into two main clusters. The first comprised six cultivars, a case of synonymy between 'Zio Sante' and 'Melograno Dolce' and identified also a case of a misnaming cultivars between 'Melograno Dente di Cavallo' and 'Dente di cavallo2'. The second cluster identified a group of three presumed synonymous cultivars: 'Acido', 'Chionna' and 'Melograno Acido'. In the end, Pagliarulo was set alone in one branch of the cluster showing to have a unique SSR profile.

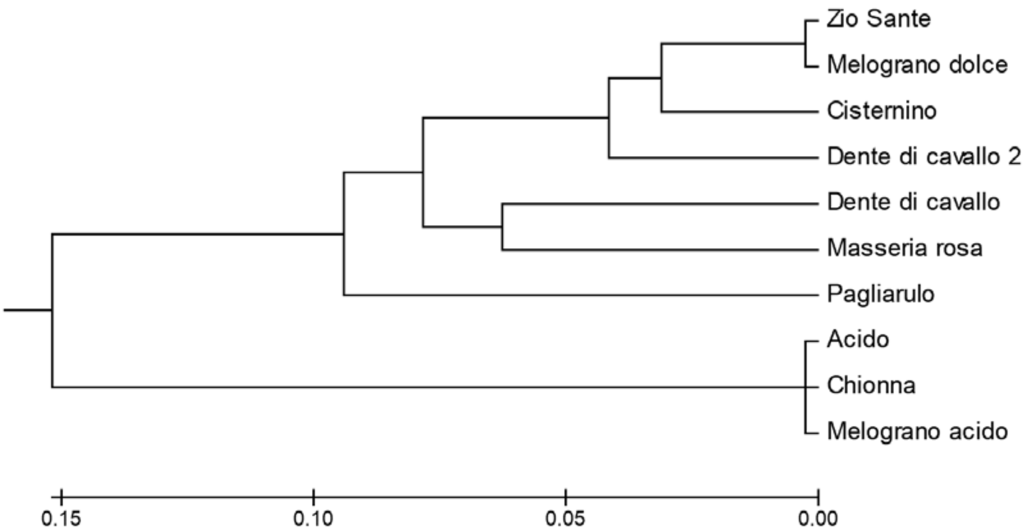

Figure 1. Dendogram obtained by cluster analysis of 10 accessions, studied at 8 SSR loci.

Chemical analysis was performed on 7 genotypes confirmed by genetic analysis (Table 1). The pH values ranged from 3.14 ('Chionna') to 4.29 ('Zio Sante'). On the other hand, 'Zio Sante' resulted the accession which had the higher TSS (>17.2 °Brix). Furthermore, titratable acidity varied from 0.82% ('Zio Sante') to 2.03% ('Chionna'). These values are similar to those reported by Fadavi et al. (2005).

Table 1. Preliminary results of chemical-nutritional analysis of pomegranate fruits collected in Apuglia.

Cultivars	TSS (°Brix)	pH	Total polyphenol (mg gallic acid 100 g^{-1})	Antioxydant activity (mg TE 100 g^{-1})	Titrable acidity (citric acid %)
Masseria Rosa	15.6	3.86	216.08±0.70	422.07±4.27	1.27±0.01
Zio Sante	17.2	4.29	169.60±0.68	369.71±1.02	0.82±0.02
Pagliarulo	15.0	3.94	162.90±3.60	400.57±1.85	0.97±0.01
Chionna	16.4	3.14	277.20±1.04	319.53±7.71	2.03±0.01
Dente di Cavallo	15.6	4.04	227.58±2.41	188.93±0.07	0.87±0.01
Cisternino	15.6	3.83	276.21±1.74	242.19±1.87	0.91±0.01
Dente di Cavallo2	12.6	3.92	233.53±7.03	171.21±0.63	1.31±0.01

The total content of polyphenol and antioxidant activity varied significantly among different ecotypes. 'Chionna', 'Cisternino' and 'Masseria Rosa' are the most interesting accessions due to high polyphenol content and antioxidant activity.

CONCLUSIONS

The use of these new microsatellite markers to characterize the first set of 10 accessions of pomegranate has demonstrated as expected to be a powerful tool for cultivar identification. At least 5 SSR loci resulted particularly suitable for DNA typing. The criteria followed to select the primers will allow to carry out multiplex analysis and, therefore, to reduce time and cost of the technique. Interestingly, preliminary chemical-nutritional analyses showed a promising quality data of some pomegranate accessions especially for fresh consumption. Further analysis must be done to confirm the results obtained by the current study on the local germplasm which is still in progress.

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