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Analytical Methods

Authentication of *Punica granatum* L.: Development of SCAR markers for the detection of 10 fruits potentially used in economically motivated adulteration

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ABSTRACT

The large commercial success of pomegranate increase the likelihood of economically motivated adulteration (EMA), which has been gradually spotted with the undeclared addition of anthocyanin-rich plants or cheaper fruit juices used as bulking and diluting agents. A method based on Sequence-Characterized Amplified Regions (SCARs) was developed to detect the presence of *Aristotelia chilensis*, *Aronia melanocarpa*, *Dioscorea alata*, *Euterpe oleracea*, *Malus* × *domestica*, *Morus nigra*, *Sambucus nigra*, *Vaccinium macrocarpon*, *Vaccinium myrtillus*, *Vitis vinifera* as bulking agents in *Punica granatum*. The method enabled the unequivocal detection of up to 1% of each adulterant, allowing the preemptive rejection of suspect samples. The recourse to such method may reduce the number of samples to be subjected to further phytochemical analyses when multiple batches have to be evaluated in a short time. *Vice versa*, it allows the cross-check of suspect batches previously tested only for their anthocyanin profile. The dimension of the amplicons is suitable for the analysis of degraded DNA obtained from stored and processed commercial material. Proper SCAR markers may represent a fast, sensitive, reliable and low-cost screening method for the authentication of processed commercial pomegranate material.

1. Introduction

Anthocyanin- and polyphenol-rich fruits are enjoying a rampant success, driven by a growing evidence of their beneficial properties (de Pascual-Teresa & Sanchez-Ballesta, 2008; He & Giusti, 2010; Zanotti et al., 2015). Among the species involved, *Punica granatum* L. is enjoying most interest, leaping from an outfashioned fruit with limited commercial appeal to a commodity traded on a worldwide scale, whose volumes grew with an impressive pace during the last decade (Rymon, 2011). Alongside of such high market value, economically motivated adulteration (EMA) has been gradually spotted in commercial pomegranate samples, with the undeclared addition of both anthocyanin-rich plants or cheaper plant material used as bulking and diluting agents in juices and herbal preparations. In particular, species more often related to pomegranate EMA are *Aristotelia chilensis* Molina (maqui berries), *Aronia melanocarpa* Elliot (black chokeberry); *Dioscorea alata* L. (purple yam) *Euterpe oleracea* Mart. (açaì), *Malus* × *domestica*, Borkh. (apple), *Morus nigra* L. (black mulberry), *Sambucus nigra* L. (elderberry), *Vaccinium macrocarpon* Aiton. (cranberry), *Vaccinium myrtillus* L. (bilberry) *Vitis vinifera* L. Stuntz., which are deliberately added to commercial batches of different nature (Boggia, Casolino, Hysenaj, Oliveri, & Zunin, 2013; Borges & Crozier, 2012; Defernez, Kemsley, & Wilson, 1995; Fischer-Zorn & Ara, 2007; Nuncio-Jáuregui, Calín-Sánchez, Hernández, & Carbonell-Barrachina, 2014; Vardin, Tay, Ozen, & Mauer, 2008; Zhang et al., 2009; Zhang, Wang, Lee, Henning, & Heber, 2009). To face adulteration concerns, various analytical methods are available to profile anthocyanins in pomegranate-containing products, for quality control and authentication purposes (Sentandreu, Navarro & Sendra, 2010; Bridle & García-Viguera, 1996; Calani et al., 2013; Obón, Díaz-García, & Castellar, 2011; Zhao, Yuan, Fang, Yin, & Feng, 2013). Although these methods offer excellent capabilities in characterizing the chemical composition of fruit products, as long as species identification is concerned they may suffer in some occasions from complications emerging from intraspecific differences, seasonal and climate, geographical and growing variability, different harvest time, processing or storage conditions and length (Faria, Magalhães, Nunes, & Oliveira, 2013; Han et al., 2012). Non chromatographic methods relying on UV detection suffer from

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poor reproducibility and from sensitivity issues related to interferences between anthocyanins and polyphenols (Borochov-Neori et al., 2011; Gil, García-Viguera, Artés, & Tomás-Barberán, 1995). Furthermore, the necessity to screen large numbers of samples increases the need for quick methods. Solely in the USA, approximately 42 million food and beverage imports are screened each year, but only 1% of them are laboratory tested for authenticity, due to laborious and time consuming nature of the requested assays (Buzby, Laurian, & Roberts, 2008). Such constraints are considered behind the existence of legal disputes and conflicts in product labeling in terms of authentication and purity of pomegranate juices. (Roberts, 2010).

For the purpose of fruit authentication, researchers have recently turned their attention also to genomic methods and some countries in the European Union are actively supporting the adoption of DNA-based evaluations for routine authentication (Primrose, Woolfe, & Rollinson, 2010). The major hindrances of such approach are the availability of unique, reliable, reproducible and discriminant markers and the diversity of potential interferents encountered in complex foods as in blends of different fruits (Cordella, Moussa, Martel, Sbirrazzuoli, & Lizzani-Cuvelier, 2002). On this regard, the recourse to SCAR markers and to proper DNA extracting protocols may allow to overcome some of these limitations owing to high sample throughput, short sample preparation, unique identification, good interlaboratory replicability and low operating costs (Dhanya & Sasikumar, 2010; Kiran, Khan, Mirza, Ram, & Abdin, 2010; Marieschi, Torelli, Poli, Sacchetti & Bruni, 2009; Marieschi, Torelli, Bianchi, & Bruni, 2010, 2011; Marieschi, Torelli, & Bruni, 2012). Regarding the authentication of anthocyanin-rich fruits, some DNA-based techniques have been already optimized, including berries and other polyphenol rich fruits, but no proper DNA-based screening is at present available for pomegranate (Han et al., 2012; Jaakola, Suokas, & Häggman, 2010; Palmieri, Bozza, & Giongo, 2009).

The objective of the present study was to develop robust SCAR markers for *P. granatum* and for ten bulking agents previously spotted in processed pomegranate products, namely *A. chilensis, A. melanocarpa*, *D. alata*, *E. oleracea*, *M.* × *domestica*, *M. nigra*, *S. nigra*, *V. macrocarpon*, *V. myrtillus, V. vinifera*. The final goal is to obtain a diagnostic tool capable to confirm the rejection of suspect samples or to reduce the number of samples to be evaluated by means of phytochemical analyses, providing useful data for further molecular diagnostic tools. The method was optimized with specific respect to a reliable application on plant material of commercial grade, in order to complement existing methods.

2. Materials and methods

2.1. Plant material

P. granatum L. (leaves and fruit pulp), *M.* × *domestica* Borkh. (leaves), *M. nigra* L. (leaves), *S. nigra* L. (leaves) and *V. vinifera* L. Stuntz. (fruit and leaves) were collected from local orchards or purchased from the market. Plants of *A. chilensis* Molina (fruits and leaves), *A. melanocarpa* Elliot (leaves), *D. alata* L. (rhizomes), *E. oleracea* Mart. (leaves), *V. macrocarpon* Aiton. (leaves), and *V. myrtillus* L. Stuntz. (leaves) were kindly supplied by the Botanical Gardens of Parma. Fresh plant material was collected and immediately freeze-dried in liquid nitrogen and stored at −80 °C until molecular analysis. Four commercial samples of pomegranate-containing products (Table 1) were acquired from local groceries in Parma (Italy) and conserved sealed at room temperature until molecular analysis.

Table 1
Commercial products.

Sample	Origin	Description	Declared ingredient list
a	Italy	Herbal tea	Pomegranate fruit (20%), Rose hip, strawberry leaves, orange fruit, hibiscus flowers and natural flavors
b	UK	Herbal tea	Rooibos, honeybush (19%), hibiscus, pomegranate (2%), raspberry pieces (1%) and acai berry pieces (1%) with natural flavors
c	Italy	Jam	Pomegranate, agave syrup, lemon juice, pectin
d	Italy	Juice mix	Strawberry puree, pomegranate juice concentrate (3.5%), elderberry puree (3%)

2.2. Extraction of PCR-compatible genomic DNA

Genomic DNA was isolated from all the above mentioned samples as previously described to increase the yield and the purity of the DNA extracted and reduce the inhibitory effects of carbohydrates and polyphenols in the subsequent PCR reactions (Marieschi et al., 2011, 2012). DNA concentration and purity ($A_{260/280}$ and $A_{260/230}$) were evaluated by spectrophotometric analysis. The suitability of DNA for RAPD analysis was also checked by ethidium bromide-stained agarose/TAE gels which allowed both to evaluate DNA integrity and further confirm DNA quantitation by visual comparison with DNA standards. Agarose gels were analyzed and quantitated with a Kodak DC40 camera (Kodak) using the Kodak digital science 1D Image analysis software (Eastman Kodak Company, Rochester, NY, USA). DNA samples were adjusted to approximately 20 ng/μL prior to using them in PCR reactions. Genomic DNA from commercial samples was isolated by means of a Nucleospin® PlantII Kit (Macherey–Nagel, Duren, Germany) according to the producer's guidelines.

2.3. RAPD analysis and marker selection

Eight potential contaminants of pomegranate juice were compared with 2 samples of *P. granatum* to find RAPD amplicons suitable to develop SCAR markers (Marieschi et al., 2009). PCR was conducted on approximately 20–40 ng of DNA template and was performed in 25 μL volume containing 67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 0.2 mM dNTPs in equimolar ratio, 1U SubTherm Taq DNA Polymerase (Fisher Molecular Biology, Trevose, PA, USA), 25 pmol of each primer. To avoid inhibition due to co-precipitation of secondary metabolites with DNA, the PCR amplification was improved through the addition of BSA 0.4% and a non ionic detergent Tween 20 0.5% (Marieschi et al., 2010). Reaction was performed as follows: 94 °C for 5 min, 40 cycles of 94 °C for 40 s, 36 °C for 40 s, 72 °C for 2 min, followed by one cycle of 72 °C for 10 min (PTC-100, MJ Research Inc.). A total of 12 random primers (Operon Technologies: OPA01, OPA03, OPA04, OPA05, OPA07 OPA09, OPA10, OPA11, OPA20, OPB19, OPB20, OPP10) were utilized for RAPD analysis. RAPD patterns were compared to select amplicons present in the contaminants and absent in *P. granatum* RAPD profiles. Most of the suitable marker bands were obtained with the primers OPA01, OPA03, OPA04, OPA05, OPA09 and are listed in Table 2.

2.3.1. Cloning and sequencing of putative RAPD markers

The selected marker bands were excised from 2% agarose gels, purified using JET-Sorb Gel Extraction Kit (Genomed, Löhne, Germany) and cloned in pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA). The transformed bacterial colonies were screened through colony PCR and clones carrying correctly sized inserts were purified and sequenced with M13 forward and M13 reverse primers (BMR Genomics, Padova, Italy).