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: 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 evaluation with 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.

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Parma, 27 May, 2015

Dear Editor,

here enclosed please find our manuscript entitled "**Authentication of *Punica granatum* L.: development of SCAR markers for the detection of 10 fruits potentially used in economically motivated adulteration**" that we are submitting for publication on *Food Chemistry*, on behalf of all authors.

In our paper we describe a method based on the development of specific and robust SCAR markers, with the aim to provide a fast and reliable DNA-based test to probe the authenticity of pomegranate in juices and other processed products, or to detect the presence of specific contaminants. In particular, ten potential and actual adulterants of *P. granatum* fruit juice (*Aristotelia chilensis, Aronia melanocarpa, Dioscorea alata, Euterpe oleracea, Malus* × *domestica, Morus nigra, Sambucus nigra, Vaccinium macrocarpon, Vaccinium myrtillus* and *Vitis vinifera*, many of them recently spotted in a number of commercial samples) may be detected if added yet at 1%. The information provided in our manuscript may be useful to develop further similar methods aimed at the detection of further cross-adulteration between these fruits. The protocol based on molecular biology is proposed as a complementary or alternative approach to conventional analyses in quality control and fraud detection in commercial pomegranate products.

Hoping that our manuscript will be eligible for publication, we convey our best regards to you and remain.

Sincerely,

Renato Bruni

***Highlights (for review)**

- A SCAR-based method was set up to detect ten bulking agents in pomegranate products.
- Specific markers allowed the rapid identification of all adulterants
- Each adulterant can be detected in amounts as low as 1%.

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

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 evaluation with 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.

Keywords: pomegranate, quality control, molecular markers, fraud detection, adulteration

1. Introduction

Anthocyanin-rich and polyphenol-rich fruits are enjoying a rampant commercial success, driven by a growing evidence of their beneficial properties (de Pascual-Teresa & Sanchez-Ballesta, 2008; He, & Giusti, 2010; Zanotti, Dall'Asta, Mena, Mele, Bruni, Ray & Del Rio, 2015). At the same time, the market is also keen to the introduction of new, exotic or scarcely known fruits to lure the attention of the consumers, resulting in the modern global success of fruits rich in anthocyanins, yet previously uncommon or available only in local markets (Devalaraja, Jain & Yadav, 2011; Dembitsky et al., 2011). Among these species, *Punica granatum* L. is enjoying most interest, leaping from an out-fashioned 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). Juices, concentrates and pulps obtained from pomegranate fruits are experiencing a soaring success in the marketplace, spurred by several features including a favourable combination of novelty, cheap availability, colour, unique taste and health properties (Mena, Gironés-Vilaplana, Moreno, & García-Viguera, 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 fruit juices used as bulking and diluting agents. In particular, species more often related to pomegranate EMA in juices, concentrates, frozen pulps and purees 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 increase volume and weight of commercial batches (Nuncio-Jáuregui, Calín-Sánchez, Hernández & Carbonell-Barrachina, 2014; Zhang, Wang, Lee, Henning, Heber, 2009; Fischer-Zorn & Ara, 2007; Boggia, Casolino, Hysenaj, Oliveri & Zunin, 2013; Borges & Crozier, 2012; Defernez, Kemsley & Wilson, 1995; Vardin, Tay, Ozen, & Mauer, 2008; Zhang, et al., 2009). To face adulteration concerns, various analytical methods based

on chromatographic and spectrometric techniques are available to profile the anthocyanin content of pomegranate-containing products, for quality control and authentication purposes (Sentandreu, Navarro & Sendra, 2010; Bridle & García-Viguera, 1996; Obón, Díaz-García & Castellar, 2011; Calani et al., 2013; 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 (Han et al., 2012; Faria, Magalhães, Nunes & Oliveira, 2013). These factors, singularly or as a whole, may sometimes complicate the unequivocal detection of specific adulterants in pomegranate products like juices or concentrates, due to the similar anthocyanin profile of other fruits and to the 10-20 fold differences found for some secondary metabolites in distinct *P. granatum* cultivars. Moreover, non chromatographic methods relying on UV detection suffer from poor reproducibility and from sensitivity issues related to interferences between anthocyanins and polyphenols (Gil, García-Viguera, Artés & Tomás-Barberán, 1995; Borochov-Neori et al., 2011). Furthermore, the necessity to screen large numbers of samples increases the need for quick methods. Solely in the USA, U.S. Department of Agriculture (USDA) and Food and Drug Administration (FDA) screen approximately 42 million food and beverage imports per 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, with particular commercial relevance, 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 DNA-based methods, designed to determine the biological identity of food material at genetic level, producing fast, accurate and sensitive methods. Some countries in the European Union are actively

supporting such approach by encouraging the adoption of DNA-based evaluations on lab-on-a-chip instruments (Primrose, Woolfe & Rollinson, 2010). Sensitive and conventional PCR techniques have been successfully applied to quality control of an increasing range of juices or soft pulp products, for instance allowing the quantification of 2.5% mandarin juice in orange juice (Palmieri, Bozza & Giongo, 2009). For routine authentication the recourse to DNA fingerprinting offers various advantages and is supported also by the gradually cheaper instrumental availability and by a steady developments in the field (Turci et al., 2010). The major hindrances are the availability of unique, reliable, reproducible and discriminant markers for the plant species involved in adulteration and the diversity of potential interferents encountered in complex foods as in blends of different fruits (Cordella et al., 2002,). For instance, plant polyphenols, sugars and the partial degradation of DNA during storage are obstacles that cannot be overlooked easily. 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 of contaminants, good interlaboratory replicability and low operating costs, as recently demonstrated also by our work (Dhanya & Sasikumar, 2010; Marieschi, Torelli, Poli, Sacchetti & Bruni, 2009; Marieschi, Torelli, Bianchi & Bruni, 2010; Marieschi, Torelli, Bianchi & Bruni, 2011; Marieschi, Torelli & Bruni, 2012; Kiran, Khan, Mirza, Ram, & Abdin, 2010). Regarding the authentication of anthocyanin-rich fruits, some DNA-based techniques have been already optimized, including berries and other polyphenol fruits rich in polyphenols, but no proper DNA-based screening allowing the fast detection of potential adulteration is at present available for pomegranate (Jaakola, Suokas & Häggman, 2010; Palmieri, Bozza & Giongo, 2009; Han et al., 2012).

Therefore, 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. Fruits and leaves from *Punica granatum* L., *Malus* × *domestica* Borkh., *Morus nigra* L., *Sambucus nigra* L. and *Vitis vinifera* L. Stuntz. were collected in local orchards or purchased from the market. Plants of *Aristotelia chilensis* Molina, *Aronia melanocarpa* Elliot, *Dioscorea alata* L., *Euterpe oleracea* Mart., *Vaccinium macrocarpon* Aiton., and *Vaccinium myrtillus* L. Stuntz. 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.

2.2 Extraction of PCR-compatible genomic DNA. Genomic DNA was isolated from plant material 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, Torelli, Bianchi, Bruni, 2011; Marieschi, Torelli & Bruni, 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 of 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.

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, Torelli, Poli, Sacchetti & Bruni, 2009). PCRs were conducted on approximately 20-40 ng of DNA template and were 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, Torelli, Bianchi, Bruni, 2010). Reaction was performed as follows: 94 °C for 5 min, 40 cycles of 94 °C for 40 sec, 36 °C for 40 sec, 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 1**.

2.4 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).

2.5 Sequence analysis. Database searches of sequence similarity were performed using the programs BlastN, BlastX, and PSI-BLAST set to standard parameters (Altschul et al., 1997). The sequences were deposited into GenBank (Benson et al., 2012) (**Table 1**).

2.6 SCAR primer design and optimization of PCR conditions. The sequence data after similarity searches were used for SCAR primers design of contaminant species, with the sole exception of *M.* × *domestica* and *S. nigra*. Given the large amount of reliable information available, for these two species, specific marker were instead constructed on intronic regions of sequences selected from GenBank database (**Table 1**). For all selected sequences, primers 20-22 nucleotides long were designed for stringent conditions of annealing temperature (about 62 °C) and did not overlap the sequence of the original RAPD primer (**Table 1**). The annealing temperature was first calculated as 4-fold the number of GC (guanidine/cytosine) plus 2-fold the number of AT (adenine/thymine). Each primer pair was tested up to 2 °C over and under its annealing temperature to obtain the optimum. PCR reactions were conducted on approximately 40 ng of DNA template, were performed in 25 μl volume containing 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.8 mM $MgCl_2$, 0.2 mM dNTPs in equimolar ratio, 1U SubTherm Taq DNA Polymerase (Fisher Molecular Biology, Trevose, PA USA), 12.5 pmol specific primers and were enhanced through the addition of BSA 0.4% and Tween 20 0.5%. Unless not otherwise specified, amplification was performed as follows: 94 °C for 5 min, 37 cycles of 94 °C for 40 sec, 62 °C for 40 sec, 72 °C for 40 sec, followed by one cycle of 72 °C for 5 min (PTC-100, MJ Research Inc.). An aliquot (10 μl) of the amplification product was resolved by electrophoresis on 1.5% agarose gel and detected by ethidium bromide staining.

2.7 Validation of the SCAR markers. DNA samples of contaminant species were analyzed with the specific primer pairs defined for each species and with *P. granatum* samples to exclude possible cross reactions leading to misinterpretation of the results. Once the primer specificity was

confirmed, SCAR analysis was conducted on genomic DNA extracted from mixtures of *P. granatum* containing 1, 2 and 5% of each contaminant species. The integrity and availability of DNA from samples for SCAR analysis was checked by performing an amplification with a couple of primers, constructed on a RAPD amplicon (OPA01Pg_{300}) obtainable from the amplification with the primer OPA01 on *P. granatum* DNA. The band, 300 bp long, was cloned and sequenced. The sequence was deposited into GenBank (accession number KP763488). The SCAR primers ScPg_{249} forward and reverse, gave rise to a 249bp amplicon, suitable as positive control for PCR reactions (**Table 1**).

3. Results and Discussion

The detection of economically motivated adulteration in soft fruit products and juices is a challenging task, due to the drastic modification to morphological traits and chemical variability occurring during fruit transformation. These factors require a substantial amount of experienced time and/or the recourse to costly technologies to achieve a timely and reliable authentication. This happens in particular when the plant material is squeezed or pressed to obtain a juice or a puree, or processed to obtain a concentrate thus increasing the possibility to conceal the addition of bulking agents. As our goal was the development of a reliable method based on molecular genetic markers, we proceeded by the identification of RAPD amplicons markers discriminating previously reported bulking or diluting agents from *P. granatum* and then strengthening the reproducibility by their transformation into SCAR markers. These markers were finally evaluated in artificial mixes of plant material at different concentrations in order to evaluate the sensitivity and the accuracy of the method.

3.1 Development of SCAR markers. We have previously analyzed 120 RAPD profiles obtained by amplification with 12 primers on DNA extracted from 2 samples of *P. granatum* and 8 of the 10 plant species indicated in literature among the most frequent adulterants found in commercial pomegranate juices, namely *A. chilensis, A. melanocarpa, D. alata, E. oleracea, M. nigra, V.*

macrocarpon, V. myrtillus, V. vinifera. By following a consolidated approach, the polymorphic bands to be cloned were selected on the basis of their reproducibility, their intensity and the absence of other bands in the proximity to avoid contamination (Marieschi, Torelli, Poli, Sacchetti & Bruni, 2009; Marieschi, Torelli, Bianchi, Bruni, 2010; Marieschi, Torelli, Bianchi, Bruni, 2011; Marieschi, Torelli & Bruni, 2012). Eight RAPD fragments, each specific for a contaminant species and absent in the RAPD profiles of the *P. granatum* samples (OPA05*Ac*$_{648}$, OPA04*Am*$_{460}$, OPA03*Da*$_{416}$, OPA09*Eo*$_{372}$, OPA03*Mn*$_{598}$, OPA09*Vma*$_{546}$, OPA05*Vmy*$_{408}$, OPA05*Vv*$_{293}$, specific for *A. chilensis, A. melanocarpa, D. alata, E. oleracea, M. nigra, V. macrocarpon, V. myrtillus* and *V. vinifera*, respectively) were selected. RAPD amplicons were cloned and sequenced and their sequences were deposited into GenBank (**Table 1**). A further RAPD amplicon (OPA01*Pg*$_{300}$) present in *P. granatum* and absent in all contaminants profiles was also selected to be used as positive control in the analysis of commercial samples. The BLASTN of the nucleotide sequences did not show similarity with any sequences in the database. These discriminating RAPD bands were subsequently converted into SCAR markers. The only exception was represented by OPA04*Am*$_{460}$ which showed partial homology with two uncharacterized mRNA sequences of *Malus* × *domestica* (Ident 77%). ClustalW analysis (**Figure 1**) showed the high identity of the OPA04*Am*$_{460}$ nucleotide sequences and the two above mentioned sequences. In this case SCAR primer pair specific for *A. melanocarpa* (Sc*Am*$_{358}$) was thus constructed on the regions of maximal heterogeneity (**Figure 1**, black boxes). For the two species *M.* × *domestica* and *S. nigra* SCAR primer pairs were obtained as described above (see Methods).

In order to increase the amplification specificity, the SCAR primers did not contain the sequences of the original RAPD primers thus giving rise to amplification products shorter than the respective original selected sequences. The relative short dimension of the amplification targets offers a further advantage, these SCAR markers being more suitable for the analysis also when DNA is partially degraded, as it may be the case with products subject to long storage conditions during any processing step. This event is likely to occur in commercial concentrates and purees and is

considered a critical point in DNA authentication of fruit derived products (Primrose, Woolfe & Rollinson, 2010).

3.2 Validation of selected markers. SCAR marker specificity was validated by testing each primer pair in amplification on DNA extracted from *P. granatum*, the species for which each specific primer pair was designed and the 10 contaminants other than the target species. None of the tested primer pairs generated an amplification product when applied to DNA of *P. granatum* and most of them did not give cross reaction when tested on the DNA of the other contaminants. The only exception was represented by ScMd_{262} designed for *M.* × *domestica* which recognized also *A. melanocarpa* and other species belonging the Maloideae subfamily of Rosaceae and could thus offer the advantage to identify more than a single contaminant (data not shown). The SCAR marker ScPg_{249} selected as positive control was further validated on DNA extracted from 11 *P. granatum* different accessions to test its uniform response at intraspecific level. The ScPg_{249} marker correctly identified *P. granatum* giving an amplicon of the expected size in each sample (**Figure 2**). This SCAR marker represents an useful tool for routine analyses of commercial pomegranate samples, allowing the evaluation of the accuracy of the whole procedure of DNA extraction and downstream assays. It may also act as a positive control by preventing the misinterpretation of false negatives due to bad quality DNA.

3.3 Sensitivity assay. After checking marker specificity, the method sensitivity was validated in order to define its limit of detection for each adulterant. To create conditions similar to those of commercial samples, artificial counterfeit mixtures where prepared by mixing fresh pomegranate tissue with the fresh material obtained from each contaminant, namely *A. chilensis, A. melanocarpa, D. alata, E. oleracea, M.* × *domestica, M. nigra, S. nigra, V. macrocarpon, V. myrtillus, V. vinifera*. **Figure 3** reports the results obtained with DNA extracted from artificial mixtures containing *P. granatum* and 1, 2 or 5% of *A. chilensis* (Panel A), *A. melanocarpa* (Panel B), *D. alata* (Panel C), *E.*

oleracea (Panel D), *M.* × *domestica* (Panel E), *M. nigra* (Panel F), *S. nigra* (Panel G), *V. macrocarpon* (Panel H), *V. myrtillus* (Panel I), *V. vinifera* (Panel J) subjected to amplification with the primer pairs for Sc*Ac*$_{336}$, Sc*Am*$_{358}$, Sc*Da*$_{185}$, Sc*Eo*$_{143}$, Sc*Md*$_{262}$, Sc*Mn*$_{293}$, Sc*Sn*$_{226}$, Sc*Vma*$_{241}$, Sc*Vmy*$_{287}$ and Sc*Vv*$_{144}$ respectively. The selected SCAR markers allowed an easy detection up to 1% of each contaminant, as shown by the amplification of an individual specific band absent in the lanes of *P. granatum* (**Figure 3**) and present both in the reactions with the individual contaminant species (Panel A-J, lanes *A.c.*, *A.m.*, *D.a.*, *E.o.*, *M.d.*, *M.n.*, *S.n.*, *V.ma.*, *V.my.*, *V.v.*) and with its mixtures with *P. granatum* (Panel A-J, lanes 1%, 2%, 5%).

4. Conclusions. A method based on SCAR markers was developed from RAPD specific for ten common bulking agents used for economically motivated adulteration of pomegranate, in order to authenticate their presence and set up a fast, sensitive, reliable and low-cost screening of *P. granatum* juices and purees. The method enabled the unequivocal detection of low amounts of contaminants up to 1%. The method meets the needs of the present market and may be used to screen large pomegranate batches before proceeding with their grading, preemptively excluding those counterfeited with bulking agents that may otherwise go undetected. At the same time, it may be enforced as a confirming tool for samples previously detected as potentially adulterated after screening of their anthocyanin profile. The availability of genetic sequences could also ease the development of technological devices like gene chip or specific kits to be enforced in routine control, as their response is not affected by phenotypic, environmental and process or storage variability. Data presented may be useful also to enforce more careful monitoring of economically motivated substitutions between different anthocyanin-rich plant materials. With proper adjustments, the SCAR markes described in our work may be used to setup similar methods to reveal further adulterations, as those recently noticed for red wine and elderberry, for bilberry and mulberry, chokeberry or elderberry (Bridle & García-Viguera, 1996; Filippini, Piovan & Caniato, 2011; Giacomelli et al., 2014; Gardana et al., 2014). Besides the obvious trade issues, deceitful

adulteration of pomegranate may also represent a matter of concern when commercial plant materials are evaluated for research purposes, since it may lead to misleading results both from the chemical, biological and nutritional standpoint.

5. References

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Table(s)

Table 1. Species-specific SCAR markers and primer specifications

Species	Orginal RAPD amplicon	GeneBank accession number	SCAR marker		SCAR primer sequence (5′→ 3′)	Calculated annealing temperature (°C)	Working annealing temperature (°C)	Amplicon lenght (bp)
Punica granatum	OPA01*Pg*$_{300}$	KP763488	Sc*Pg*$_{249}$	For	ACATTATGCCAAGGGCTCCC	62	60	249
				Rev	CATAGGAGTTCTTGGGGAGG	62		
Aristotelia chilensis	OPA05*Ac*$_{684}$	KP763489	Sc*Ac*$_{336}$	For	ATGCAGCACCAGAAACCAGC	62	62	336
				Rev	GTGGCATTGCTAACTCCAGG	62		
Aronia melanocarpa	OPA04*Am*$_{460}$	KP763487	Sc*Am*$_{358}$	For	CCTTGATAATTGGTCTCGAGAT	62	62	358
				Rev	TTCAGCCTTGGTCTTGGTACT	62		
Dioscorea alata	OPA03*Da*$_{416}$	JX239749	Sc*Da*$_{185}$	For	CAGTATGTTGCTCCCACTGG	62	62	185
				Rev	ACCATGTTCATTGAGGGAAGC	62		
Euterpe oleracea	OPA09*Eo*$_{372}$	KP763492	Sc*Eo*$_{143}$	For	TGGAGTGTCTAAGGTGACGC	62	62	143
				Rev	ACCTCCTTGTGGTACTCTGG	62		
Malus × *domestica*	-	KM105163.1	Sc*Md*$_{262}$	For	TCAAGGACAGAGACTCGAGG	62	62	262
				Rev	GTGATCTGGAAGATGGAAAGG	62		
Morus nigra	OPA03*Mn*$_{598}$	KP939268	Sc*Mn*$_{293}$	For	GTTCTTTCCATGCATGCCATG	62	62	293
				Rev	CTCAAAATCACAGAGCCTACC	62		
Sambucus nigra	-	FJ805282.1	Sc*Sn*$_{226}$	For	CAAGTGCATGCGTTCTATTCC	62	62	226
				Rev	GACTTCAGACCTAACTCTACC	62		
Vaccinium macrocarpon	OPA09*Vma*$_{546}$	KP763490	Sc*Vma*$_{241}$	For	GGGCATCCTCTCTGATTTGC	62	62	241
				Rev	ATCATCCAAGAGGGACAGGC	62		
Vaccinium myrtillus	OPA05V*my*$_{408}$	KP939269	ScV*my*$_{287}$	For	TGTTTGGTAGCACGAGGTCG	62	62	287
				Rev	GGCTAGTGGTCTACATGAGG	62		
Vitis vinifera	OPA05*Vv*$_{293}$	KP763491	Sc*Vv*$_{144}$	For	GATGAGAATATGCCTTGAGGC	62	60	144
				Rev	GCAAATAAGAGAGGGAGATGG	62		

Figure(s)

Figure 1

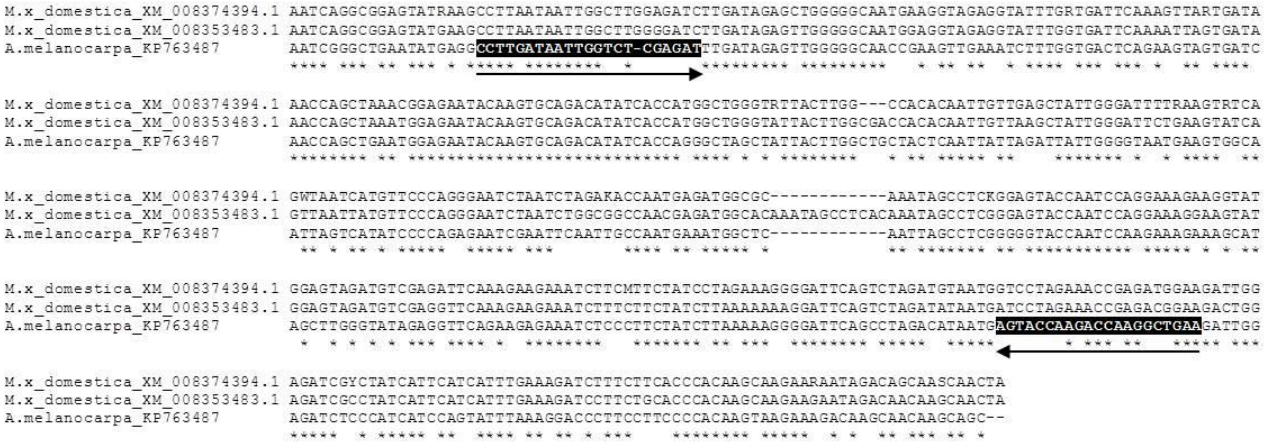
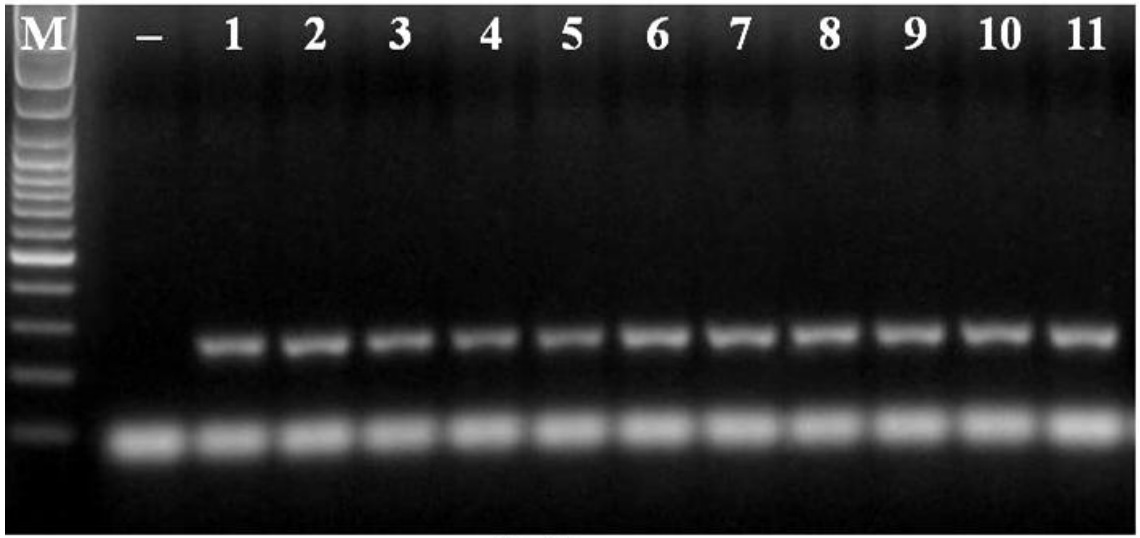


Figure 2



$ScPg_{249}$

Figure 3

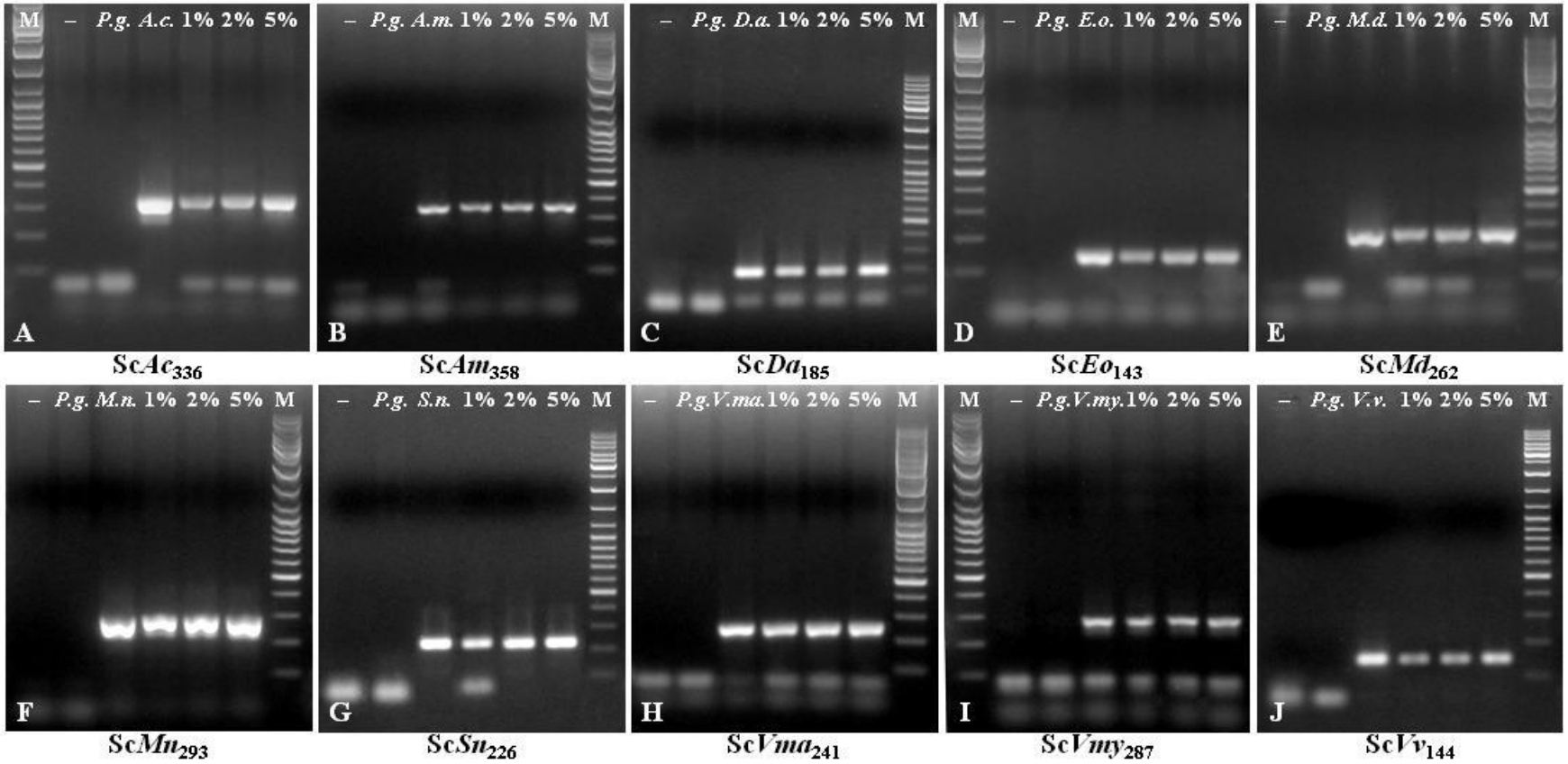

M – P.g. A.c. 1% 2% 5%
A
ScAc336
– P.g. A.m. 1% 2% 5% M
B
ScAm358
– P.g. D.a. 1% 2% 5% M
C
ScDa185
M – P.g. E.o. 1% 2% 5%
D
ScEo143
– P.g. M.d. 1% 2% 5% M
E
ScMd262
– P.g. M.n. 1% 2% 5% M
F
ScMn293
– P.g. S.n. 1% 2% 5% M
G
ScSn226
– P.g.V.ma.1% 2% 5% M
H
ScVma241
M – P.g.V.my.1% 2% 5%
I
ScVmy287
– P.g. V.v. 1% 2% 5% M
J
ScVv144

Figure 1.

ClustalW alignment of the RAPD fragments OPA04*Am*$_{460}$ selected for *Aronia melanocarpa* and homologue sequences resulted from Blast analysis. Asterisks below the alignment indicate the nucleotides perfect matching. Black boxes highlight two regions of high heterogeneity among the sequence of *A. melanocarpa* and the homologues, these regions were selected to design forward and reverse primers (black arrows) for PCR amplification of the *A. melanocarpa* specific SCAR marker (Sc*Am*$_{357}$).

Figure 2.

Sc*Pg*$_{249}$ SCAR marker validation on fresh leaves samples from different *Punica granatum* accessions. PCR performed with primer pair specific for *Punica granatum* in order to validate the identity of samples and to certify PCR availability of DNA: M, 100 bp DNA ladder; -, negative control, amplification with no template DNA; 1-11, DNA from different accessions of *P. granatum*.

Figure 3. SCAR markers sensitivity assay. Analyses were conducted on DNA extracted from individual samples and mixtures of *Punica granatum* and each contaminant species. Fresh material of every single contaminant species were mixed in three different percentages (shown at the top of the panels) with fresh material of *P. granatum*, before extracting genomic DNA. In all panels: -, negative control, amplification with no template DNA; *P.g.*, DNA from *P. granatum*, as further negative control; M, 100 bp DNA ladder. **A-** PCR performed with Sc*Ac*$_{336}$ primer pair specific for *Aristotelia chilensis*: *A.c.*, DNA from *A. chilensis*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *A. chilensis*. **B-** PCR performed with Sc*Am*$_{358}$ primer pair specific for *Aronia melanocarpa*: *A.m.*, DNA from *A. melanocarpa*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *A. melanocarpa*. **C-** PCR performed with Sc*Da*$_{185}$ primer pair specific for *Dioscorea alata*: *D.a.*, DNA from *D. alata*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *D. alata*. **D-** PCR performed with Sc*Eo*$_{143}$ primer pair specific for

Euterpe oleracea: DNA from *E. oleracea*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *E. oleracea*. **E-** PCR performed with Sc*Md*$_{262}$ primer pair specific for *Malus* × *domestica*: *M.d.*, DNA from *M.* × *domestica*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *M.* × *domestica*. **F-** PCR performed with Sc*Mn*$_{293}$ primer pair specific for *Morus nigra*: *M.n.*, DNA from *M. nigra*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *M. nigra*. **G-** PCR performed with Sc*Sn*$_{226}$ primer pair specific for *Sambucus nigra*: *S.n.*, DNA from *S. nigra*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *S. nigra*. **H**- PCR performed with Sc*Vma*$_{546}$ primer pair specific for *Vaccinium macrocarpon*: *V.ma.*, DNA from *V. macrocarpon*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *V. macrocarpon*. **I**- PCR performed with Sc*Vmy*$_{287}$ primer pair specific for *Vaccinium mirtyllus*: *V.my.*, DNA from *V. myrtillus*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *V. myrtillus*. **L**- PCR performed with Sc*Vv*$_{144}$ primer pair specific for *Vitis vinifera*: *V.v.*, DNA from *V. vinifera*, as positive control; 1%, 2%, 5%, DNA from mixtures of *P. granatum* and *V. vinifera*.