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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%.

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

3

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25 **Abstract**

26 A method based on Sequence-Characterized Amplified Regions (SCARs) was developed to detect
27 the presence of *Aristotelia chilensis*, *Aronia melanocarpa*, *Dioscorea alata*, *Euterpe oleracea*,
28 *Malus × domestica*, *Morus nigra*, *Sambucus nigra*, *Vaccinium macrocarpon*, *Vaccinium myrtillus*,
29 *Vitis vinifera* as bulking agents in *Punica granatum*. The method enabled the unequivocal detection
30 of up to 1% of each adulterant, allowing the preemptive rejection of suspect samples. The recourse
31 to such method may reduce the number of samples to be subjected to further evaluation with
32 phytochemical analyses when multiple batches have to be evaluated in a short time. *Vice versa*, it
33 allows the cross-check of suspect batches previously tested only for their anthocyanin profile. The
34 dimension of the amplicons is suitable for the analysis of degraded DNA obtained from stored and
35 processed commercial material. Proper SCAR markers may represent a fast, sensitive, reliable and
36 low-cost screening method for the authentication of processed commercial pomegranate material.

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

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51 **1. Introduction**

52 Anthocyanin-rich and polyphenol-rich fruits are enjoying a rampant commercial success, driven by
53 a growing evidence of their beneficial properties (de Pascual-Teresa & Sanchez-Ballesta, 2008; He,
54 & Giusti, 2010; Zanotti, Dall'Asta, Mena, Mele, Bruni, Ray & Del Rio, 2015). At the same time, the
55 market is also keen to the introduction of new, exotic or scarcely known fruits to lure the attention
56 of the consumers, resulting in the modern global success of fruits rich in anthocyanins, yet
57 previously uncommon or available only in local markets (Devalaraja, Jain & Yadav, 2011;
58 Dembitsky et al., 2011). Among these species, *Punica granatum* L. is enjoying most interest,
59 leaping from an out-fashioned fruit with limited commercial appeal to a commodity traded on a
60 worldwide scale, whose volumes grew with an impressive pace during the last decade (Rymon,
61 2011). Juices, concentrates and pulps obtained from pomegranate fruits are experiencing a soaring
62 success in the marketplace, spurred by several features including a favourable combination of
63 novelty, cheap availability, colour, unique taste and health properties (Mena, Gironés-Vilaplana,
64 Moreno, & García-Viguera, 2011). Alongside of such high market value, economically motivated
65 adulteration (EMA) has been gradually spotted in commercial pomegranate samples, with the
66 undeclared addition of both anthocyanin-rich plants or cheaper fruit juices used as bulking and
67 diluting agents. In particular, species more often related to pomegranate EMA in juices,
68 concentrates, frozen pulps and purees are *Aristotelia chilensis* Molina (maqui berries), *Aronia*
69 *melanocarpa* Elliot (black chokeberry); *Dioscorea alata* L. (purple yam) *Euterpe oleracea* Mart.
70 (açai), *Malus × domestica*, Borkh. (apple), *Morus nigra* L. (black mulberry), *Sambucus nigra* L.
71 (elderberry), *Vaccinium macrocarpon* Aiton. (cranberry), *Vaccinium myrtillus* L. (bilberry) *Vitis*
72 *vinifera* L. Stuntz., which are deliberately added to increase volume and weight of commercial
73 batches (Nuncio-Jáuregui, Calín-Sánchez, Hernández & Carbonell-Barrachina, 2014; Zhang,
74 Wang, Lee, Henning, Heber, 2009; Fischer-Zorn & Ara, 2007; Boggia, Casolino, Hysenaj, Oliveri
75 & Zunin, 2013; Borges & Crozier, 2012; Defernez, Kemsley & Wilson, 1995; Vardin, Tay, Ozen, &
76 Mauer, 2008; Zhang, et al., 2009). To face adulteration concerns, various analytical methods based

77 on chromatographic and spectrometric techniques are available to profile the anthocyanin content of
78 pomegranate-containing products, for quality control and authentication purposes (Sentandreu,
79 Navarro & Sendra, 2010; Bridle & García-Viguera, 1996; Obón, Díaz-García & Castellar, 2011;
80 Calani et al., 2013; Zhao, Yuan, Fang, Yin & Feng, 2013). Although these methods offer excellent
81 capabilities in characterizing the chemical composition of fruit products, as long as species
82 identification is concerned they may suffer in some occasions from complications emerging from
83 intraspecific differences, seasonal and climate, geographical and growing variability, different
84 harvest time, processing or storage conditions and length (Han et al., 2012; Faria, Magalhães,
85 Nunes & Oliveira, 2013). These factors, singularly or as a whole, may sometimes complicate the
86 unequivocal detection of specific adulterants in pomegranate products like juices or concentrates,
87 due to the similar anthocyanin profile of other fruits and to the 10-20 fold differences found for
88 some secondary metabolites in distinct *P. granatum* cultivars. Moreover, non chromatographic
89 methods relying on UV detection suffer from poor reproducibility and from sensitivity issues
90 related to interferences between anthocyanins and polyphenols (Gil, García-Viguera, Artés &
91 Tomás-Barberán, 1995; Borochoy-Neori et al., 2011). Furthermore, the necessity to screen large
92 numbers of samples increases the need for quick methods. Solely in the USA, U.S. Department of
93 Agriculture (USDA) and Food and Drug Administration (FDA) screen approximately 42 million
94 food and beverage imports per year, but only 1% of them are laboratory tested for authenticity, due
95 to laborious and time consuming nature of the requested assays (Buzby, Laurian & Roberts, 2008).
96 Such constraints, with particular commercial relevance, are considered behind the existence of legal
97 disputes and conflicts in product labeling in terms of authentication and purity of pomegranate
98 juices. (Roberts, 2010).

99

100 For the purpose of fruit authentication, researchers have recently turned their attention also to DNA-
101 based methods, designed to determine the biological identity of food material at genetic level,
102 producing fast, accurate and sensitive methods. Some countries in the European Union are actively

103 supporting such approach by encouraging the adoption of DNA-based evaluations on lab-on-a-chip
104 instruments (Primrose, Woolfe & Rollinson, 2010). Sensitive and conventional PCR techniques
105 have been successfully applied to quality control of an increasing range of juices or soft pulp
106 products, for instance allowing the quantification of 2.5% mandarin juice in orange juice (Palmieri,
107 Bozza & Giongo, 2009). For routine authentication the recourse to DNA fingerprinting offers
108 various advantages and is supported also by the gradually cheaper instrumental availability and by a
109 steady developments in the field (Turci et al., 2010). The major hindrances are the availability of
110 unique, reliable, reproducible and discriminant markers for the plant species involved in
111 adulteration and the diversity of potential interferences encountered in complex foods as in blends of
112 different fruits (Cordella et al., 2002,). For instance, plant polyphenols, sugars and the partial
113 degradation of DNA during storage are obstacles that cannot be overlooked easily. On this regard,
114 the recourse to SCAR markers and to proper DNA extracting protocols may allow to overcome
115 some of these limitations owing to high sample throughput, short sample preparation, unique
116 identification of contaminants, good interlaboratory replicability and low operating costs, as
117 recently demonstrated also by our work (Dhanya & Sasikumar, 2010; Marieschi, Torelli, Poli,
118 Sacchetti & Bruni, 2009; Marieschi, Torelli, Bianchi & Bruni, 2010; Marieschi, Torelli, Bianchi &
119 Bruni, 2011; Marieschi, Torelli & Bruni, 2012; Kiran, Khan, Mirza, Ram, & Abdin, 2010).
120 Regarding the authentication of anthocyanin-rich fruits, some DNA-based techniques have been
121 already optimized, including berries and other polyphenol fruits rich in polyphenols, but no proper
122 DNA-based screening allowing the fast detection of potential adulteration is at present available for
123 pomegranate (Jaakola, Suokas & Häggman, 2010; Palmieri, Bozza & Giongo, 2009; Han et al.,
124 2012).

125

126 Therefore, the objective of the present study was to develop robust SCAR markers for *P. granatum*
127 and for ten bulking agents previously spotted in processed pomegranate products, namely *A.*
128 *chilensis*, *A. melanocarpa*, *D. alata*, *E. oleracea*, *M. × domestica*, *M. nigra*, *S. nigra*, *V.*

129 *macrocarpon*, *V. myrtillus*, *V. vinifera*. The final goal is to obtain a diagnostic tool capable to
130 confirm the rejection of suspect samples or to reduce the number of samples to be evaluated by
131 means of phytochemical analyses, providing useful data for further molecular diagnostic tools. The
132 method was optimized with specific respect to a reliable application on plant material of
133 commercial grade, in order to complement existing methods.

134

135 **2. Materials and Methods**

136 **2.1 Plant material.** Fruits and leaves from *Punica granatum* L., *Malus × domestica* Borkh.,
137 *Morus nigra* L., *Sambucus nigra* L. and *Vitis vinifera* L. Stuntz. were collected in local orchards or
138 purchased from the market. Plants of *Aristotelia chilensis* Molina, *Aronia melanocarpa* Elliot,
139 *Dioscorea alata* L., *Euterpe oleracea* Mart., *Vaccinium macrocarpon* Aiton., and *Vaccinium*
140 *myrtillus* L. Stuntz. were kindly supplied by the Botanical Gardens of Parma. Fresh plant material
141 was collected and immediately freeze-dried in liquid nitrogen and stored at -80 °C until molecular
142 analysis.

143

144 **2.2 Extraction of PCR-compatible genomic DNA.** Genomic DNA was isolated from plant
145 material as previously described to increase the yield and the purity of the DNA extracted and
146 reduce the inhibitory effects of carbohydrates and polyphenols in the subsequent PCR reactions
147 (Marieschi, Torelli, Bianchi, Bruni, 2011; Marieschi, Torelli & Bruni, 2012). DNA concentration
148 and purity ($A_{260/280}$ and $A_{260/230}$) were evaluated by spectrophotometric analysis. The suitability of
149 DNA for RAPD analysis was also checked by ethidium bromide-stained agarose/TAE gels which
150 allowed both to evaluate DNA integrity and further confirm of DNA quantitation by visual
151 comparison with DNA standards. Agarose gels were analyzed and quantitated with a Kodak DC40
152 camera (Kodak) using the Kodak digital science 1D Image analysis software (Eastman Kodak
153 Company, Rochester, NY, USA). DNA samples were adjusted to approximately 20 ng/μL prior to
154 using them in PCR reactions.

155

156 **2.3 RAPD analysis and marker selection.** Eight potential contaminants of pomegranate juice
157 were compared with 2 samples of *P. granatum* to find RAPD amplicons suitable to develop SCAR
158 markers (Marieschi, Torelli, Poli, Sacchetti & Bruni, 2009). PCRs were conducted on
159 approximately 20-40 ng of DNA template and were performed in 25 µl volume containing 67 mM
160 Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 0.01% Tween 20, 2 mM MgCl₂, 0.2 mM dNTPs in
161 equimolar ratio, 1U SubTherm Taq DNA Polymerase (Fisher Molecular Biology, Trevose, PA,
162 USA), 25 pmol of each primer. To avoid inhibition due to co-precipitation of secondary
163 metabolites with DNA, the PCR amplification was improved through the addition of BSA 0.4%
164 and a non ionic detergent Tween 20 0.5% (Marieschi, Torelli, Bianchi, Bruni, 2010). Reaction was
165 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
166 min, followed by one cycle of 72 °C for 10 min (PTC-100, MJ Research Inc.). A total of 12
167 random primers (Operon Technologies: OPA01, OPA03, OPA04, OPA05, OPA07 OPA09, OPA10,
168 OPA11, OPA20, OPB19, OPB20, OPP10) were utilized for RAPD analysis. RAPD patterns were
169 compared to select amplicons present in the contaminants and absent in *P. granatum* RAPD
170 profiles. Most of the suitable marker bands were obtained with the primers OPA01, OPA03,
171 OPA04, OPA05, OPA09 and are listed in **Table 1**.

172

173 **2.4 Cloning and sequencing of putative RAPD markers.** The selected marker bands were excised
174 from 2% agarose gels, purified using JET-Sorb Gel Extraction Kit (Genomed, Löhne, Germany)
175 and cloned in pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA). The transformed
176 bacterial colonies were screened through colony PCR and clones carrying correctly sized inserts
177 were purified and sequenced with M13 forward and M13 reverse primers (BMR Genomics, Padova,
178 Italy).

179

180

181 **2.5 Sequence analysis.** Database searches of sequence similarity were performed using the
182 programs BlastN, BlastX, and PSI-BLAST set to standard parameters (Altschul et al., 1997). The
183 sequences were deposited into GenBank (Benson et al., 2012) (**Table 1**).

184

185 **2.6 SCAR primer design and optimization of PCR conditions.** The sequence data after
186 similarity searches were used for SCAR primers design of contaminant species, with the sole
187 exception of *M. × domestica* and *S. nigra*. Given the large amount of reliable information
188 available, for these two species, specific marker were instead constructed on intronic regions of
189 sequences selected from GenBank database (**Table 1**). For all selected sequences, primers 20-22
190 nucleotides long were designed for stringent conditions of annealing temperature (about 62 °C)
191 and did not overlap the sequence of the original RAPD primer (**Table 1**). The annealing
192 temperature was first calculated as 4-fold the number of GC (guanidine/cytosine) plus 2-fold the
193 number of AT (adenine/thymine). Each primer pair was tested up to 2 °C over and under its
194 annealing temperature to obtain the optimum. PCR reactions were conducted on approximately 40
195 ng of DNA template, were performed in 25 µl volume containing 67 mM Tris-HCl (pH 8.8), 16.6
196 mM (NH₄)₂SO₄, 0.01% Tween 20, 1.8 mM MgCl₂, 0.2 mM dNTPs in equimolar ratio, 1U
197 SubTherm Taq DNA Polymerase (Fisher Molecular Biology, Trevose, PA USA), 12.5 pmol
198 specific primers and were enhanced through the addition of BSA 0.4% and Tween 20 0.5%. Unless
199 not otherwise specified, amplification was performed as follows: 94 °C for 5 min, 37 cycles of 94
200 °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-
201 100, MJ Research Inc.). An aliquot (10 µl) of the amplification product was resolved by
202 electrophoresis on 1.5% agarose gel and detected by ethidium bromide staining.

203

204 **2.7 Validation of the SCAR markers.** DNA samples of contaminant species were analyzed with
205 the specific primer pairs defined for each species and with *P. granatum* samples to exclude
206 possible cross reactions leading to misinterpretation of the results. Once the primer specificity was

207 confirmed, SCAR analysis was conducted on genomic DNA extracted from mixtures of *P.*
208 *granatum* containing 1, 2 and 5% of each contaminant species. The integrity and availability of
209 DNA from samples for SCAR analysis was checked by performing an amplification with a couple
210 of primers, constructed on a RAPD amplicon (OPA01P_{g300}) obtainable from the amplification with
211 the primer OPA01 on *P. granatum* DNA. The band, 300 bp long, was cloned and sequenced. The
212 sequence was deposited into GenBank (accession number KP763488). The SCAR primers ScP_{g249}
213 forward and reverse, gave rise to a 249bp amplicon, suitable as positive control for PCR reactions
214 (**Table 1**).

215

216 **3. Results and Discussion**

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

229 **3.1 Development of SCAR markers.** We have previously analyzed 120 RAPD profiles obtained
230 by amplification with 12 primers on DNA extracted from 2 samples of *P. granatum* and 8 of the 10
231 plant species indicated in literature among the most frequent adulterants found in commercial
232 pomegranate juices, namely *A. chilensis*, *A. melanocarpa*, *D. alata*, *E. oleracea*, *M. nigra*, *V.*

233 *macrocarpon*, *V. myrtillus*, *V. vinifera*. By following a consolidated approach, the polymorphic
234 bands to be cloned were selected on the basis of their reproducibility, their intensity and the absence
235 of other bands in the proximity to avoid contamination (Marieschi, Torelli, Poli, Sacchetti & Bruni,
236 2009; Marieschi, Torelli, Bianchi, Bruni, 2010; Marieschi, Torelli, Bianchi, Bruni, 2011; Marieschi,
237 Torelli & Bruni, 2012). Eight RAPD fragments, each specific for a contaminant species and absent
238 in the RAPD profiles of the *P. granatum* samples (OPA05Ac₆₄₈, OPA04Am₄₆₀, OPA03Da₄₁₆,
239 OPA09Eo₃₇₂, OPA03Mn₅₉₈, OPA09Vma₅₄₆, OPA05Vmy₄₀₈, OPA05Vv₂₉₃, specific for *A. chilensis*, *A.*
240 *melanocarpa*, *D. alata*, *E. oleracea*, *M. nigra*, *V. macrocarpon*, *V. myrtillus* and *V. vinifera*,
241 respectively) were selected. RAPD amplicons were cloned and sequenced and their sequences were
242 deposited into GenBank (**Table 1**). A further RAPD amplicon (OPA01Pg₃₀₀) present in *P. granatum*
243 and absent in all contaminants profiles was also selected to be used as positive control in the
244 analysis of commercial samples. The BLASTN of the nucleotide sequences did not show similarity
245 with any sequences in the database. These discriminating RAPD bands were subsequently
246 converted into SCAR markers. The only exception was represented by OPA04Am₄₆₀ which showed
247 partial homology with two uncharacterized mRNA sequences of *Malus × domestica* (Ident 77%).
248 ClustalW analysis (**Figure 1**) showed the high identity of the OPA04Am₄₆₀ nucleotide sequences
249 and the two above mentioned sequences. In this case SCAR primer pair specific for *A. melanocarpa*
250 (ScAm₃₅₈) was thus constructed on the regions of maximal heterogeneity (**Figure 1**, black boxes).
251 For the two species *M. × domestica* and *S. nigra* SCAR primer pairs were obtained as described
252 above (see Methods).

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

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

261

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

277

278 **3.3 Sensitivity assay.** After checking marker specificity, the method sensitivity was validated in
279 order to define its limit of detection for each adulterant. To create conditions similar to those of
280 commercial samples, artificial counterfeit mixtures were prepared by mixing fresh pomegranate
281 tissue with the fresh material obtained from each contaminant, namely *A. chilensis*, *A. melanocarpa*,
282 *D. alata*, *E. oleracea*, *M. × domestica*, *M. nigra*, *S. nigra*, *V. macrocarpon*, *V. myrtillus*, *V. vinifera*.
283 **Figure 3** reports the results obtained with DNA extracted from artificial mixtures containing *P.*
284 *granatum* and 1, 2 or 5% of *A. chilensis* (Panel A), *A. melanocarpa* (Panel B), *D. alata* (Panel C), *E.*

285 *oleracea* (Panel D), *M. × domestica* (Panel E), *M. nigra* (Panel F), *S. nigra* (Panel G), *V.*
286 *macrocarpon* (Panel H), *V. myrtillus* (Panel I), *V. vinifera* (Panel J) subjected to amplification with
287 the primer pairs for ScAc₃₃₆, ScAm₃₅₈, ScDa₁₈₅, ScEo₁₄₃, ScMd₂₆₂, ScMn₂₉₃, ScSn₂₂₆, ScVma₂₄₁,
288 ScVmy₂₈₇ and ScVv₁₄₄ respectively. The selected SCAR markers allowed an easy detection up to 1%
289 of each contaminant, as shown by the amplification of an individual specific band absent in the
290 lanes of *P. granatum* (**Figure 3**) and present both in the reactions with the individual contaminant
291 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
292 mixtures with *P. granatum* (Panel A-J, lanes 1%, 2%, 5%).

293

294 **4. Conclusions.** A method based on SCAR markers was developed from RAPD specific for ten
295 common bulking agents used for economically motivated adulteration of pomegranate, in order to
296 authenticate their presence and set up a fast, sensitive, reliable and low-cost screening of *P.*
297 *granatum* juices and purees. The method enabled the unequivocal detection of low amounts of
298 contaminants up to 1%. The method meets the needs of the present market and may be used to
299 screen large pomegranate batches before proceeding with their grading, preemptively excluding
300 those counterfeited with bulking agents that may otherwise go undetected. At the same time, it may
301 be enforced as a confirming tool for samples previously detected as potentially adulterated after
302 screening of their anthocyanin profile. The availability of genetic sequences could also ease the
303 development of technological devices like gene chip or specific kits to be enforced in routine
304 control, as their response is not affected by phenotypic, environmental and process or storage
305 variability. Data presented may be useful also to enforce more careful monitoring of economically
306 motivated substitutions between different anthocyanin-rich plant materials. With proper
307 adjustments, the SCAR markers described in our work may be used to setup similar methods to
308 reveal further adulterations, as those recently noticed for red wine and elderberry, for bilberry and
309 mulberry, chokeberry or elderberry (Bridle & García-Viguera, 1996; Filippini, Piovan & Caniato,
310 2011; Giacomelli et al., 2014; Gardana et al., 2014). Besides the obvious trade issues, deceitful

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

314

315 **5. References**

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Table 1. Species-specific SCAR markers and primer specifications

Species	Original RAPD amplicon	GeneBank accession number	SCAR marker		SCAR primer sequence (5' → 3')	Calculated annealing temperature (°C)	Working annealing temperature (°C)	Amplicon length (bp)
<i>Punica granatum</i>	OPA01Pg ₃₀₀	KP763488	ScPg ₂₄₉	For	ACATTATGCCAAGGGCTCCC	62	60	249
				Rev	CATAGGAGTTCTTGGGGAGG	62		
<i>Aristotelia chilensis</i>	OPA05Ac ₆₈₄	KP763489	ScAc ₃₃₆	For	ATGCAGCACCAGAAACCAGC	62	62	336
				Rev	GTGGCATTGCTAACTCCAGG	62		
<i>Aronia melanocarpa</i>	OPA04Am ₄₆₀	KP763487	ScAm ₃₅₈	For	CCTTGATAAATTGGTCTCGAGAT	62	62	358
				Rev	TTCAGCCTTGGTCTTGGTACT	62		
<i>Dioscorea alata</i>	OPA03Da ₄₁₆	JX239749	ScDa ₁₈₅	For	CAGTATGTTGCTCCCCTGG	62	62	185
				Rev	ACCATGTTCATTGAGGGAAGC	62		
<i>Euterpe oleracea</i>	OPA09Eo ₃₇₂	KP763492	ScEo ₁₄₃	For	TGGAGTGTCTAAGGTGACGC	62	62	143
				Rev	ACCTCCTTGTGGTACTCTGG	62		
<i>Malus × domestica</i>	-	KM105163.1	ScMd ₂₆₂	For	TCAAGGACAGAGACTCGAGG	62	62	262
				Rev	GTGATCTGGAAGATGGAAAGG	62		
<i>Morus nigra</i>	OPA03Mn ₅₉₈	KP939268	ScMn ₂₉₃	For	GTTCTTTCCATGCATGCCATG	62	62	293
				Rev	CTCAAAATCACAGAGCCTACC	62		
<i>Sambucus nigra</i>	-	FJ805282.1	ScSn ₂₂₆	For	CAAGTGCATGCGTTCTATTCC	62	62	226
				Rev	GACTTCAGACCTAACTCTACC	62		
<i>Vaccinium macrocarpon</i>	OPA09Vma ₅₄₆	KP763490	ScVma ₂₄₁	For	GGGCATCCTCTCTGATTTGC	62	62	241
				Rev	ATCATCCAAGAGGGACAGGC	62		
<i>Vaccinium myrtillus</i>	OPA05Vmy ₄₀₈	KP939269	ScVmy ₂₈₇	For	TGTTTGGTAGCACGAGGTTCG	62	62	287
				Rev	GGCTAGTGGTCTACATGAGG	62		
<i>Vitis vinifera</i>	OPA05Vv ₂₉₃	KP763491	ScVv ₁₄₄	For	GATGAGAATATGCCTTGAGGC	62	60	144
				Rev	GCAAATAAGAGAGGGAGATGG	62		

Figure 2

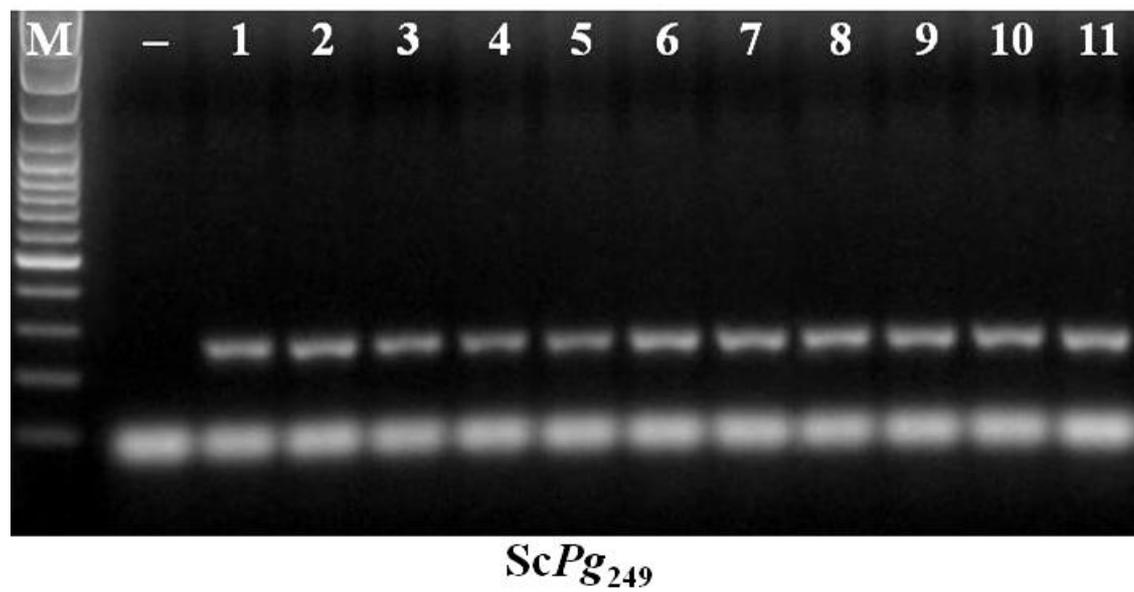


Figure 3

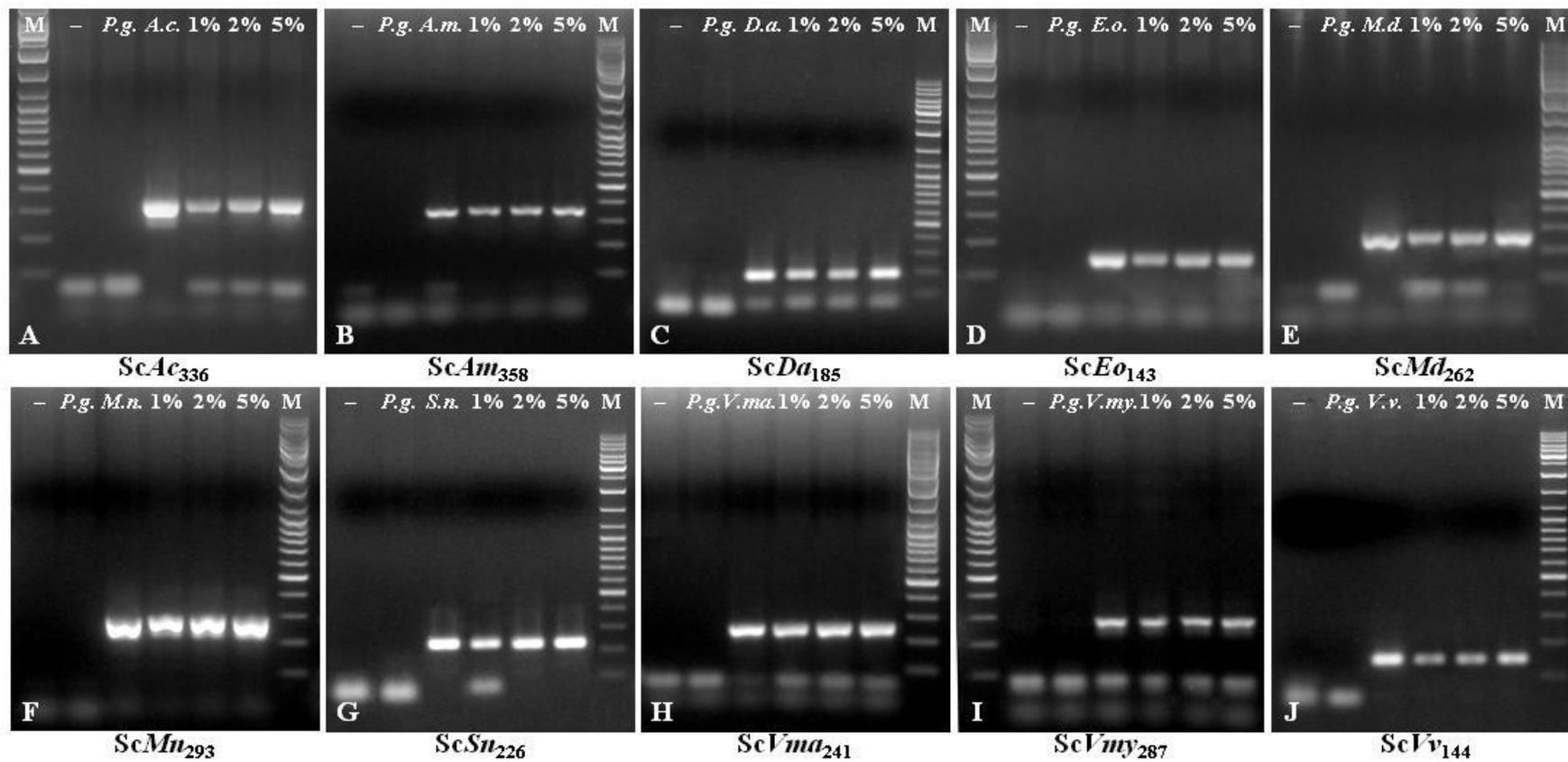


Figure 1.

ClustalW alignment of the RAPD fragments OPA04Am₄₆₀ 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 (ScAm₃₅₇).

Figure 2.

ScPg₂₄₉ 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 ScAc₃₃₆ primer pair specific for *Aristolelia 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 ScAm₃₅₈ 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 ScDa₁₈₅ 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 ScEo₁₄₃ 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 ScMd₂₆₂ 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 ScMn₂₉₃ 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 ScSn₂₂₆ 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 ScVma₅₄₆ 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 ScVmy₂₈₇ primer pair specific for *Vaccinium myrtillus*: *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 ScVv₁₄₄ 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*.