

1                   **Characterization and Identification of Markers Associated with**  
2 **Morphological and Biochemical Traits of Pomegranate Fruit Using SCoT and**  
3 **SSR Markers**

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6 **Abstract**

7                   This research aimed to identify markers related to morphological and biochemical traits in  
8 pomegranate using 15 SCoT and 6 pairs of SSR markers. Based on the results obtained from  
9 association analysis using mixed linear modeling (MLM) method, a total of 169 markers related  
10 to the studied traits were identified among which 149 were based on SCoT markers and 20 relied  
11 on SSR markers. The obtained results showed that SCoT35<sub>1200</sub> loci associated with vitamin C,  
12 titratable acidity, peel soluble solid and aril, PGCT001<sub>100</sub> associated to fruit juice pH, titratable  
13 acidity, aril soluble solid, peel soluble solid and total aril total phenolic content. In addition,  
14 SCoT1<sub>1800</sub>, SCoT21<sub>100</sub>, SCoT5<sub>900</sub>, SCoT23<sub>900</sub>, PGCT001<sub>105</sub> and PGCT002<sub>200</sub> loci were highly  
15 associated only with fruit diameter, fruit crown length, fruit crown diameter, titratable acidity, seed  
16 hardness and fruit juice pH, respectively, which could be considered as informative markers in  
17 breeding programs for pomegranate.

18 **Keywords:** Association analysis, Informative markers, Pomegranate, Population structure, SSR  
19 and SCoT.

20 **Abbreviations:** SCoT: Start Codon Targeted, SSR: Simple Sequence Repeat.

21  
22 **Introduction**

23                   Pomegranate (*Punica granatum* L.) belongs to the Punicaceae family and due to having  
24 phenolic compounds, anthocyanin and vitamin C, its fruits have high nutritional and  
25 pharmaceutical value (Karimi and Mirdehghan, 2013). Genetic maps provide information on the  
26 order, position and relative location of different genes based on genetic distance and breeders can  
27 use these association maps to help the selection process. Since in breeding programs, the aim is to  
28 select appropriate plants in terms of the considered trait, any method that can help rapid selection

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29 of that trait could shorten the modification period. This phenomenon is especially important when  
30 a trait requires a long time to be expressed or the plant is cross-pollination (Adawy et al., 2004).  
31 Determining plant traits and conducting genetic characterization are fundamental steps in the  
32 development of effective plant breeding programs. These approaches enable the identification of  
33 superior genotypes and provide insights into the genetic basis of key agronomic traits (Mishra et  
34 al., 2025). Access to genetic resources and identification of morphologic traits are the first steps  
35 of any pomegranate breeding program. Several studies have been conducted on the genetic  
36 structure and diversity of pomegranate with morphological markers (Parashuram et al., 2022;  
37 Karimi and Mirdehghan , 2013) as well as molecular markers such as RAPD (Zamani *et al.*, 2010),  
38 SSR (Zarei and Sahraroo, 2018; Patil et al., 2020) and SCoT (Sadeghi Seresht et al., 2023;  
39 Shahsavari et al., 2022). Due to the limitations of morphologic markers, DNA-based molecular  
40 markers have been able to address problems of morphologic markers by providing a significant  
41 number of markers and eliminating environmental effects. Also, molecular markers can select and  
42 create genetic maps that are of special significance in programs related to fruit tree breeding  
43 (Adawy et al., 2004). Association analysis is another positioning method. In this method, the  
44 relationship between the phenotype and genotype of the plant is directly applied to achieve and  
45 identify chromosome regions involved in controlling the quantitative traits in natural and  
46 germplasm populations (Myles et al., 2009). Few studies have been conducted on the identification  
47 of markers related to quantitative and qualitative traits of pomegranate. In a study, the relationship  
48 between RAPD markers and the traits of pomegranate cv. ‘Malase-Torshe-Saveh’ was investigated  
49 and it was found that the most important informative markers for soft seeded, taste index, peel  
50 thickness and anthocyanin were TIBMBB-14<sub>1100</sub>, OPAE-10<sub>850</sub>, OPG-11<sub>1060</sub> and TIBMBB-14<sub>700</sub>,  
51 respectively (Zamani et al., 2010). Singh et al., (2015) applied 44 SSR loci to identify markers  
52 associated with fruit traits in 88 genotypes of wild and cultivated pomegranate and found that 4  
53 markers were related to fruit weight, TA and sensitivity to bacterial blight. Basaki et al., (2011)  
54 investigated the relationships among 18 qualitative traits and 24 SSR loci in some local Iranian  
55 pomegranate species and introduced one informative marker (MP26) which was highly associated  
56 with at least one of these traits. In addition, two linkage maps in pomegranate were constructed  
57 using AFLP (Sarkhosh et al., 2012) and SNP (Harel-Beja et al., 2015) molecular markers.  
58 Although relationships among several markers with quantitative traits have been reported for  
59 pomegranate, due to the lack of comprehensive and complete reports on the genomic sequence and

60 the relationships of markers with important phenotypic traits, applications of these linkage maps  
61 to accelerate breeding programs in pomegranate have been postponing. So far, no comprehensive  
62 research has been reported on the association analysis of pomegranate covering important  
63 morphological and biochemical fruit traits and the majority of reports have been limited to a few  
64 morphological and biochemical traits. Therefore, this research identified informative markers  
65 associated with morphological and biochemical traits using SCoT and SSR molecular markers.

## 66 **Materials and Methods**

67 The objective of this study was the identification of SCoT and SSR markers loci associated with  
68 important morphological and biochemical traits 23 genotypes of Iranian pomegranate (Table 1)  
69 (Sadeghi Seresht et al., 2023).

## 70 71 **Evaluation of Quantitative Traits of Fruits**

72 To evaluate the morphological and biochemical traits of the 23 genotypes' fruits, a factorial  
73 experiment was conducted based on a Completely Randomized Design (CRD) with three  
74 replications, where each replication included 5 fruits from each genotype. Fruit weight, peel  
75 weight, aril weight, and seed weight were determined with a digital scale (g). The dimensions of  
76 fruit, aril, and seed, as well as peel thickness, were measured using a digital caliper with an  
77 accuracy of 0.01 mm. Peel firmness, aril firmness, and seed hardness were assessed using a digital  
78 hardness tester (Lutron FG5020, Taiwan) following the method of Pongener et al. (2011). Fruit  
79 juice pH was determined with a digital pH meter (Labtron pH-110) as described by Karimi and  
80 Mirdehghan (2013). Vitamin C content was quantified by titration with potassium iodide in the  
81 presence of 1% starch indicator and expressed as mg vitamin C per 100 mL fruit juice (Nafees et  
82 al., 2018). Titratable acidity was determined by titration with 0.1 N sodium hydroxide and  
83 expressed as mg citric acid per 100 g fruit juice (Fawole and Opara, 2013). Total soluble solids  
84 (TSS) in the fruit peel were quantified by extracting 0.5 g fresh tissue with 95% ethanol, mixing  
85 0.1 mL of the extract with 3 mL anthrone, incubating the mixture in a hot water bath for 10 min,  
86 and measuring absorbance at 625 nm using a spectrophotometer (PG Instruments Ltd T80  
87 UV/VIS, Alma Park, England) (Irigoyen et al., 1992). Fruit juice TSS was measured with a  
88 refractometer (PAL-1 Atago, Japan) and expressed as °Brix (AOAC, 2000). **Maturity indices were**  
89 **determined by dividing the TSS with Titrable acidity (Caliskan and Bayazit, 2013).** Total phenolic  
90 content of the peel was determined using the method of Singelton et al. (1996). Total phenolics of

91 the aril were measured following the method of Ayala-Zavala (2004) at 760 nm. Anthocyanin  
92 contents of peel and aril were determined according to Rapisarda et al. (2000). Descriptive  
93 statistics parameters related to the morphological and biochemical traits, including Mean values,  
94 minimum and maximum values, standard deviation, and coefficient of variation, were calculated  
95 using SPSS software version 24.

#### 96 **Molecular Evaluation**

97 Genomic DNA was extracted from young leaf tissues using Murry and Thompson (1980)  
98 method. The quality and quantity of the extracted DNA were determined using spectrophotometry  
99 at wavelengths of 260 and 280 nm, and by electrophoresis on a 1% agarose gel. Furthermore, to  
100 standardize the DNA concentration, the samples were adjusted to a concentration of 20 ng/μL  
101 (nanograms per microliter). Polymerase chain reaction (PCR) amplifications of SCoT and SSR  
102 loci were performed following the optimized protocols of Malekzadeh et al. (2018) and Soriano et  
103 al. (2010), respectively. Amplification products of SCoT primers were electrophoresed on 1%  
104 agarose gels at a constant voltage of 120 V for approximately 2 h to achieve clear band resolution  
105 (Malekzadeh et al., 2018). In contrast, SSR amplification products were resolved on 6%  
106 polyacrylamide gels and subsequently visualized using silver nitrate staining according to the  
107 procedure of Sambrook et al. (2001), which provided high sensitivity for detecting allelic  
108 polymorphisms. Finally, all gels were documented using a digital gel imaging system (UVP,  
109 Germany) to ensure accurate scoring of bands for subsequent analyses.

110

#### 111 **Data Analysis**

112 The genetic structure of the studied pomegranate genotypes was evaluated using STRUCTURE  
113 software (version 2.3.4). To infer the most likely number of genetic clusters, the assumed  
114 subpopulation parameter (K) was set to range from 1 to 10. In this context, K represents the number  
115 of assumed subpopulations or distinct genetic clusters that the software tests for in the genetic  
116 data. For each K value, independent runs were performed, consisting of a burn-in period of 100,000  
117 iterations to minimize the effect of initial conditions, followed by 10,000 replications of the Monte  
118 Carlo Markov Chain (MCMC). The analysis was carried out under the admixture model with  
119 correlated allele frequencies, which allows for the possibility that individuals may have mixed  
120 ancestry. The optimal number of subpopulations (K) was determined using the  $\Delta K$  method

121 proposed by Evanno et al. (2005). To further explore marker–trait associations, a mixed linear  
122 model (MLM) framework was implemented in TASSEL software (version 5.2.12), integrating  
123 both population structure (Q matrix) and kinship (K matrix) into the analysis to reduce spurious  
124 associations. Additionally, descriptive statistical parameters, as well as correlation analyses among  
125 morphological and biochemical traits, were conducted using SPSS software version 24 (SPSS Inc.,  
126 Chicago, IL, USA) (Norusis, 1998; SPSS, 2012), providing complementary insights into  
127 phenotypic variation and inter-trait relationships.

128

## 129 **Results**

### 130 **Descriptive Statistics**

131 The results showed a high phenotype diversity of important morphological and biochemical traits  
132 in 23 pomegranate genotypes (data not shown). The coefficient of variation was the lowest for the  
133 TSS of aril (CV = 11.63%), while it was the highest for titratable acidity (CV = 54.10 %). Based  
134 on the obtained results, higher phenotypic diversity was obtained from biochemical traits (with an  
135 average variation coefficient of 29.79%) compared to that of morphological traits (with an average  
136 variation coefficient of 25.58%).

### 137 **Analysis of Population Structure**

138 To evaluate the genetic structure and diversity of the studied pomegranate genotypes, 15 SCoT  
139 and 6 SSR markers with appropriate distribution across the genome. In total, 237 loci were  
140 proliferated and among these loci, 213 were related to SCoT markers and 24 belonged to SSR  
141 markers. Based on genetic structure analysis, four genetic groups (k=4) were found in the studied  
142 germplasm in both the SCoT and the SSR markers. The mean expected heterozygosity and mean  
143  $F_{st}$  for the SCoT marker were 0.26 and 0.35 and for the SSR marker were 0.36 and 0.20,  
144 respectively (data not shown). The mean proportion of membership of the genotypes to the inferred  
145 groups was higher than 0.7. Of 23 studied pomegranate genotypes with SCoT marker, 3 (13.4%),  
146 5 (21.73%), 3 (13.04%) and 9 (39.3%) genotypes were assigned to subgroups Q1, Q2, Q3 and Q4,  
147 respectively. Furthermore, 3 genotypes of Tabrizi, Voshik Malas Saravan and Bargmordi were  
148 identified as mixed genotypes (Fig. 1a). Based on the analysis results of SSR marker of all  
149 genotypes showed  $q < 0.90$  and were identified as admixed (Fig. 1-b).

150

151 **Association Analysis Using SCoT Molecular Marker**

152 In total, from the 213 loci produced by the SCoT marker, 149 loci showed a significant  
153 association with the studied traits at a 5% probability level among which, 53 loci were specifically  
154 associated with one of the studied traits. The highest number of loci (21) were identified for the  
155 traits of fruit crown length and TSS of peel and the least loci number (5) was identified for the  
156 traits of seed length and peel thickness (Table 2). The justified phenotypic variations ( $R^2$ ) in  
157 cumulative informative markers were in the range of 2% (aril anthocyanin) to 50% (peel TSS).  
158 Primers SCoT12 and SCoT31, presented maximum (23) and minimum (5) numbers of significant  
159 association with traits, respectively. For each trait, multiple loci with significant association were  
160 identified where the loci with the highest phenotypic variation (coefficient of determination) were  
161 introduced as the informative marker for that trait. Based on the obtained results, a total of 17 loci  
162 were identified as associated with fruit weight, with the most important locus being SCoT21400  
163 which accounted for 29% of the variance in the phenotype of this trait followed by SCoT22700  
164 locus which justified 25% of the phenotypic variations. For traits corresponding to the weight of  
165 peel, aril and seed, primers SCoT22, SCoT35, and SCoT30 each one with 5, 3, and 4 loci showed  
166 a higher association for expression of these traits more than the other markers. Loci SCoT31400,  
167 SCoT22700, SCoT3500 and SCoT301700 with 29%, 29% and 25% of phenotypic variations  
168 respectively, showed a significant association with the weight of peel, aril and seed. Also, most of  
169 the loci associated with fruit weight were common to traits of weight of peel, aril and seed. 16 loci  
170 were highly associated with fruit length, such that the percentage of phenotypic variation explained  
171 by these markers ranged between 10 to 20%. The highest phenotypic variations were obtained for  
172 SCoT12750. Among the loci associated, SCoT121400 was considered a common marker among the  
173 traits of fruit length and weight of peel and aril. 15 loci could provide a significant association with  
174 fruit diameter. Among them, SCoT5200 with phenotypic variations of 22% was the most effective  
175 locus on this trait, while SCoT11800 was specifically associated with fruit diameter. Also,  
176 SCoT19550 was common for fruit length and diameter traits. 21 loci were associated with fruit  
177 crown. SCoT7400 and SCoT7500 with phenotypic variations of 43% played stronger roles in the  
178 justification of phenotypic variance of this trait. Also, these two loci were identified to be common  
179 for fruit weight. Two loci SCoT211000 and SCoT32600 specifically associated with fruit crown  
180 length. Based on the obtained results, SCoT121400 was identified as the most informative marker  
181 with the fruit crown diameter with phenotypic variations of 17%, although this locus was common

182 between traits of fruit crown diameter and fruit length and the fruit weight. Also, among the studied  
183 markers SCoT5<sub>900</sub> was specifically associated with fruit crown diameter. SCoT5<sub>600</sub> and SCoT11<sub>700</sub>  
184 determined 41% and 28% of phenotypic variations in aril length and aril diameter traits,  
185 respectively and were identified as the most informative markers of these traits. Also, two loci  
186 SCoT3<sub>550</sub> and SCoT23<sub>600</sub> were common between aril length and aril diameter traits and the  
187 remaining markers were separated between these two traits. Based on the obtained results,  
188 SCoT5<sub>1400</sub>, SCoT12<sub>500</sub> and SCoT13<sub>1600</sub> played more important roles in accounted phenotypic  
189 variations of seed length with phenotypic variations of 12%. 5 loci were found to be associated  
190 with seed diameter trait among which locus SCoT3<sub>200</sub> with phenotypic variations of 15% was the  
191 most informative marker. Also, all markers associated with seed length were commonly associated  
192 with seed diameter. Based on the results of association analysis, 5 loci exhibited significant  
193 associations with peel thickness trait. SCoT7<sub>1000</sub> and SCoT30<sub>1200</sub> played more important roles in  
194 the justification of phenotypic variation of this trait with phenotypic variations of 23% and 20%,  
195 respectively. Also, SCoT19<sub>1400</sub> was commonly associated with peel thickness and seed weight and  
196 the remaining markers for this trait were separated from other traits. Peel firmness and aril firmness  
197 traits were associated with SCoT12<sub>600</sub> and SCoT12<sub>1000</sub> with a coefficient of determination 32%  
198 and 21% for the justification of phenotypic variation, respectively. In the aril firmness trait, 7 loci  
199 (SCoT3<sub>700</sub>, SCoT3<sub>800</sub>, SCoT5<sub>1600</sub>, SCoT7<sub>700</sub>, SCoT13<sub>600</sub>, SCoT21<sub>1400</sub> and SCoT30<sub>500</sub>) were  
200 identified which were not associated with any other morphological and biochemical traits and were  
201 specific for this trait. Also, SCoT3<sub>550</sub> and SCoT23<sub>600</sub> were commonly associated with traits  
202 connected to aril including diameter, length and firmness. 11 loci were significantly associated  
203 with seed hardness. The locus SCoT30<sub>850</sub> was an important marker in the justification of seed  
204 hardness with 28% of phenotypic variations. In the present study, 15 loci exhibited significant  
205 associations with vitamin C. SCoT35<sub>500</sub> was introduced as an informative marker that justified  
206 15% of the phenotypic variations. Among the 13 loci associated with fruit juice pH, locus  
207 SCoT5<sub>1200</sub> which accounted for 39% of phenotypic variations was considered the most important  
208 informative marker of this trait, although this locus was common for peel firmness. In the present  
209 study, two SCoTs (SCoT3<sub>850</sub> and SCoT12<sub>1600</sub>) exhibited significant associations with titratable  
210 acidity with phenotypic variations of 40% to 27%, respectively. Maturity indices were found to be  
211 more associated with SCoT3 than any other primer. SCoT3<sub>1000</sub> with maximum phenotypic  
212 variations (33%) was introduced as the informative marker of this trait. Also, 6 loci were common

213 between titratable acidity and maturity indices and the remaining markers were separated for these  
214 traits. In relation to peel TSS, 5 loci were found to be specific among which SCoT12<sub>500</sub> with  
215 phenotypic variations of 70% and maximum significance level (P=0.008) was considered as an  
216 informative marker. Among the studied loci, SCoT12<sub>500</sub> was found to be commonly associated  
217 with seed traits (weight, length and diameter) and SCoT35<sub>900</sub> was commonly associated with the  
218 weight of fruit, peel, and aril as well as seed hardness and vitamin C. In the present study, 15 loci  
219 were significantly associated with aril TSS. The highest phenotypic variations (36%) belonged to  
220 SCoT32<sub>800</sub>. 6 loci were common between peel and aril TSS traits and the remaining markers were  
221 separated between these traits. Loci SCoT30<sub>1700</sub> and SCoT32<sub>1000</sub>, which accounted for 20% of the  
222 phenotypic variations of peel total phenolic content, were considered the most informative markers  
223 for this trait. For this trait, SCoT13<sub>1100</sub> was common among peel total phenolic content, vitamin C  
224 and peel TSS. Furthermore, SCoT23<sub>600</sub> with phenotypic variations of 20% and significance level  
225 of p=0.008 was identified as an informative marker for describing aril total phenolic content.  
226 SCoT5<sub>300</sub>, SCoT11<sub>1800</sub>, SCoT19<sub>200</sub> and SCoT19<sub>700</sub> were specifically associated with aril total  
227 phenolic content. SCoT12<sub>300</sub> and SCoT19<sub>750</sub> were commonly associated with aril total phenolic  
228 content and aril and peel TSS. The most important marker for peel and aril anthocyanin was found  
229 to be SCoT32<sub>1400</sub> and SCoT32<sub>1100</sub> which justified 41% and 23% of phenotypic variations in these  
230 traits. SCoT11<sub>1000</sub> was common for aril and peel anthocyanin and aril total phenolic content and  
231 SCoT32<sub>1100</sub> was common for aril and peel anthocyanin and the remaining traits for these two traits  
232 were separated. SCoT3<sub>500</sub>, SCoT12<sub>1200</sub> and SCoT30<sub>800</sub> were specifically identified for peel and aril  
233 anthocyanin.

234

### 235 **Association Analysis using SSR Molecular Marker**

236 Based on the results, in total, 20 loci from 6 microsatellite markers were associated with at least  
237 one of the traits related to the properties of fruit, aril and seed at a probability level of 5% using  
238 genotypic values (BLUP). The maximum number of loci (11) was identified for fruit juice pH and  
239 minimum locus number (1) was identified for total seed weight, fruit crown diameter, aril diameter,  
240 seed length and diameter, peel and aril firmness and peel total phenolic content (Table 2).

241 Based on SSR data, phenotypic variations ( $R^2$ ) in cumulative informative markers were in the  
242 range of 9% (fruit juice pH) to 45% (fruit crown length). Primers PGCT017 and PGCT019  
243 presented the maximum (16) and minimum (5) number of associations with traits, respectively. In

244 the present study, primers PGCT005 and PGCT020, each with 2 loci, showed more association  
245 with fruit weight and fruit weight. The most important loci associated with the weight of fruit, aril  
246 and seed were PGCT017<sub>155</sub> with a coefficient of determination 36%, 27% and 18% for the  
247 justification of phenotypic variations in these traits, respectively. Also, loci associated with fruit  
248 weight were commonly associated with aril weight. PGCT005<sub>100</sub> loci played more important roles  
249 in justifying phenotypic variations (16%) of fruit length. 4 loci were associated with fruit diameter.  
250 PGCT001<sub>90</sub> with phenotypic variations of 25% was the most informative marker of this trait. For  
251 this trait, loci PGCT005<sub>130</sub> and PGCT020<sub>140</sub> were common with the weight of fruit and aril. Fruit  
252 crown length and fruit crown diameter presented the maximum association with the PGCT017  
253 marker. Also, loci PGCT017<sub>170</sub> and PGCT017<sub>190</sub> were the main informative markers for the  
254 expression of fruit crown length and diameter with phenotypic variations of 45% and 24%,  
255 respectively. PGCT017<sub>140</sub> was found to be the most informative marker for aril diameter which  
256 accounted for 16% of phenotypic variations. Regarding the length and diameter of the seed, the  
257 maximum percent of phenotypic variations (22%) was observed for the PGCT020<sub>100</sub> locus. In this  
258 study, three loci (PGCT002<sub>115</sub>, PGCT019<sub>240</sub> and PGCT019<sub>254</sub>) were identified to have a  
259 significant association with peel thickness among which PGCT002<sub>115</sub> had the highest phenotypic  
260 variations (22%) which was considered as the informative marker. Peel firmness and aril firmness  
261 were associated with PGCT020<sub>120</sub> and PGCT017<sub>155</sub>, respectively, which explained 30% of the  
262 phenotypic variations, although these two loci were common with the weight of fruit, aril and seed.  
263 4 loci (PGCT001<sub>105</sub>, PGCT001<sub>120</sub>, PGCT005<sub>100</sub> and PGCT017<sub>170</sub>) were highly associated with  
264 seed hardness. Among these PGCT017<sub>170</sub>, which accounted for 27% of the phenotypic variations,  
265 was introduced as the informative marker for seed hardness. Although this locus was also  
266 commonly associated with fruit diameter and fruit crown length. Also, PGCT001<sub>105</sub> was not  
267 associated with any of the morphological and biochemical traits of fruits and was only associated  
268 with seed hardness. Fruit juice pH with the PGCT020 marker presented a stronger association with  
269 compared to other markers. Among these associated loci, PGCT017<sub>155</sub>, which accounted for 21%  
270 of the phenotypic variations was identified as the most important informative marker for this trait.  
271 This locus was common with fruit weight, aril weight, seed weight, and aril firmness. Also,  
272 PGCT020<sub>200</sub> was specifically associated with fruit juice pH. Based on the obtained results, 4 loci  
273 (PGCT001<sub>100</sub>, PGCT005<sub>100</sub>, PGCT017<sub>140</sub> and PGCT020<sub>140</sub>) were significantly associated with  
274 titratable acidity among which maximum phenotypic variations (27%) belonged to PGCT005<sub>100</sub>.

275 This locus was commonly and significantly associated with seed hardness. 3 loci (PGCT005<sub>100</sub>,  
276 PGCT017<sub>140</sub> and PGCT019<sub>254</sub>) were associated with maturity indices which, PGCT019<sub>254</sub>, which  
277 accounted for 24% of the phenotypic variations, were identified as the informative marker  
278 although this locus was also commonly and associated with peel thickness. Five loci (PGCT001<sub>100</sub>,  
279 PGCT002<sub>115</sub>, PGCT017<sub>140</sub>, PGCT017<sub>190</sub> and PGCT019<sub>240</sub>) have been associated with peel TSS  
280 among which PGCT002<sub>115</sub> and PGCT017<sub>140</sub> were the most important informative markers which  
281 justified 31% of the phenotypic variations for this trait. These loci were common with peel  
282 thickness and aril diameter, respectively. Locus PGCT001<sub>100</sub> with 40% of phenotypic variations,  
283 showed a significant association with aril TSS. Also, all of the loci associated with aril TSS were  
284 common to peel TSS. Based on the results, one locus (PGCT019<sub>240</sub>) was associated with peel total  
285 phenolic content and 2 loci (PGCT001<sub>100</sub> and PGCT020<sub>100</sub>) were associated with aril total phenolic  
286 content were identified as informative markers for this trait. Primers PGCT017 with 3 loci showed  
287 a higher association for expression of peel and aril anthocyanin content compared to other markers.  
288 Loci PGCT017<sub>190</sub> and PGCT017<sub>140</sub> with 25% of phenotypic variations, showed a significant  
289 association with peel and aril anthocyanin, respectively.

## 290 **Discussion**

291 Climate change, water shortage, and serious temperature oscillation have reduced the yield of  
292 pomegranate. Therefore, investigation of local genotypes with diverse beneficial attributes seems  
293 essential to develop and protect the cultivars and use them as parents in breeding programs  
294 (Pirsevedi et al., 2010). Based on the results obtained, the coefficient of variation (CV) ranged  
295 from 11.63% to 54.10% across the 25 quantitative and qualitative traits analyzed. The highest CV  
296 was observed for titratable acidity, while the lowest CV was recorded for the soluble solids content  
297 of the aril. This observation is consistent with the findings of Karapetsi et al., (2021), whose study  
298 also reported a higher CV (30.85%) for titratable acidity among the traits investigated. The  
299 findings of this study showed that the studied pomegranate germplasm had high diversity in terms  
300 of most investigated traits where this diversity can be useful in association analysis of  
301 pomegranate. The occurrence of recombination during the evolutionary history of natural  
302 populations with high diversity results in the linkage disequilibrium in the genome. Therefore, in  
303 association analysis, factors related to phenotypic polymorphism can be better searched in natural  
304 populations than in populations resulting from the crossing of two specific parents due to their

305 higher diversity. The results obtained are according to the findings of Sadeghi Seresht et al.,  
306 (2023), Karimi and Mirdehghan, (2013); Khadivi et al., (2020) and Parashuram et al., (2022)  
307 reporting the presence of high genetic diversity in pomegranate. Knowledge of the population  
308 structure is a prerequisite for association mapping studies to avoid making spurious relationship  
309 identification among markers and factors controlling certain traits and obtain reliable results (Patil  
310 et al., 2020). Based on the results obtained, the SSR markers were not able to separate the genotype  
311 of the studied pomegranate and high admixture level was seen among genotypes while the SCoT  
312 marker separated most of the genotypes which could be due to the insufficient number of SSR  
313 markers used in this research or insufficient coverage of SSR markers compared to SCoT markers.  
314 In a research, Patil et al., (2020) used 21 SSR pairs to study the population structure among 42  
315 diverse pomegranate genotypes and reported that based on population structure genotypes could  
316 be divided into 4 groups. Similarly, Luo et al., (2018) determined the genetic variation and  
317 population structure of 136 pomegranate varieties using 13 SSR markers and found three  
318 subpopulations. The genetic study of quantitative traits is one of the main objectives of modern  
319 plant breeding, which is carried out by locating genes that control traits. The phenotypic variation  
320 in quantitative traits is influenced, by several genes of small effect and environmental factors, and  
321 the interaction of the two, which makes breeding about these traits difficult (Stich and Melchinger,  
322 2010). In the present study, using SCoT and SSR markers, a total of 169 significant associations  
323 were identified among gene locus and morphological and biochemical traits among which 149  
324 significant associations belonged to SCoT markers and 20 significant associations belonged to  
325 SSR markers. Differences in the number and type of the identified locus could be related to the  
326 nature of the studied traits as well as the type and nature of the applied markers. In this study,  
327 phenotypic variations for the studied traits with SCoT markers varied from 2 to 50% and that for  
328 SSR markers ranged from 9 to 45%. The highest value of  $R^2$  in SCoT markers was observed for  
329 the association of SCoT12500 locus with peel TSS and in SSR markers was obtained for the  
330 association of PGCT017170 locus with fruit crown length. High  $R^2$  values indicated a more  
331 significant association of a trait with a marker. Therefore, in the current study, markers with higher  
332  $R^2$  values were used for the identification and sequencing of genes coding these traits (. Also, due  
333 to the long juvenile period of pomegranate, promising genotypes could be selected in the early  
334 stages of growth which could accelerate pomegranate breeding programs. In the present study,  
335 some loci presented significant associations with more than one trait in the fruit. The association

336 of a marker with multiple specific traits could be due to the nature of the inheritance of quantitative,  
337 polygenic traits and pleiotropic effects (Lou et al., 2015). Pleiotropic occurs when a gene can  
338 simultaneously play roles in the expression of multiple phenotypic traits. Also, inter-related QTLs  
339 which control different traits can result in the generation of a common marker for multiple  
340 associated traits (Lal et al., 2018). These results were similar to previous studies on walnut (Arab  
341 et al., 2019) and pistachio (Mirzaei et al., 2006). They reported that the use of informative markers,  
342 especially markers with a particular chromosomal location, made it possible to primarily and  
343 effectively select genotypes with high-performance. Lower values of phenotypic variation of some  
344 locus related to the trait showed that a small part of the variation in the traits was generated through  
345 the identified location and environmental effects played more important roles than genetic effects  
346 in the variations of these traits. Loci SCoT3<sub>100</sub>, SCoT3<sub>550</sub>, SCoT3<sub>1400</sub>, SCoT14<sub>900</sub>, SCoT19<sub>1900</sub>,  
347 SCoT30<sub>750</sub>, SCoT31<sub>500</sub> and SCoT31<sub>600</sub> in SCoT markers and loci PGCT001<sub>120</sub>, PGCT017<sub>155</sub> and  
348 PGCT0020<sub>120</sub> in SSR marker were associated with more than one morphological trait. Traits such  
349 as peel and aril colors, fruit shape and fruit weight (250 to 300 g on average), and fruit juice volume  
350 and taste are considered the major objectives of pomegranate breeding. Therefore, the  
351 identification of common markers caused by pleiotropic effects or association of genomic regions  
352 involved in controlling these traits makes simultaneous breeding of these traits possible in breeding  
353 programs (Jun et al., 2008).

354 Resistance against pests and diseases is among the most important factors that should be  
355 considered in pomegranate breeding programs. The sensitivity of fruits to this pest (carob moth)  
356 is different among different pomegranate cultivars. Fruits with black and thick peel, which contain  
357 higher contents of polyphenol compounds, are less attacked by the pest due to disturb the  
358 movement of larvae inside the fruit. Also, in cultivars with closed crown, pests cannot egg inside  
359 the crown and therefore, these genotypes are more resistant to this pest. Hence, a closed crown can  
360 be considered as an improved trait in pomegranate (Zolfaghari et al., 2018). In this study,  
361 positive correlations were observed between fruit crown length, peel thickness and fruit crown  
362 diameter with phenol peel. Also, locus PGCT0019<sub>240</sub> was common between peel thickness and  
363 peel phenol traits. Therefore, this marker could be used as an informative marker in selecting  
364 pomegranate seedlings resistant to carob moth in the early stages of pomegranate breeding which  
365 requires reassessing this marker in a pomegranate population. The phenolic compounds,  
366 anthocyanin content, secondary metabolites and defensive metabolites existing in pomegranate

367 fruits act as nutrition inhibitors, preventers and anti-digestion compounds and decrease the  
368 population growth index by elongating immature stages of the pest. Abedi et al., (2019) reported  
369 that the biochemical compounds present in pomegranate, including total phenol content,  
370 flavonoids, anthocyanin and acidity presented a negative and significant correlation with  
371 parameters related to the life table of carob tree moth in pomegranate. Similar results were reported  
372 by Zarei et al. (2013), who stated that the amounts of photochemical compounds and secondary  
373 metabolites in pomegranate were negatively correlated with the growth period of the pest and were  
374 also important factors in the identification of resistant and sensitive cultivars to this pest.

375 Seed hardness and resistance to scorching and cracking are among other breeding traits in  
376 pomegranate. It has been reported that the crossing of hard-seeded and soft-seeded cultivars results  
377 in decreased seed hardness. In addition, resistance to scorching and cracking in pomegranate is  
378 strongly affected by environmental effects and fruit peel flexibility. It has been reported that early-  
379 maturing cultivars and those with thicker peel are more resistant to this problem than late-maturing  
380 cultivars and those with thinner peel (Hoseinbeigi et al., 2019). Therefore, loci SCoT30<sub>850</sub> and  
381 PGCT017<sub>170</sub> associated with seed hardness and loci SCoT7<sub>1000</sub> and PGCT002<sub>115</sub> associated with  
382 peel thickness could be informative marker for pomegranate breeding in line with the above goals.  
383 Soluble solids, titratable acidity and the ratio of these two traits, known as maturity indices, are  
384 among the important qualitative traits of pomegranate fruits that contribute to acceptance by  
385 consumers through generating a favorable taste in fruit and determining the harvesting time and  
386 maturity indices of the fruit. Loci SCoT3<sub>850</sub> and PGCT001<sub>100</sub> in soluble solids, locus SCoT32<sub>800</sub>  
387 in titratable acidity and locus SCoT3<sub>1000</sub> in fruit maturity indices were identified as informative  
388 markers. Vitamin C, phenolic compounds and anthocyanin are among the antioxidant compounds,  
389 that are favorable for human health and play an important role in preventing and treating various  
390 diseases such as cancer; therefore, they are important from a medicinal point of view. Furthermore,  
391 measuring these traits is difficult and costly. Locus SCoT32<sub>1200</sub> was associated with vitamin C,  
392 titratable acidity and peel and aril TSS, PGCT001<sub>100</sub> was highly associated with fruit juice pH,  
393 titratable acidity, aril TSS, peel TSS and aril total phenolic content, PGCT017<sub>140</sub> was associated  
394 with titratable acidity, maturity indices, aril and peel TSS and aril and peel anthocyanin, and  
395 PGCT001<sub>120</sub> and PGCT017<sub>155</sub> were highly associated with fruit weight, aril weight, seed hardness,  
396 fruit length, fruit juice pH and peel anthocyanin. Therefore, these loci could be used as informative  
397 markers in breeding programs to produce cultivars with maximum medicinal characteristics. Since

398 the ultimate goal of most breeding programs is to increase trait characteristics affecting fruit  
399 performance, these genes or genetic regions related to these traits might be affected by  
400 environmental conditions in expressing their maximum potential. On the other hand, achieving an  
401 acceptable performance is not a result of the expression of genes independently and in fact, is result  
402 of the function of the related genomic regions. Therefore, the identification of such regions  
403 associated with important traits related to function and determining their association stability in  
404 different environments is of special importance (Forcada et al., 2019).

### 405 **Conclusions**

406 Pomegranate is widely recognized as a valuable fruit crop due to its strong antioxidant activity  
407 and high levels of bioactive, anti-carcinogenic compounds. Improving fruit yield and enhancing  
408 the concentration of biochemical constituents are key objectives in pomegranate breeding  
409 programs; however, the long juvenile phase and the high costs associated with evaluation make  
410 conventional breeding particularly challenging. Consequently, the identification of informative  
411 molecular markers linked to these traits is crucial to facilitate breeding efficiency. In the present  
412 study, loci such as SCoT12<sub>500</sub> and PGCT017<sub>170</sub> explained considerable proportions of phenotypic  
413 variation, suggesting their utility as primary indicators for indirect selection through marker-trait  
414 associations. Moreover, SCoT35<sub>900</sub> exhibited significant associations with multiple traits including  
415 fruit weight, peel weight, aril weight, fruit length, seed hardness, vitamin C content, and peel TSS.  
416 Similarly, SCoT32<sub>1200</sub> was linked with vitamin C, titratable acidity, and peel and aril TSS;  
417 PGCT001<sub>100</sub> was associated with fruit juice pH, titratable acidity, aril and peel TSS, and aril  
418 phenolic content; PGCT017<sub>140</sub> was correlated with titratable acidity, taste index, peel and aril TSS,  
419 as well as peel and aril anthocyanins; while PGCT001<sub>120</sub> and PGCT017<sub>155</sub> were related to fruit  
420 weight, aril weight, seed hardness, fruit length, fruit juice pH, and peel anthocyanin. In addition,  
421 several loci—SCoT11<sub>800</sub>, SCoT21<sub>100</sub>, SCoT5<sub>900</sub>, SCoT23<sub>900</sub>, PGCT001<sub>105</sub>, and PGCT020<sub>200</sub>—were  
422 found to be trait-specific, being associated exclusively with fruit diameter, crown length, crown  
423 diameter, titratable acidity, seed hardness, and fruit juice pH, respectively. These loci, therefore,  
424 hold promise as informative markers for the preliminary selection of hybrid cultivars and the  
425 construction of genetic maps in pomegranate.

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579 **Table 1.** List and code of pomegranate genotypes used in this study (Sadeghi Seresht *et al.*, 2023).

NO.	Cultivar	Code	NO.	Cultivar	Code
1	Malas Mumtaz Saveh	AIMS	13	Alak	AIAL
2	Tabrizi	AITA	14	Ghojogh Qom	AIGH
3	Yousef Khani	AIYO	15	Shirin-e-Shahvar	AISS
4	Taft Tabas soski	AITT	16	Zhagh Aghda	AIZH
5	Bajestani	AIBA	17	Poost Siah	AIPO
6	Shirin Ghermeze Zabol	AIPA	18	Bihaste Ravar	AIBR
7	Bihaste-e-Khafr Jahrom	AIBK	19	Shishe Kabe	AISK
8	Voshik Malas Saravan	AIVO	20	Anare Shekari	AIAS
9	Bargmordi	AIBG	21	Kolbad	AIKO
10	Oude Pooste Ghermez	AIOU	22	Gol beh Behshahr	AIGO
11	Ashkezar	AIAS	23	Sefid Zodras Shirin	AISF
12	Shirin Zodras	AISH			

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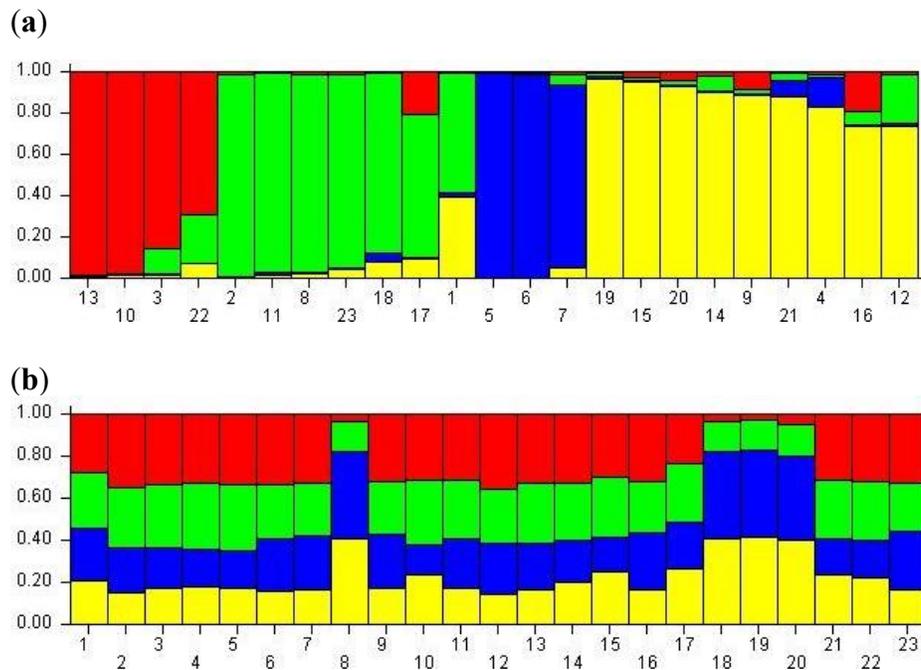
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**Fig.1.** Bayesian Model-Based Cluster Analysis of the 23 Studied Iranian Pomegranate Genotypes Based on 15 SCoT Markers (a) and 6 Pairs of SSR Primers (b). Red color indicates Structure I, green color indicates Structure II, blue color indicates Structure III, and yellow color indicates Structure IV.  $\Delta K$  computation results were tested at  $K = 1-10$  and supported  $K = 4$ . The vertical axis shows the coefficient of membership (or 'probability of assignment') of each individual to each cluster.

615 **Table 2.** Identified informative markers for morphological and biochemical traits in the studied  
616 pomegranate genotypes using SCoT and SSR markers based on mixed linear model (MLM).

No.	Trait	Unit	SCoT markers			SSR markers		
			Marker	P-value	R <sup>2</sup>	Marker	P-value	R <sup>2</sup>
1	Fruit Total Weight (FrW)	g	SCoT21 <sub>400</sub>	0.01	0.29	PGCT017 <sub>155</sub>	0.01	0.36
2	Peel Total Weight (PeW)	g	SCoT3 <sub>1400</sub>	0.02	0.29	-	-	-
3	Aril Total Weight (ArW)	g	SCoT22 <sub>700</sub>	0.02	0.29	PGCT017 <sub>155</sub>	0.02	0.27
4	Seed Total Weight (SeW)	g	SCoT30 <sub>1700</sub>	0.01	0.25	PGCT017 <sub>155</sub>	0.04	0.18
5	Fruit Length (FrL)	mm	SCoT12 <sub>750</sub>	0.02	0.20	PGCT005 <sub>100</sub>	0.05	0.16
6	Fruit Diameter (FrD)	mm	SCoT7 <sub>1300</sub>	0.03	0.23	PGCT001 <sub>90</sub>	0.02	0.25
7	Fruit Crown Length (FrCL)	mm	SCoT7 <sub>400</sub> SCoT7 <sub>500</sub>	0.01	0.43	PGCT017 <sub>170</sub>	0.01	0.45
8	Fruit Crown Diameter (FrCD)	mm	SCoT12 <sub>1400</sub>	0.03	0.17	PGCT017 <sub>190</sub>	0.05	0.24
9	Aril Length (ArL)	mm	SCoT5 <sub>600</sub>	0.01	0.41	-	-	-
10	Aril Diameter (ArD)	mm	SCoT19 <sub>550</sub>	0.05	0.25	PGCT017 <sub>140</sub>	0.06	0.16
11	Seed Length (SeL)	mm	SCoT13 <sub>1600</sub>	0.07	0.13	PGCT020 <sub>100</sub>	0.07	0.22
12	Seed Diameter (SeD)	mm	SCoT3 <sub>200</sub>	0.05	0.15	PGCT020 <sub>100</sub>	0.07	0.22
13	Peel Thickness (PeT)	mm	SCoT7 <sub>1000</sub>	0.04	0.23	PGCT002 <sub>115</sub>	0.03	0.22
14	Peel Firmness (PeF)	N	SCoT12 <sub>600</sub>	0.02	0.32	PGCT020 <sub>120</sub>	0.03	0.30
15	Aril Firmness (ArF)	N	SCoT12 <sub>1000</sub>	0.02	0.21	PGCT017 <sub>155</sub>	0.02	0.30
16	Seed Hardness (SeH)	N	SCoT30 <sub>850</sub>	0.01	0.28	PGCT017 <sub>170</sub>	0.02	0.27
17	pH of Juice (pHJ)	-	SCoT5 <sub>1200</sub>	0.01	0.39	PGCT017 <sub>155</sub>	0.01	0.21
18	Vitamin C (VC)	mg <sup>-1</sup> gfw	SCoT35 <sub>500</sub>	0.02	0.15	-	-	-
19	Titrateable Acidity (TA)	percent	SCoT3 <sub>850</sub>	0.008	0.44	PGCT005 <sub>100</sub>	0.03	0.27
20	ArTSS/TA (MI)	percent	SCoT3 <sub>1000</sub>	0.01	0.33	PGCT019 <sub>254</sub>	0.04	0.24
21	Peel Total Soluble Solid (PeTSS)	mg <sup>-1</sup> gfw	SCoT12 <sub>500</sub>	0.008	0.50	PGCT002 <sub>115</sub> PGCT017 <sub>140</sub>	0.03	0.31
22	Aril Total Soluble Solid (ArTSS)	Brix	SCoT32 <sub>800</sub>	0.01	0.36	PGCT001 <sub>100</sub>	0.01	0.40
23	Peel Phenol (PeP)	mg <sup>-1</sup> gfw	SCoT32 <sub>1000</sub>	0.05	0.20	PGCT019 <sub>240</sub>	0.09	0.17
24	Aril Phenol (ArP)	mg <sup>-1</sup> gfw	SCoT32 <sub>1400</sub>	0.01	0.41	PGCT020 <sub>100</sub>	0.07	0.15
25	Peel Anthocyanin (PeA)	mg <sup>-1</sup> gfw	SCoT32 <sub>1100</sub>	0.03	0.23	PGCT017 <sub>190</sub>	0.04	0.25
26	Aril Anthocyanin (ArA)	mg <sup>-1</sup> gfw	SCoT32 <sub>1400</sub>	0.004	0.20	PGCT017 <sub>140</sub>	0.03	0.25

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630 ارزیابی مولکولی و شناسایی نشانگرهای مرتبط با صفات مورفولوژیکی و بیوشیمیایی میوه انار با  
631 استفاده از نشانگرهای SCoT و SSR

632  
633 الهام صادقی سرشت، حمید رضا کریمی، خلیل ملک زاده، علی اکبر محمدی میریک، سید حسین میردهقان، و محمد صادق  
634 جمعه یزدیان

#### چکیده

637 این مطالعه به منظور شناسایی نشانگرهای مرتبط با برخی صفات مورفولوژیکی و بیوشیمیایی در 23 ژنوتیپ انار با استفاده  
638 از 15 آغازگر SCoT و 6 جفت آغازگر SSR انجام شد. براساس نتایج حاصل از تجزیه ارتباطی به روش MLM، در  
639 مجموع 169 مکان ژنی مرتبط با صفات مورد مطالعه شناسایی شد که در این بین 149 مکان ژنی مبتنی بر نشانگر SCoT  
640 و 20 مکان ژنی مبتنی بر نشانگر SSR بود. نتایج نشان داد که مکان ژنی SCoT35900 با صفات وزن کل میوه و پوست و  
641 آریل، طول میوه، سختی دانه، ویتامین ث و مواد جامد محلول پوست، مکان ژنی SCoT321200 با صفات ویتامین ث، اسیدیت  
642 قابل تیتراسیون، مواد جامد محلول پوست و آریل، مکان ژنی PGCT001100 با صفات pH، اسیدیت قابل تیتراسیون، مواد  
643 جامد محلول آریل و پوست و محتوی فنل کل آریل ارتباط معنی داری نشان دادند. علاوه بر این مکان های ژنی SCoT1800،  
644 SCoT21100 و SCoT5900، SCoT23900، PGCT001105 و PGCT0020200 به ترتیب فقط با صفات قطر میوه، طول  
645 تاج میوه، قطر تاج میوه، اسیدیت قابل تیتراسیون، سختی دانه و pH آب میوه همبستگی معنی داری نشان دادند که می توان از  
646 این مکان های ژنی تحت عنوان نشانگر های آگاهی بخش در برنامه های اصلاحی انار استفاده کرد.

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