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Research Article

Genetic and chemical diversity analyses in tale grapes (*Vitis vinifera* L.)

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Abstract

Vitis vinifera L. is one of the economically important plant crops worldwide which is a valuable food source for humans. This precious plant species has several local varieties and accessions which are continuously under selection and cultivation. Due to these human activities, the grape faces genetic homogeneity and erosion. Therefore, it is important to investigate available genetic diversity in grape plants all over the world. We aimed to study the genetic structure and diversity as well as chemical differences of seven grape cultivars in the country. We used SSR, and SRAP molecular markers for genetic diversity analyses, as well as biochemical traits. Both molecular markers showed a medium to moderate genetic variability in the studied grape cultivars (about 20% genetic polymorphism). Similarly, both molecular markers differentiated the studied cultivars into two genetic groups. AMOVA indicated significant genetic difference in these cultivars. ANOVA analysis of flavonols (quercetin, myricetin, kaempferol, and rutin) contents of seeds extract by HPLC indicated the significant difference ($P < 0.01$) among grape cultivars. PCA biplot of cultivars based on chemical features separated these cultivars into two major groups according to their flavone and flavonoid contents. Pairwise Mantel tests performed between molecular and chemical data showed a significant association between SSR and SRAP data, but no significant association was obtained between either SSR or SRAP data with chemical features in grape cultivars studied. A heat-map constructed based on combined molecular and chemical data revealed that some of the studied grape cultivars are distinct in their genetic and chemical features.

Keywords: flavonol; HPLC; SRAP; SSR; *Vitis vinifera*

Introduction

The grape is one of the earliest domesticated fruit crops which was widely cultivated in different regions through the world which is prized for its fruit and nutritional values. A Near East origin for the grape was suggested that thousands of grape cultivars in use today were generated by vegetative propagation and through crossing elite and desirable local cultivars (Mylets *et al.*, 2011). However, based on archaeological evidence, the mountainous areas between the Caspian and the Black Sea were proposed as the most likely centre of grape domestication by (Ebadi *et al.*, 2019).

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(Mylets *et al.,* 2011) presented evidence for introgression among local *sylvestris* grapes within Europe and reported a high level of genetic diversity for these cultivars, which is associated with a weak domestication bottleneck followed by thousands of years of widespread vegetative propagation.

Grape cultivars are cultivated in many parts of Iran and are considered an economically important crop in the country. The highest percentage of area under grape cultivation in the country occurs in Takestan (8.2%), Mamasani (5.9%), Shiraz (3.9%), and Urmeya (3.6%) (Figure 1).

Grape is one of the most important plants which products fruit contains a large amount of soluble flavonoids. These compounds are the most important natural antioxidants in the fruit. In addition to phenolic acids (mainly benzoic and hydroxynamic acids), there are flavonoids in the fruit and seed of grapes include tannins, anthocyanins, and flavons (Braidot *et al.*, 2008).

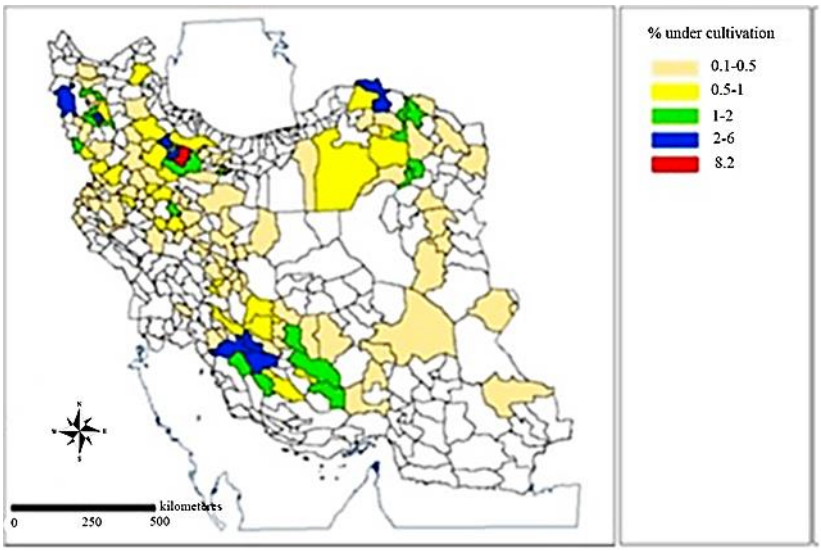

Figure 1. Iran map showing the area under cultivation of grape plants

Flavonols effectively reduce the harmful effects of oxidative stress due to ultraviolet light and intense light (Haselgrove *et al.*, 2000). In white grapes, flavonols are the yellow pigments which are directly involved. In red grapes, anthocyanins are more abundant in the exocarp than in other flavonoids.

The biosynthesis of phenolic compounds in V. vinifera is genetically controlled, and the differences in grape cultivars are sometimes significant enough to make it possible to use the phenolic composition of grapes as a tool for cultivar originality and differentiation.

According to (Mylets *et al.*, 2011), although a substantial genetic diversity is present in the grape cultivars subsequent to domestication, there was limited exploration of this diversity. Therefore, it is necessary to explore the genetic diversity of grape cultivars in different geographical areas and produce data on these important plant species for future conservation and breeding. Moreover, grape plants contain important chemical constituents like flavones, and flavonoids and they are also investigated for. varietal. Chemical difference in many countries (see for example, (Rolle *et al.*, 2013; Oprica *et al.*, 2016)).

Molecular markers were extensively used in genetic fingerprinting and genetic diversity studies in different plant cultivars and cultivars, including grapes. These molecular markers include ISSR, SSR, SNP, etc. markers (see for example, (Di Gaspero *et al.*, 2010; Mylets *et al.*, 2011; Gismondi *et al.*, 2014; Koohdar *et al.*, 2015; Khadivi *et al.*, 2017; Ebadi *et al.*, 2019; Tabasi *et al.*, 2020)).

Therefore, this study aimed to investigate both genetic as well as chemical diversity in seven grape cultivars of Iran and to illustrate if the molecular and chemical features of these cultivars are associated with each other.

We used simple sequence repeats (SSRs) as well as simple repeat amplified polymorphic (SRAP) molecular markers for cultivar genetic study, as these markers are very efficient in genetic finger printing of many crop plants, including grape (Riaz *et al.*, 2018; Gholami *et al.*, 2020; Ibrahim *et al.*, 2020; Partovi *et al.*, 2020).

Materials and Methods

Plant materials

We studied 21 plants of seven grape cultivars of Iran (Table 1). All samples were collected during 2019–2020 from the 10-year-old trees of a vineyard garden located in the city of Brojerd province Lorestan.

Table 1. Characteristics of seeds size and fruit colour of studied grape cultivars

No.	The name of cultivars	Seeds size	Fruit colour
1	'Keshmeshi'	Small	Light Green
2	'Monnagha'	Large	Light Green
3	'Sahebi'	Large	Red
4	'Yaghooti'	Small	Red
5	'Asgari'	Small	Light Green
6	'Fakhri'	Large	Light Green
7	'Black'	Large	Red

Molecular studies

DNA extraction and PCR procedure

Fresh leaves were put to dry in silica gel powder. Cetyltrimethyl-ammonium bromide -activated charcoal protocol (CTAB) was applied to extract the genomic DNA. The extraction was done by activating char-coal and polyvinyl pyrrolidone (PVP) to bind polyphenols during extraction (Krisman *et al.*, 2006). The quality and quantity of the. extracted DNA was checked by running on 0.8% agarose as well as Nanodrop spectrophotometer. The PCR reactions for both molecular markers were carried out in a 25 µl volume mixture containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 1 U of Taq DNA polymerase (Bioron, Germany). We used a T100 thermal cycler (BioRad, USA), for PCR amplification. Five SRAP primer pairs including forward primers: Me1, Me2, Me3, Me4, Me5 and reverse primers: Em1, Em2, Em3, Em4, Em5 were used (Table 2) (Feng *et al.*, 2014).

Table 2. Primer pairs in SRAP marker

Primer code	Type	Sequence 5′-3′
Me1	Forward	TGA GTC CAA ACC GGA TA
Me2	Forward	TGA GTC CAA ACC GGA GC
Me3	Forward	TGAGTCCAAACCGGAAT
Me4	Forward	TGAGTCCAAACCGGACC
Me5	Forward	TGAGTCCAAACCGGAAG
Em1	Reverse	GAC TGC GTA CGA ATT AAT
Em2	Reverse	GAC TGC GTA CGA ATT TGC
Em3	Reverse	GAC TGC GTA CGA ATT GAC
Em4	Reverse	GAC TGC GTA CGA ATT TGA
Em5	Reverse	GAC TGC GTA CGA ATT AAC

The PCR reaction used for SRAP marker was: the initial denaturation at 94 °C for 5 min, 36 cycles of denaturation at 94 °C for 1 min, annealing at 54-56 °C for 1 min, and the extension at 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min. Similarly, four pairs of SSR primers including forward (VVMD5, VVMD7, VVMD36 and VrZAG64) and revers primers (VVMD5, VVMD7, VVMD36, VrZAG64) were amplified by following PCR reaction: the initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 52-55 °C for 1min, and the extension at 72 °C for 1.30 min. The final extension was carried out at 72 °C for 10 min. The result of amplification was run on 2% agarose gel, followed by GelRed™ Nucleic Acid Gel Staining. A 100-base pair (bp) molecular size ladder (Fermentas, Germany) was used to determine the fragments' size.

Biochemical studies

We used the cultivars' seeds to measure biochemical traits such as total phenol, total anthocyanins and total flavonoids andflavonols (quercetin, myricetin, rutin, kaempferol*1000).

Total phenol assay

The Folin-Ciocalteu method was used to measure the amount of total phenol. In Folin-Ciocalteu solution acts as the reagent and gallic acid works as the standard, to draw the curve. 0.5 g of seed powder was mixed with 20 ml of 80% methanol and placed on a shaker for 24 hours and were centrifuged at 3000 rpm for 15 minutes, and then the upper solution was separated and reached a volume of 5 ml with 80% metanol. 0.2 ml of extract was mixed with 1.8 ml of distilled water and 0.2 ml of dilute Folin-Ciocalteu reagent and after 5 minutes, 3 ml of 7% sodium carbonate was added to the mixture. After 90 minutes the adsorption was read at 750 nm. The phenolic compounds were measured using high performance liquid chromatography (Ordoez *et al.*, 2006).

Total flavonoids assay

Flavonoids were measured according to Anderson *et al.* (2006) method. 0.5 ml of metanolic extract was mixed with 1.5 ml of 80% methanol, 0.1 ml of 1% aluminum chloride, 0.1 mm of potassium acetate and 2.8 mm of distilled water were added. After one hour the absorption was read at 415 nm by spectrophotometer. Total flavonoid content was finally calculated by comparing with the standard quercetin curve.

Total anthocyanins assay

Anthocyanins content was determined according to (Nogues *et al.*, 2000). Seed (1 g) were homogenized with HCl methanol (99:1 v: v) and kept for 24 h in dark at 4 °C. Solutions were then centrifuged at 13000×g for 15 minutes. The content of anthocyanins was determined from the absorbance at 530 nm. The content of anthocyanins was measured using the extinction coefficient of anthocyanins (ε= 33000 cm2 M-1) according to the formula (A = εbc).

HPLC analysis

0.5 g of seed were hydrolysed with 5 mL of methanol 80%, and shaken for 24h, then centrifuged at 13500×g for 15 min, and the supernatants were topped up to 5mL by methanol 80%. The extracts were filtered via 0.45 ×m nylon filters and stored at 4 °C for further analysis.

Quercetin, Kaempferol, Myricetin, and Rutin standards were provided by Sigma (USA), and prepared in 80% methanol. For each individual compounds, the calibration curves were drawn using different concentrations of standards.

The analysis of Quercetin, Kaempferol, Myricetin, and Rutin was done using Agilent 1260 infinity ii HPLC system (Agilent, USA), covering Binary Pump, Vial sampler with integrated column compartment, 6.0 µL heater and sample cooler, Diode Array Detector WR with a standard 10-mm flow cell, 1100 Autosampler, 1100 Thermostatted Column Compartment, 1100 Diode Array Detector with a standard 10-mm flow cell,

and Agilent OpenLAB CDS Version 2.1. The separation of compounds was carried out using Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 μm. The used mobile phase was methanol: 200, acetonitrile: 100, acetic acid: 10, phosphoric acid: 10, and 200 mL of water. The injection volume was 20 μL by a flow rate of 0.6 ml/min. The integrated peak areas of the sample against the standard curves were used for quantification of compounds, in terms of mg/ ml.

Data analyses

For molecular studies, SSR and SRAP bands obtained were treated as binary characters (Podani, 2000) and used for further analyses. DCA (Dentrented correspondence analysis) was used to evaluate the suitability of molecular bands obtained. for cultivar genetic study. Discriminant power of the bands obtained was determined by Gst analysis as performed in POPGENE program. The number of private bands versus common bands and genetic diversity parameters were determined by GeneAlex 4.2 (Peakall *et al.*, 2006). Grouping of the genotypes was done by WARD clustering and PCoA (Principal coordinate analysis), as well as PCA (Principal components analysis) ordination methods as performed by PAST (Hammer *et al.*, 2001). AMOVA was performed in Gene Alex to determine any significant genetic difference among the studied cultivars. For chemical studies, ANOVA was performed in PAST program to determine any significant difference for all chemical contents studied among grape cultivars. Grouping of the genotypes was done by WARD clustering and PCA (Principal components analysis), as well as PCA biplot ordination methods as performed by PAST (Hammer *et al.*, 2001).

For combined molecular and chemical studies, Pair-wise Mantel tests performed after 10000 permutations between molecular markers and chemical data in PAST program as well as, a heat-map was constructed based on combined molecular and chemical data by R package.

Results

SRAP analyses

We obtained in total 125 SRAP bands in grape trees studied. The number of bands varied from 54 in cultivar 2 ('Monnagha'), to 84, in cultivar 1 ('Keshmeshi'). Private bands (2-6) were observed in cultivars 1, 2, 4, ('Keshmeshi',' Monnagha', 'Yaghooti') and 6 ('Fakhri'), which may be considered as local and cultivar specific bands.

The suitability of SRAP bands for grape cultivar genetic study was determined by DCA plot (Figure 2). The plot shows a well-scattered distribution of SSR loci, which indicated these loci are from different regions of the genome and are not clustered to each other. Such loci are useful in. cultivar genetic studies.

Discriminating power analysis (Gst) of SRAP data revealed that out of 125 loci, about 30% have a high discrimination power (Gst>0.90), and can efficiently differentiate grape plants cultivars from each other and are of genetic fingerprinting potential (Table 3).

PCoA plot (Figure 3) of the studied grape plants cultivars based on SRAP data, separated the replicates of these cultivars from each other, which indicates their genetic differentiation. This is well documented by significant AMOVA (Phi'PT = 0.80, P = 0.01), obtained too. AMOVA based on SRAP also revealed that 68% of total genetic variability occurs due to among cultivar genetic difference, while 32% occurs due to within cultivar genetic variability.

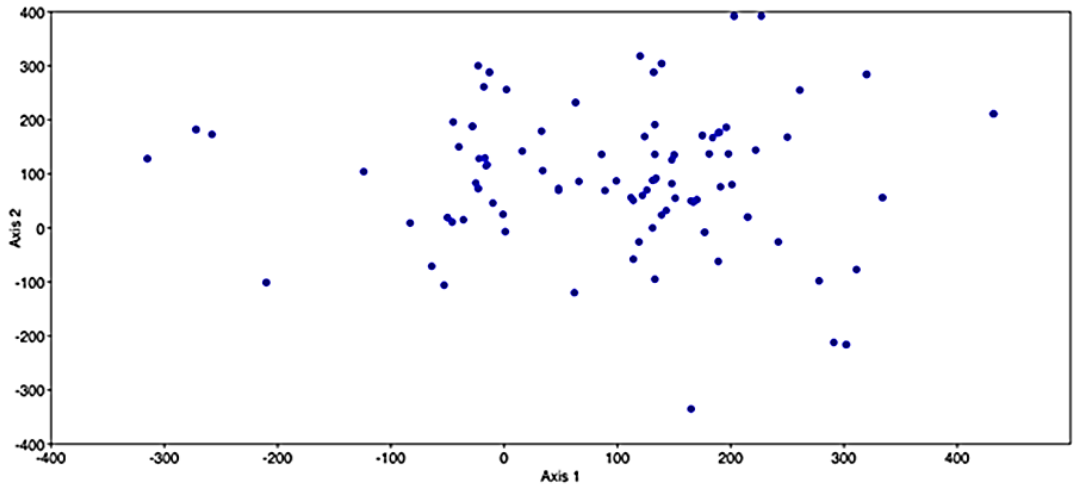

Figure 2. DCA plot of SRAP bands/loci in grape cultivars showing well-scattered distribution of loci obtained

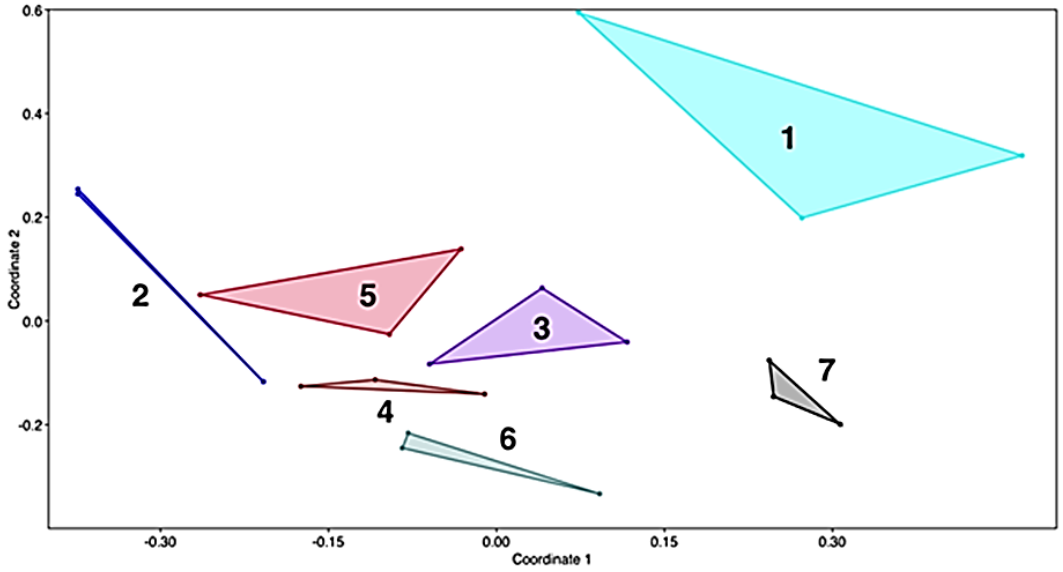

Figure 3. PCoA plot of grape cultivars based on SRAP data showing the cultivars' genetic differentiation (The numbers of 1-7 are in Table 1)

Table 3. Discrimination power (Gst value) of SRAP loci versus migration index (Nm), in grape plants cultivars studied (The numbers of 1-7 are in Table 1)

Locus	Sample	Ht	Hs	Gst	Nm*
Locus2	21	0.4809	0.0428	0.9110	0.0489
Locus5	21	0.4959	0.0428	0.9137	0.0472
Locus6	21	0.4898	0.0000	1.0000	0.0000
Locus7	21	0.2449	0.0000	1.0000	0.0000
Locus8	21	0.4898	0.0000	1.0000	0.0000
Locus9	21	0.4898	0.0000	1.0000	0.0000
Locus27	21	0.2449	0.0000	1.0000	0.0000
Locus30	21	0.4082	0.0000	1.0000	0.0000
Locus31	21	0.4898	0.0000	1.0000	0.0000

Locus32	21	0.2449	0.0000	1.0000	0.0000
Locus33	21	0.4082	0.0000	1.0000	0.0000
Locus34	21	0.4082	0.0000	1.0000	0.0000
Locus35	21	0.4898	0.0000	1.0000	0.0000
Locus36	21	0.2449	0.0000	1.0000	0.0000
Locus37	21	0.4809	0.0000	1.0000	0.0000
Locus42	21	0.4082	0.0000	1.0000	0.0000
Locus45	21	0.4809	0.0000	1.0000	0.0000
Locus50	21	0.4293	0.0000	1.0000	0.0000
Locus52	21	0.2449	0.0000	1.0000	0.0000
Locus53	21	0.4082	0.0000	1.0000	0.0000
Locus55	21	0.2449	0.0000	1.0000	0.0000
Locus60	21	0.4959	0.0428	0.9137	0.0472
Locus61	21	0.4082	0.0000	1.0000	0.0000
Locus62	21	0.4898	0.0000	1.0000	0.0000
Locus63	21	0.2449	0.0000	1.0000	0.0000
Locus64	21	0.4809	0.0428	0.9110	0.0489
Locus65	21	0.4082	0.0000	1.0000	0.0000
Locus69	21	0.2449	0.0000	1.0000	0.0000
Locus70	21	0.2449	0.0000	1.0000	0.0000
Locus71	21	0.7082	0.0000	1.0000	0.0000
Locus73	21	0.2449	0.0000	1.0000	0.0000
Locus88	21	0.2449	0.0000	1.0000	0.0000
Locus89	21	0.4082	0.0000	1.0000	0.0000
Locus100	21	0.2449	0.0000	1.0000	0.0000
Locus101	21	0.4809	0.0428	0.9110	0.0489
Locus112	21	0.4082	0.0000	1.0000	0.0000
Locus113	21	0.4293	0.0428	0.9003	0.0554
Mean	21	0.3962	0.0921	0.7675	0.1514

SRAP analyses

Genetic diversity of grape plants based on SRAP data

Data with regard to genetic diversity parameters determined in grape plants are presented in Table 4.

The range of polymorphism percentage varied from 1.60 in cultivar No. 4 ('Yaghooti'), to 43.2 in cultivar No. 5 ('Asgari'). The mean value for polymorphism percentage obtained was 22.40%, while the mean value of Nei' gene diversity for SRAP data was about 0.1. Both these values indicate a low degree of genetic diversity in grape plants cultivars investigated, but yet appreciable for further breeding studies if accompanied by some degree of morphological and agronomical desirable traits variation.

Table 4. Genetic diversity parameters in grape cultivars (The numbers of 1-7 are in Table 1)

Cultivars	Na	Ne	I	He	uHe	P%
'Keshmeshi'	0.968	1.240	0.185	0.129	0.155	29.600
'Monnagha'	0.520	1.059	0.050	0.034	0.041	8.800
'Sahebi'	0.800	1.175	0.140	0.097	0.116	23.200
'Yaghooti'	0.488	1.007	0.008	0.005	0.006	1.600
'Asgari'	1.008	1.303	0.252	0.172	0.206	43.200

'Fakhri'	0.728	1.223	0.166	0.117	0.141	25.600
'Black'	0.832	1.152	0.136	0.091	0.109	24.800

Nei genetic distance determined in the grape cultivars varied from 0.342 between cultivars 4 ('Yaghooti') and 5 ('Asgari'), to 0.778 between cultivars 2 ('Monnagha') and 7 ('Black') (Table 5).

Table 5. Nei' genetic distance of grape cultivars studied based on SRAP data (The numbers of 1-7 are in Table 1)

'Keshmeshi'	'Monnagha'	'Sahebi'	'Yaghooti'	'Asgari'	'Fakhri'	'Black'	Cultivars
0.000							'Keshmeshi'
0.742	0.000						'Monnagha'
0.360	0.446	0.000					'Sahebi'
0.635	0.617	0.466	0.000				'Yaghooti'
0.491	0.404	0.408	0.342	0.000			'Asgari'
0.531	0.658	0.423	0.498	0.410	0.000		'Fakhri'
0.455	0.778	0.510	0.510	0.429	0.374	0.000	'Black'

Grouping of the cultivars based on SRAP data

Ward clustering (Figure 4), based on SRAP markers placed grape cultivars in two major clusters. Cultivars 1-3 ('Keshmeshi', 'Monnagha', 'Sahebi'), showed a higher level of genetic similarity and comprised the first major cluster, while cultivars 4-7 ('Yaghooti', 'Asgari', 'Fakhri', 'Black') formed the second major cluster.

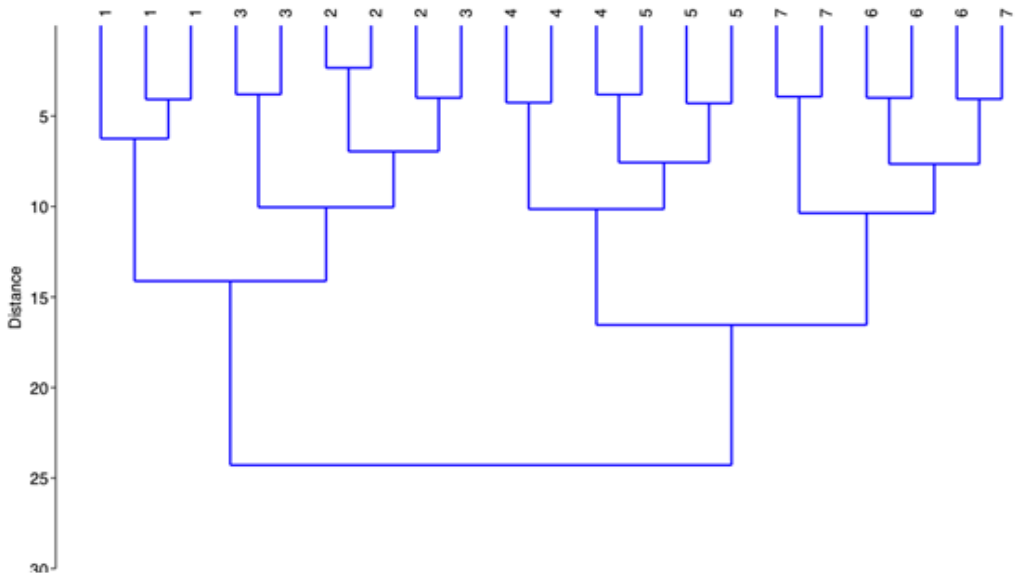

Figure 4. WARD dendrogram of grape cultivars based on SRAP data (The numbers of 1-7 are in Table 1)

SSR data analyses

We obtained 13 SSR bands in grape cultivars studied. Only 1 private SSR band was observed in cultivars 1 ('Keshmeshi') and 7 ('Black'). Most of SSR loci showed high level of discriminating power (Gst>0.70) in grape cultivars studied (Table 6).

The suitability of SSR bands for grape cultivar genetic studies was also determined by DCA plot (Figure not shown). The plot shows a well-scattered distribution of SSR loci, and these loci are useful in cultivar genetic study.

Table 6. Discriminating value (Gst) of SSR bands obtained in grape cultivars (The numbers of 1-7 are in Table1)

Locus	Sample	Ht	Hs	Gst	Nm*
Locus1	21	0.3239	0.0697	0.7847	0.1372
Locus2	21	0.3239	0.0697	0.7847	0.1372
Locus3	21	0.3239	0.0697	0.7847	0.1372
Locus4	21	0.2449	0.0000	1.0000	0.0000
Locus5	21	0.3239	0.0697	0.7847	0.1372
Locus6	21	0.3239	0.0697	0.7847	0.1372
Locus7	21	0.4526	0.0697	0.8460	0.0910
Locus8	21	0.4631	0.2520	0.4559	0.5968
Locus9	21	0.1135	0.0697	0.3856	0.7968
Locus10	21	0.2449	0.0000	1.0000	0.0000
Locus11	21	0.2123	0.1394	0.3434	0.9562
Locus12	21	0.3239	0.0697	0.7847	0.1372
Locus13	21	0.4653	0.0697	0.8501	0.0881
Mean	21	0.3184	0.0784	0.7539	0.1632

Genetic diversity of grape plants based on SSR data

Data with regard to genetic diversity parameters determined in grape plants are presented in Table 7.

Table 7. Genetic diversity parameters in grape cultivars (The numbers of 1-7 are in Table 1)

Cultivars	Na	Ne	I	He	uHe	%P
'Keshmeshi'	0.846	1.253	0.194	0.136	0.163	30.70%
'Monnagha'	0.385	1.000	0.000	0.000	0.000	0.00%
'Sahebi'	0.615	1.220	0.157	0.113	0.135	23.70%
'Yaghooti'	0.385	1.000	0.000	0.000	0.000	0.00%
'Asgari'	0.615	1.220	0.157	0.113	0.135	23.08%
'Fakhri'	0.769	1.293	0.210	0.150	0.180	30.77%
'Black'	0.385	1.073	0.052	0.038	0.045	7.69%

The mean value for polymorphism percentage obtained was 16.5%, with cultivars 1 ('Keshmeshi') and 6 ('Fakhri'), showing the highest percentage of genetic diversity (30.7%). The mean values obtained for polymorphism percentage = 16.5, He = 0.07, and Ne = 0.04, based on SSR data, are very much alike what was obtained by SRAP molecular markers. Therefore, these genetic diversity parameters, altogether indicate a low genetic diversity in grape cultivars studied.

Nei' genetic distance determined among grape cultivars based on SSR data (Table 8), showed the highest degree of genetic distance (0.80), between cultivars 1 ('Keshmeshi') and 7 ('Black'), followed by cultivars 1 ('Keshmeshi') and 4 ('Yaghooti') (0.71).

Table 8. Nei' genetic distance determined among the studied grape cultivars based on SSR data (The numbers of 1-7 are in Table 1).

'Keshmeshi'	'Monnagha'	'Sahebi'	'Yaghooti'	'Asgari'	'Fakhri'	'Black'	Cultivars
0.000							'Keshmeshi'
0.581	0.000						'Monnagha'
0.198	0.338	0.000					'Sahebi'
0.714	0.368	0.338	0.000				'Yaghooti'
0.426	0.161	0.214	0.161	0.000			'Asgari'
0.413	0.404	0.239	0.100	0.112	0.000		'Fakhri'
0.809	0.541	0.407	0.541	0.327	0.506	0.000	'Black'

WARD dendrogram of grape cultivars based on SSR data (Figure 5), separated grape cultivars into distinct major clusters. Cultivars 1-3 ('Keshmeshi', 'Monnagha', 'Sahebi') comprised the first cluster, while cultivars 4-7('Yaghooti', 'Asgari', 'Fakhri', 'Black') formed the second major cluster. The cultivars genetic affinity based on SSR data is very much similar to what we obtained by SRAP molecular markers. This will be presented in more detail by Mantel test result in the following sections.

AMOVA based on SSR data produced significant genetic difference among grape cultivars (PhiPT = 0.71, P = 0.001). It also revealed that 71% of total genetic variability occurs due to among cultivar genetic difference, while 29% occurs due to within cultivar genetic variability. These results indicate the presence of genetic difference within grape plant germ plasm, which can be used in future breeding program.

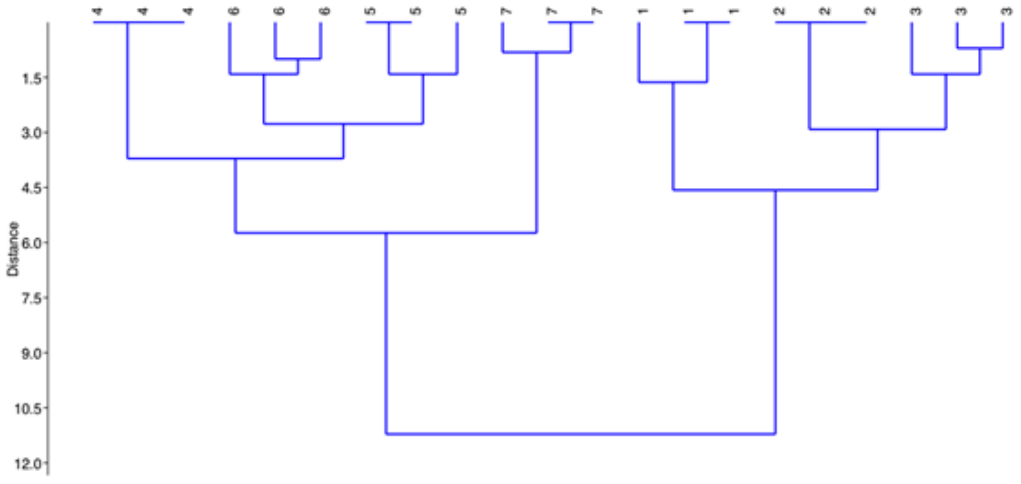

Figure 5. WARD dendrogram of the studied grape cultivars based on SSR data (The numbers of 1-7 are in Table 1)

Chemical analyses

Details of biochemical traits such as total phenol, total anthocyanins and total flavonoids and flavonols are given in Table 9. The highest total phenol was obtained in 'Sahebi' cultivar, while 'Asgari' cultivar had the lowest value for the same. Similarly, the highest value for total flavonoid occurred in 'Sahebi' cultivar, while 'Yaghoti' cultivars had lowest value for the same parameter. The lowest amount of total anthocyanin was observed in 'Monnagha' cultivar while the highest amount occurred in 'Keshmehi' cultivar. With regard to Quercetin and Myricetin content, 'Asgari' cultivar showed the lowest value for both, while the highest value for these chemicals was observed in 'Yaghoti' and 'Monnagha' cultivars, respectively. The highest value for rutin and kaempferol was observed in 'Asgari' and 'Monnagha' cultivars, while, 'Yaghoti' and 'Black' cultivars contained the lowest vale for the same parameters, respectively.

Table 9. Total phenol, flavonoids, anthocyanins and flavonols (quercetin, myricetin, rutin, kaempferol) contents of seven grape cultivars seeds. Data are means of three replicates with standard errors (Mean±SE)

Cultivar	Total phenol (mg galic acid/ gDW)	Total flavonoids (mg quercetin/ gDW)	Total anthocyanins (µM/ gDW)	Quercetin (mg/ml)	Myricetin (mg/ml)	Rutin (mg/ml)	Kaempferol (µg/ml)
'Keshmehi'	33.71±0.99	21.29±4.92	0.67±0.02	0.63±0.29	0.79±0.14	109.79±0.44	1.50±0.29
'Monnagh'	44.85±1.66	39.82±3.05	0.40±0.17	1.32±0.20	2.97±0.78	106.93±0.98	3.90±0.21
'Sahebi'	54.04±3.02	50.23±0.41	0.62±0.02	3.53±0.24	2.20±0.04	105.48±0.28	2.70±0.00
'Yaghoti'	33.34±0.61	20.78±3.89	0.44±0.05	4.93±0.23	1.83±0.04	104.46±0.28	0.79±0.11
'Asgari'	32.53±1.08	23.16±3.77	0.62±0.03	0.50±0.13	0.07±0.31	110.65±0.17	1.33±0.21
'Fakhri'	47.37±2.67	34.19±0.25	0.58±0.04	3.53±0.14	1.61±0.23	106.08±0.09	1.00±0.57
'Black'	45.83±0.49	45.27±2.05	0.51±0.07	1.94±0.06	0.83±0.15	108.45±0.34	0.60±0.14

ANOVA produced significant difference for all chemical contents studied among grape cultivars ($P<0.01$). Each cultivar showed a higher mean value for one or few chemicals compared to the others. Ward Dendrogram of the studied cultivars, based on chemical contents of seeds, separated cultivars 1, 4, 5 ('Keshmeshi', 'Yaghooti', 'Asgari'), from the cultivars 2, 3, 6, 7 ('Monnagha', 'Sahebi', 'Fakhri', 'Black') under the influence of seeds size (Figure 6). However, details of these major clusters differ from what we obtained for both molecular markers.

PCA analysis of chemical data revealed that the first two PCA axes comprise about 96% of total variance among the studied grape cultivars. In the first PCA axis with about 89% of total variation, flavonoid and total phenol content showed the highest correlation value (>0.90), while, in the second PCA axis with about 7% of total variation, Quercetin ($r = 0.53$), and Rutin ($r = -0.53989$), were positively and negatively correlated characters. Therefore, these are differentiating chemical characteristics in grape cultivars studied.

PCA biplot (Figure 7), revealed that chemical features separated the studied cultivars in two main groups and chemical characteristics like total phenol and flavonoid content differentiate grape cultivars 2, 3, 6, and 7, while the rest of chemical features separated cultivars 'Keshmeshi', 'Yaghooti' and 'Asgari'.

Association between molecular markers and chemical content

Pair-wise Mantel tests performed after 10000 permutations between SSR markers and chemical data, produced no significant association ($R = 0.10$, $P = 0.11$). Similarly, the same test produced no significant association between SRAP markers and chemical data ($R = 0.056$, $P = 0.26$). However, the Mantel test produced a significant association between SSR and SRAP molecular markers ($R = 0.25$, $P < 0.01$). These results indicate that, both SRAP and SSR molecular markers show genetic affinity of the studied grape cultivars in a similar fashion. However, the genetic distance of these cultivars is not correlated with their difference in chemical content. i.e., the grape cultivars relationship based on molecular data differs from the same obtained from chemical data.

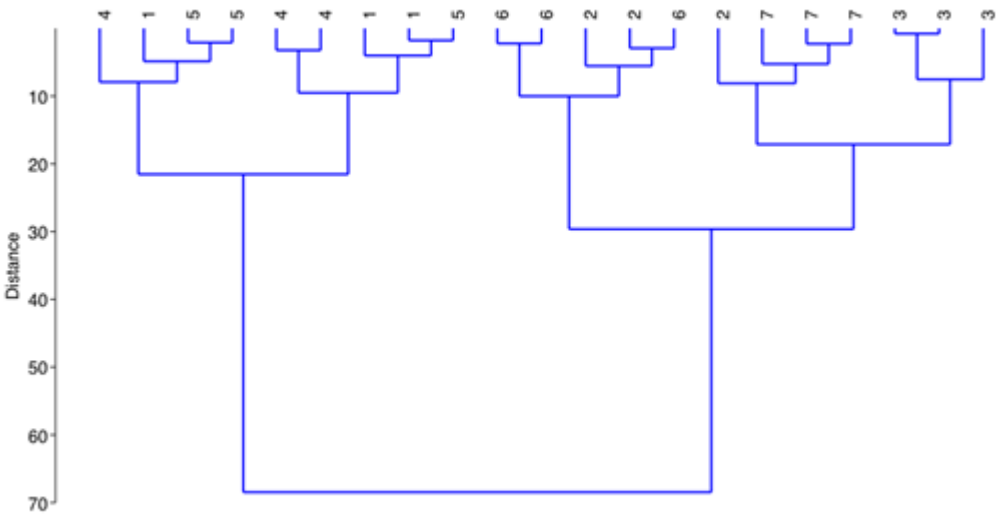

Figure 6. WARD dendrogram of grape cultivars based on chemical data (The numbers of 1-7 are in Table 1

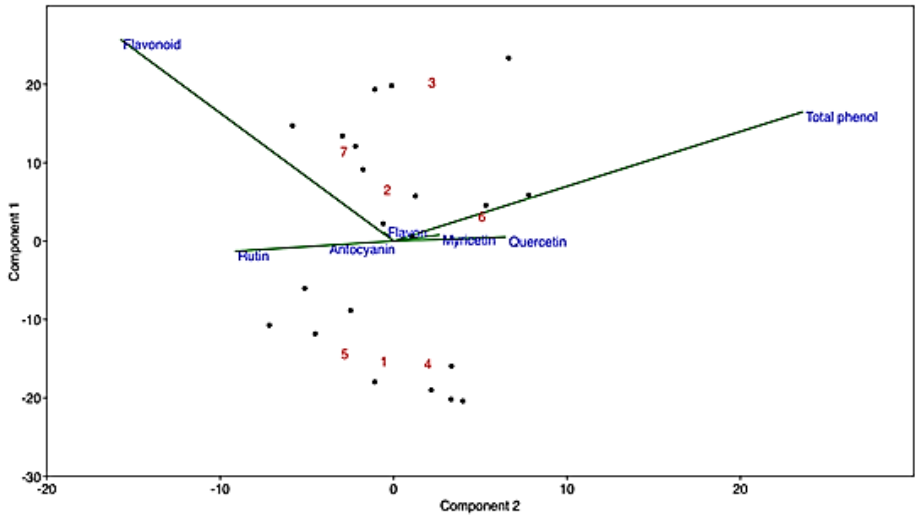

Figure 7. PCA-biplot of grape cultivars based on chemical data (The numbers of 1-7 are in Table1)

Combined data analyses

A Heat-map was constructed based on combined molecular and chemical data after proper data coding (Figure 8). The heat-map revealed almost separation of each cultivar based on combined data. This indicates that grape cultivars have specific genetic and chemical features. This particularly fits to the cultivars 'Keshmeshi', 'Asgari', and 'Black', which stands separate from the other studied grape cultivars. Cultivars' specific features may be utilized in future breeding and crossing of grapes.

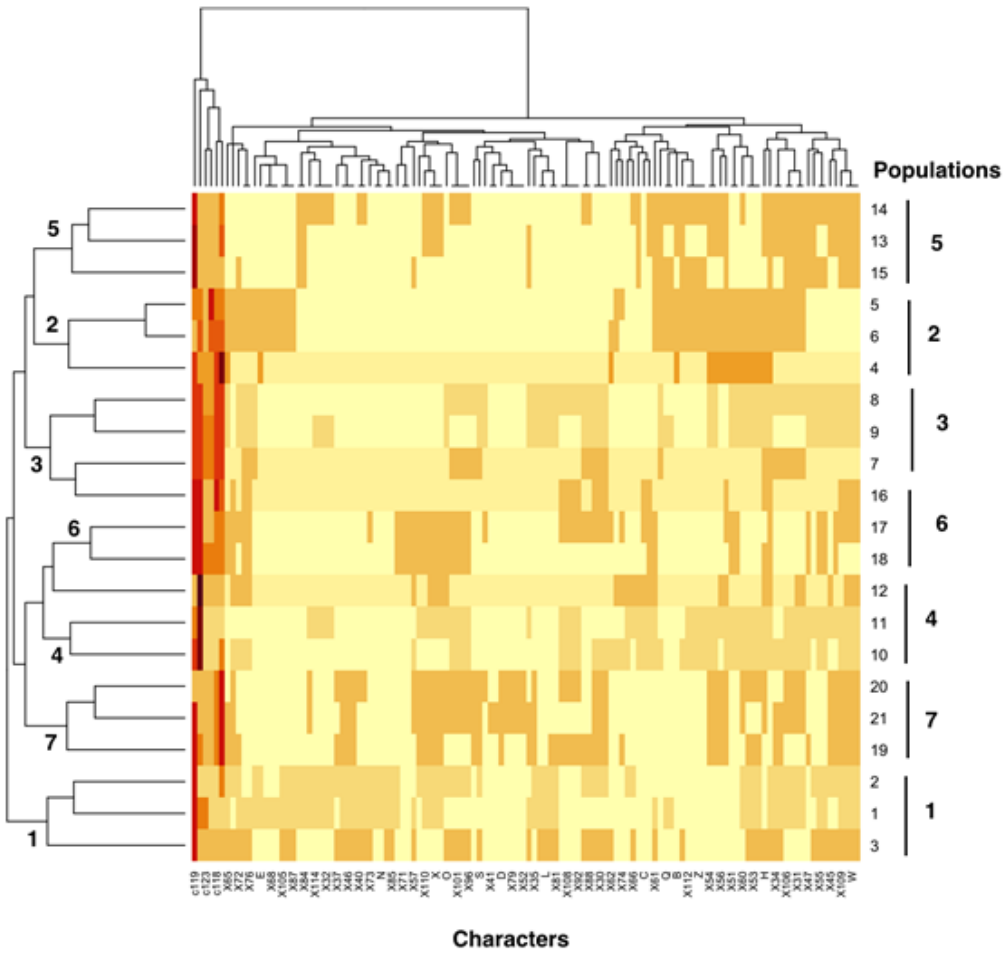

Figure 8. Heat-map of grape cultivars based on combined molecular and chemical data

Discussion

Genetic diversity

We obtained a low-medium level of mean genetic diversity in the limited number of grape cultivars studied. This may be due to the process of grape domestication or local cultivation practice, or it me be the result of continuous cultivation/ selection for a desirable agronomy feature. However, Mylets *et al.* (2011) by studying over 1000 grape plant accessions by high via sequencing concluded that in general, the reduction of genetic diversity in the grapes which is attributable to domestication and breeding appears to be weak on a genome-wide scale.

We found some private SRAP and SSR bands in the studied grape cultivars which may represent local genetic differentiation. Such genetic changes may result in morphological or agronomical characteristics, which should be investigated in future work. According to Mylets *et al.* (2011), a few notable changes in morphology have emerged since grape domestication, including perfect flowers, larger berry sizes, higher sugar content, and a wide range of berry colours which may be related to some genomic regions. They reported that GWA (Genome wide association) study for berry colour identified a 5-Mb region on chromosome 2 that encompasses a group of MYB transcription factor genes known to be the major determinants of grape colour. They also observed a strong signal of positive selection for white grapes around this locus.

We obtained the mean genetic polymorphism percentage of 16.48, and the mean Ne value of 1.15 based on SSR markers in grape cultivars studied. Our result is in agreement with the study made by Khadivi *et al.* (2017) who reported Ne ranging from 1.34 to 2.00 with almost the same mean value. However, they reported a higher genetic diversity in all their samples (PIC values ranged from 0.49 to 0.87, and the mean expected heterozygosity (He) was 0.43). These higher values of genetic diversity may be due to the presence of foreign cultivars in their study or may be due to our material local selection and inbreeding.

Genetic differentiation of grape cultivars

We presented a significant genetic difference (AMOVA, P =0.01) among the studied local grape cultivars. Ebadi *et al.* (2019) performed their molecular study based on SSR and SNP data on a collection of 35 important grape genotypes from Iran and 10 representative European cultivars. They did not have replicates for the studied local and foreign cultivars and only reported genetic differences between Iranian and European accessions.

We observed a high discriminating power (Gst>0.90) in SRAP and SSR molecular markers, and they can be considered efficient in genetic finger printing of grape cultivars. Gismondi *et al.* (2014) and Khadivi *et al.* (2017), also reported a high discrimination power for SSR markers in grape cultivars (0.74, and. 0.80, respectively).

Significant AMOVA observed among grape cultivars studied is due to the same differentiating loci obtained. It shows that despite cross-pollination in grapes, these cultivars maintained their genetic integrity and did not take part in major gene flow events with each other.

Chemical contents difference

The flavonoids of different grape cultivars showed that the exocarp and mesocarp flavonols of red and white grape fruits contain different flavanols such as kaempferol, quercetin, isorhamnetin, myrisetin, laricetrinand, syringetin (Montealegre *et al.*, 2006) as well as glycosidic derivatives are similar to Isorhamnetin-3-O-glucoside (Cheynier *et al.*, 2003). Grape seeds also have high amounts of flavan-3-ols (Cadot *et al.*, 2006; Pinelo *et al.*, 2006).

The present studies show that rutin is the major constituent among grape seed flavonols. While this substance is rare in exocarp and mesocarp (Castillo-Muoz *et al.*, 2009).

Grape cultivars studied significantly differed in their chemical contents, and they were scattered into two major groups based on the chemical data obtained. In a similar study, Oprica *et al.* (2016) investigated total polyphenols and flavonoids content in skin, pulps, and seeds of three grape cultivars from Romania. They reported the highest content of these chemicals in the seeds, followed by skins and pulps. In general, they found no considerable variation in the amounts of total polyphenols and flavonoids among the three grape berry cultivars; however, these levels do differ significantly within the three analysed parts, seed, skin, and pulp.

Rolle *et al.* (2013) studied the chemical constituents and mechanical properties of different black table grape varieties. They used spectrophotometric and HPLC methods and texture analysis tests to evaluate colour index, sugars and acid composition, phenolic characteristics, and mechanical properties of skin and the pulp of berries and reported a significant difference in hydroxycinnamic acid, anthocyanin content, and mechanical properties.

PCA analysis identified chemical features that differentiated our grape samples into two major groups. Rolle *et al.* (2013) also used PCA to analyse the chemical constituents and mechanical properties of different black table grape varieties and showed that the texture profile analysis parameters (hardness, cohesiveness, gumminess, and resilience) and berry skin characteristics were the best indices to differentiate their grape samples. The present results show that there is no positive correlation between the genetic results and the profile of grain flavanols.

Grouping of the cultivars by WARD clustering, based on chemical contents, separated cultivars with small seeds from cultivars with large seeds. Most studies showed that the exocarp and mesocarp flavonol profiles of grapefruit have not been useful to identify and differentiate red and white grape cultivars in some cases (Cantos *et al.*, 2001; Castillo-Muoz *et al.*, 2009). Perhaps one of the reasons for the discrepancy between grape genotypes and flavonoid profiles is that these compounds are strongly influenced by biotic and abiotic conditions (Braidot *et al.*, 2008) the total flavonol content cannot be a characteristic for distinguishing grape cultivars.

Conclusions

In conclusion, we presented both genetic and chemical differences of seven grape cultivars of our country. Some of these cultivars have specific features which can be used for crossing experiments and future grape breeding. Genetic information is of immense importance in breeding many crops, including the grape (di Gaspero *et al.*, 2010). Having in hand the genetic data, the grape breeders have the opportunity to select plants based on DNA sequences within or near the gene that controls a desirable trait rather than handling their phenotypes. Marker-assisted selection in grape can find novel genotypes that meet the demand for grape breeders and consumers.

Authors' Contributions

Vahid Yakhchi: data collection and lab work, wrote the paper; Hossein Abbaspour: contributed in analysis tools or data; Maryam Peyvandi: Conceived and designed the experiments; analysed and interpreted the data; wrote the paper; Ahmad Majd: Analysed and interpreted the data; Zahra Noormohammadi: Analysed and interpreted the data, edited the paper.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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