MOLECULAR ANALYSIS OF GRAPEVINE GERMPLASM BY SSR (SIMPLE SEQUENCE REPEATS) IN DIYARBAKIR PROVINCE, TURKEY

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Abstract. This study was aimed to identify the grape cultivars growing in Diyarbakır. A total of 45 genotypes were analyzed using 7 microsatellite loci. The numbers of alleles per locus ranged between 7 and 10, whereby VVS2 and VrZAG47 had the highest and VVMD7, VrZAG62, and VrZAG79 had the lowest number of alleles. The expected and observed heterozygosity were 0.77 and 0.73, respectively. Clustering analysis was performed using the UPGMA method (Unweighted Pair-Group Method using Arithmetic means). A dendrogram was constructed based on the genetic similarity among the genotypes, which indicated 5 distinct groups, with each group involving numerous subgroups. Two additional genotypes that are used as reference genotypes around the world, Cabernet Sauvignon and Merlot, were also analyzed and were classified into a separate subgroup and differentiated from the other 43 genotypes. In conclusion, synonyms and homonyms were detected among some of the genotypes analyzed in the study.
Keywords: *Vitis vinifera L., molecular analysis, genotype, similarity index, homonym, synonym*

Introduction

Grape (*Vitis vinifera* L.) is one of the most important fruit in the world. Turkey has a long history of grape cultivation. Diyarbakir province, located in the Southeastern Anatolian Region in Turkey, has an important position in Turkey with regard to its plant diversity. Grapevine, in particular, is a leading perennial garden plant well adapted to this region. The region also features various forms of grapes including table grapes, wine grapes, and grapes for drying. Kaplan (1994) conducted an ampelographic study using the classic method for naming the grapes growing in this region. The researcher demonstrated the rich genotypic diversity in the region and also noted that the naming of grapes is complicated mainly due to the complexity of the synonyms used for grapes. Accordingly, it is commonly known that the use of different names for plants based on regional variation leads to significant problems and confusion in terms of the correspondence of these names to those used in studies and to those mentioned in each phase of production. These problems can only be resolved by the use of molecular markers of polymorphism.

Microsatellite, a highly powerful type of DNA markers, provides a unique genetic profile for every cultivar, permitting unambiguous identification that is not affected by environment, disease, or farming methods (Meredith, 2001). Since the first grape microsatellites were reported by Thomas and Scott (1993), many more microsatellites have been developed for characterization of *Vitis* germplasm (Bowes et al., 1996, 1999; Sefc et al., 1999; Dı Gaspero et al., 2000; Scott et al., 2000; Dı Gaspero et al., 2005).

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Microsatellite markers have been extensively used for genotyping and the determination of synonyms and homonyms of grape genotypes (Costantini et al., 2005; Karaağaç, 2006; Cipriani et al., 2010; Emanuelli et al., 2013; Alifragkis et al., 2015; Maletic et al., 2015 Maul et al., 2015; Li et al., 2017; Zequim Maia et al., 2018; Van Heerden et al., 2018) and for pedigree analysis and for investigating the parentage of cultivars and genome mapping (Meredith et al., 1996; Sefc et al., 1998; Grando et al., 2003; Vouıllamoz et al., 2004; Adam-Blondon et al., 2004; Akkak, 2007; Huber, 2016; Dong et al., 2018). These markers have also been used for the identification of chimaeras of grapes (Franks et al., 2002; Riaz et al., 2002; Hocquigny et al., 2004; Boz et al., 2011). Moreover, these SSR markers have recently been successfully used for the protection of the germplasms of wild grapevine (*Vitis vinifera* ssp. silvestris) and the elucidation of the historical development of grapevine (Schneider et al., 2015; Zdunić et al., 2017; Butorac et al., 2018).

The aim of this study was to identify the grape cultivars growing in Diyarbakır based on the DNA profiles of the grape cultivars transplanted to the Tekirdağ National Germplasm Repository Vineyard in order to protect the germplasms of these grapes and to provide an accurate genetic identification for these cultivars. With the aims stated above, we performed genetic identification of 43 grape cultivars growing in Diyarbakır Province, where gene potential is remarkably high.

Materials and methods

Materials

The study was conducted at Ankara University Agriculture Faculty Horticulture Department. The materials used in the study consisted of 36 genotype samples that were collected from Diyarbakir Province and its districts (D) and all the 7 genotype samples of the cultivars that had been transplanted to the Tekirdağ National Germplasm Repository Vineyard several years earlier (TD). One-year-old seedlings with 3-5 buds were obtained from each genotype and were planted in polyethylene tubes filled with a 2:2:1 mixture of perlite, turf, and powder and then germinated in greenhouse conditions until the buds were rooted. In addition to D and TD genotypes, two additional genotypes that are used as reference genotypes around the world, Cabernet Sauvignon and Merlot, were also analyzed.

Table 1 presents the microsatellites used for the characterization of the genotypes analyzed in the study.

Methods

DNA was extracted from the young leaves of cultivars collected during the summer season, using the method proposed by Lodhi et al. (1994). The DNA concentration was adjusted to 30 ng/µl for polymerase chain reaction (PCR) amplification.

A total of 7 SSR primers were used in the study including the 6 microsatellite loci, VVS2, VVMD5, VVMD7, VVMD27, VrZAG62 and VrZAG79, which were used in a previous GENRES 081 European Union (EU) research project and are currently accepted as minimum standards around the world, and another primer, VrZAG47, which was used in our previous studies and was proven to be a polymorphic primer. Forward primers of each primer pair were labeled with fluorescent dyes including Fam (blue), Vic (green), and Ned (yellow) (*Table 1*).

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Table 1. *Primers used for the study*

Primer	5′-3′	Base sequences of primers		Reference
VVS2	F	VIC-CAG CCC GTA AAT GTA TCC ATC	Vic	Thomas and Scott (1993)
	R	AAA TTC AAA ATT CTA ATT CAA CTG G		
VVMD5	F	6-FAM-CTA GAG CTA CGC CAA TCC AA	Fam	Bowers et al. (1996, 1999)
	R	TAT ACC AAA AAT CAT ATT CCT AAA		
VVMD7	F	NED-AGA GTT GCG GAG AAC AGG AT	Ned	
	R	CGA ACC TTC ACA CGC TTG AT		
VVMD27	F	NED-GTA CCA GAT CTG AAT ACA TCC GTA AGT	Ned	
	R	ACG GGT ATA GAG CAA ACG GTG T		
VrZAG47	F	VIC-GGTCTGAATACATCCGTAAGTATAT	Vic	Sefc et al. (1999)
	R	ACGGTGTGCTCTCATTGTCATTGAC		
VrZAG62	F	6-FAM-GGT GAA ATG GGC ACC GAA CAC ACG C	Fam	
	R	CCA TGT CTC TCC TCA GCT TCT CAG C		
VrZAG79	F	6-FAM-AGA TTG TGG AGG AGG GAA CAA ACC G	Fam	
	R	TGC CCC CAT TTT CAA ACT CCC TTC C		

DNA amplification was performed using GeneAmp PCR System 9700 with EU-Applied Biossystems, and PCR optimization was achieved for each cultivar. PCR amplification was performed in a reaction volume of 20 µl containing 5 µl of DNA, 2 µl of 10X Buffer, 1.2 µl of $MgCl_2$, 0.6 µl of dNTP, 1 µl of primer 1, 1 µl of primer 2, 0.2 µl of GoldTaq (0.5 U), and 9 of µl distilled water. Touchdown PCR was performed using the following cycling conditions: 95 °C for 10 min, 94 °C for 30 s, and 52 °C for VVS2, VVMD5, and VVMD7, 58 °C for VVMD27, 55 °C for VrZAG47, and 62 °C for VrZAG62 and VrZAG79 for 30 s, depending on the rate of primer annealing, with a decrease of 0.2 °C/cycle. After 25 cycles, 15 additional cycles were performed with a reduction of 5 °C from the primer annealing temperature, finally followed by holding at 72 °C for 40 min.

To screen for amplification of the fragments in the loci analyzed, a minimum of 10 samples representing each locus were separated on agarose gel, and the amplified fragments were subjected to sequencing using ABI Prism 3730 automated DNA sequencer with GeneScan™ 500 LIZ™ dye Size Standard. The resulting data were analyzed, visualized, and processed using GeneMapper v 3.7 software. The allele size per locus was represented as peak levels.

A total of 45 grapevine genotypes were analyzed in 7 SSR loci and the genetic similarity between the genotypes was calculated using the Microsat software (Minch et al., 1995) and the genetic parameters (number of alleles per locus [n], allele frequency, expected heterozygosity [H_e], observed heterozygosity [H_o], parentage, null allele frequency (r), and probability of identity [PI]) were calculated using the IDENTITY 1.0 software (Wagner and Sefc, 1999). The dendrograms were constructed and visualized using the NTSys software (version 2.02g, Exeter Software, Setauket, NY). Clustering analysis was performed using the UPGMA method (Unweighted Pair-Group Method using Arithmetic means).

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Results

The analysis of 45 genotypes (including 43 grapevine genotypes collected from Diyarbakir Province and 2 reference genotypes) characterized by 7 microsatellite markers using the Gene Mapper v. 3.7 software, including peak levels, allele size, and basepair per locus (*Table 2*).

Table 2. *Allele sizes of the genotypes characterized by 7 microsatellite loci*

No	Genotype	VVS2		VVMD5		VVMD7		VVMD27		VrZAG47		VrZAG62		VrZAG79	
1	*D* Mikeri	121	141	230	232	246	246	181	191	155	157	186	198	244	248
2	*D* Balcani	141	149	222	228	244	244	177	191	155	169	190	202	240	246
3	*D* Hatunparmağı (white)	139	143	232	242	236	244	191	191	169	169	186	202	244	248
4	*D* Vilki	131	133	230	232	240	244	181	181	159	159	186	190	246	246
5	*D* Şitu	133	149	222	230	236	244	179	183	157	161	190	194	248	248
6	*D* Kızılbanki	131	141	232	236	240	246	181	191	159	169	188	188	244	246
7	*D* Kohar	139	149	224	230	244	246	183	183	157	161	186	202	248	248
8	*D* Hasani	131	141	232	236	246	246	181	191	159	169	188	198	244	248
9	*D* Asuri	141	149	222	228	244	244	177	191	155	169	190	202	240	246
10	*D* Zerik	133	153	230	232	244	244	191	191	157	169	190	198	248	254
11	*D* Ağek	141	149	228	242	244	252	179	191	157	169	202	202	236	244
12	*D* Gençmehmet	131	141	230	232	244	244	181	191	159	169	190	202	244	246
13	*D* Şaraplık	149	155	228	234	246	252	177	191	155	169	192	202	244	246
14	*D* Iskıcuna	141	155	230	242	244	246	177	191	155	169	186	192	246	248
15	*D* Merir	133	139	230	236	244	244	181	181	159	169	186	190	246	254
16	*D* Abderi	131	133	230	232	240	244	181	181	159	159	186	190	246	246
17	*D* Tahannebi	131	131	232	232	244	246	177	191	155	169	198	202	244	246
18	*D* Morek	149	155	228	234	246	252	177	191	155	169	192	202	244	246
19	*D* Vanki	131	141	232	236	246	246	181	191	159	169	188	188	244	248
20	*D* Şamuzli	131	155	230	234	244	244	191	191	169	169	190	202	240	254
21	*D* Şirelik	131	131	232	232	244	246	177	191	155	169	198	202	244	244
22	*D* Karik	133	155	222	232	244	246	191	191	169	169	190	198	244	248
23	*D* Mazrumi	131	131	232	232	244	246	191	191	155	169	198	202	244	246
24	*D* Belelük	139	155	226	228	246	246	177	177	155	155	192	192	246	248
25	*D* İm küçük	121	143	228	242	246	246	183	183	155	161	198	198	246	248
26	*D* Siyahgıldun	143	155	228	232	246	246	183	183	155	161	198	198	246	248
27	*D* İm büyük	141	149	222	228	244	244	177	191	155	169	190	202	240	246
28	*D* Beyazgıldun	133	143	222	232	246	246	183	191	161	169	198	198	246	248
29	*D* Amorku	133	149	222	242	236	246	177	191	155	169	194	198	244	246
30	*D* Avkenek	139	149	228	234	244	244	191	191	169	169	190	202	254	254
31	*D* İstanbullu	131	131	232	232	244	246	177	191	155	169	198	202	244	246
32	*D* Şekeri	131	131	222	222	244	262	181	181	155	159	186	198	240	254
33	*D* Şarabi	149	155	232	234	244	246	183	191	161	169	190	198	248	256
34	*D* Hatunparmağı (black)	121	121	232	242	236	252	185	191	163	169	202	202	244	246
35	*D* Kışgıldun	133	149	222	230	236	244	179	183	157	161	190	194	248	248
36	*D* Kabarcık	131	141	230	230	244	250	175	177	153	155	190	202	244	244
37	*TD* Siyahüzüm	133	155	222	232	244	246	181	191	159	169	190	198	244	246
38	*TD* Şarabi	141	149	228	234	244	252	177	191	155	169	202	202	244	246
39	*TD* İsimsiz	133	133	228	236	244	246	191	191	169	169	198	202	240	254
40	*TD* Vanki	139	149	228	234	244	244	191	191	169	169	190	202	254	254
41	*TD* Tahannebi	131	155	230	234	244	244	191	191	169	169	190	202	254	254
42	*TD* Abderi	131	133	230	232	240	244	181	181	159	159	186	190	246	246
43	*TD* Abdullah	131	133	230	232	240	244	181	181	159	159	186	190	246	246
44	Cabernet Sauvignon	137	149	228	236	236	236	171	185	151	165	186	192	244	244
45	Merlot	137	149	222	232	236	244	185	187	165	167	192	192	256	256

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Genetic parameters of 45 genotypes (i.e. number of alleles per locus [n], allele frequency, expected heterozygosity [H_e], observed heterozygosity [H_o], parentage, null allele frequency (r), and PI) were calculated for each of the 7 loci using the IDENTITY 1.0 software (Wagner and Sefc, 1999). Genetic diversity was calculated based on the following formula: the expected heterozygosity (H_e) = $1-\Sigma p_i^2$, where p_i refers to the frequency of individual alleles (Nei, 1987). The observed heterozygosity was considered as the ratio of the number of heterozygous genotypes to the total number of genotypes analyzed. The frequency of null alleles was calculated based on the following formula: $(H_e - H_o)/(1 + H_e)$ (Brookfield, 1996). PI was defined as the probability that two randomly selected samples have the same SSR profile and was calculated based on the following formula: $\Sigma p_i^4 + \Sigma\ \Sigma(2p_ip_j)^2$, where p_i and p_j indicate the frequencies of alleles *i* and *j*, respectively (Paetkau et al., 1995).

In total, 59 alleles were identified in 7 loci, whereby the total number of alleles per locus ranged between 7 and 10 and the mean number of allele per locus was 8.43. The highest numbers of alleles were detected in VVS2 and VrZAG47 and the lowest were detected in VVMD7, VrZAG62, and VrZAG79. The expected and observed heterozygosity were 0.77 and 0.73, respectively. The PI value per locus was higher than 0.05, the value proposed by Sefc et al. (2001). The PI value detected in our study implicates that the microsatellite markers used in this study are highly polymorphic for genotypic analysis of grapevine.

Genetic similarity between the genotypes was calculated using the Microsat software based on the following formula: genetic distance (D), *D* = 1-(proportion of shared alleles) (dissimilarity). The resulting value was then converted to genetic similarity index. Genotypes with a value of 1.000 had the highest similarity index and were considered as synonym cultivars, some other genotypes had a value of 0.929 and were considered to be genetically similar, and the remaining samples genotypes that had no shared alleles or no genetic similarity in any locus were considered to be genetically dissimilar.

Dendrograms were constructed and visualized using the NTSys software (version 2.02g, Exeter Software, Setauket, NY). Clustering analysis was performed using the UPGMA method. The genotypes of the samples collected from Diyarbakır were grouped based on the proportion of shared alleles. The dendrogram of the 43 genotypes indicated 5 distinct groups, with each group involving numerous subgroups. The 2 reference genotypes were classified into a separate subgroup and were differentiated from the other 43 genotypes. Genotypes with a similarity index of 1.000 were classified as synonym cultivars on the dendrogram. Overall, the dendrogram indicated that the genotypes of the samples collected from Diyarbakır were not remarkably dissimilar and even showed close relationship with each other. Moreover, numerous synonyms and homonyms were detected among the accessions in the dendrogram (*Figure 1*).

Discussion

In the present study, a total of 59 alleles were identified in the 45 genotypes characterized by 7 loci, whereby the total number of alleles per locus ranged between 7 (VVMD5, VrZAG62, and VrZAG79) and 10 (VVS2 and VrZAG47) and the mean number of alleles per locus was 8.43. *Table 4* presents the number of alleles per locus reported by previous studies. In contrast, the studies by Crespan and Milani (2001), Dangl et al. (2001) and Hvarleva et al. (2004) were conducted with lower numbers of

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genotypes and thus detected lower mean numbers of alleles compared to our study. In our study, the number of alleles per locus ranged between 7 and 10 (*Table 3*), which are higher than the numbers reported by Dong et al. (2018) and Zequim Maia et al. (2018). Dong et al. (2018) analyzed 34 grape genotypes using 15 SSR markers and found that the number of alleles per locus ranged between 1 and 8. Zequim Maia et al. (2018) analyzed 69 grape genotypes and reported that the number of alleles per locus ranged between 1.94 and 2. This difference is associated with the large number of low-frequency alleles in large-scale sample sets (Laiadi et al., 2009). However, our study was similar to those reported by Akkak et al. (2005), Costantini et al. (2005), Vouillamoz et al. (2006), and Li et al. (2017) with regard to the number of alleles per locus (*Table 4*).

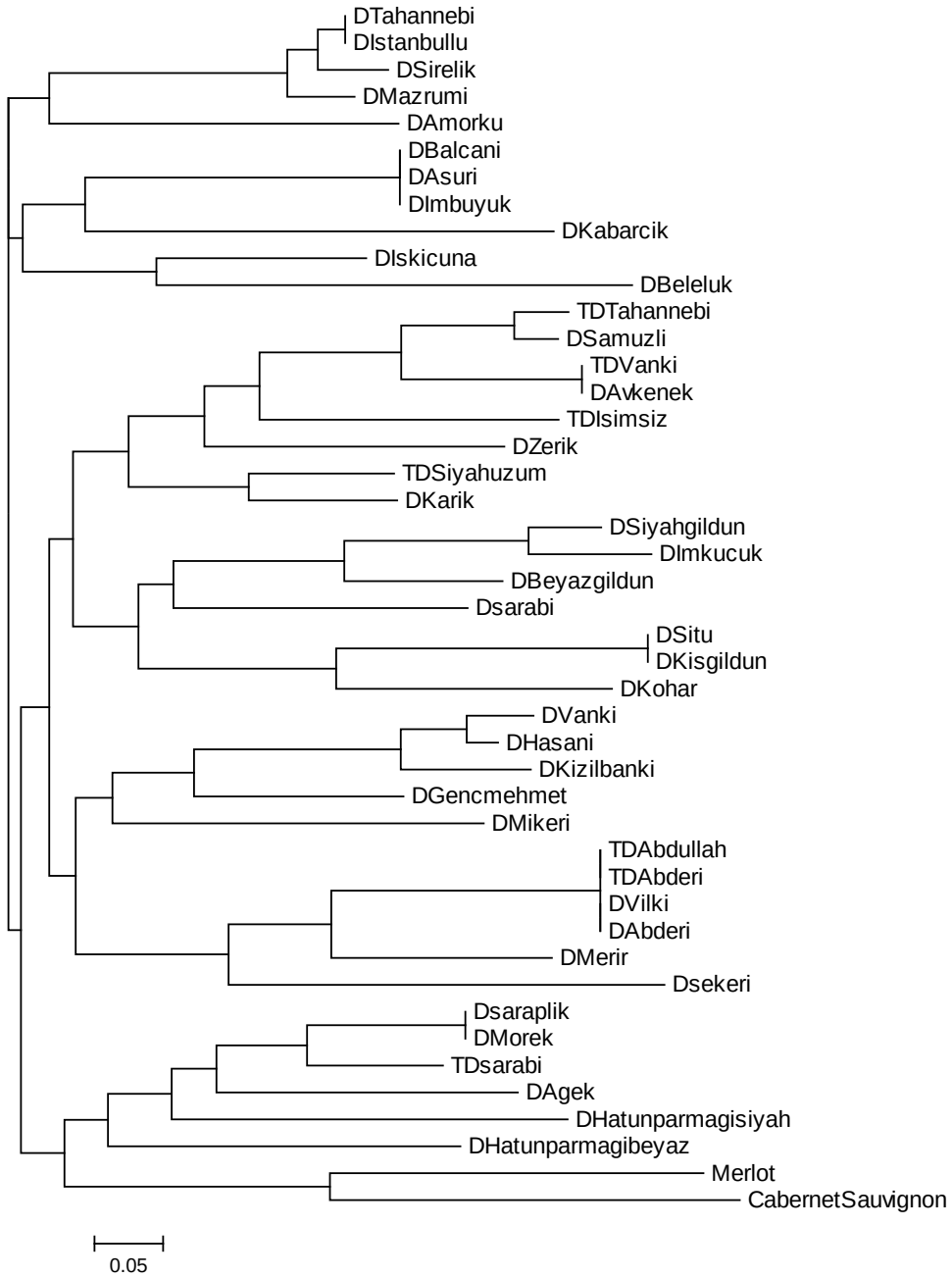

Figure 1. *Dendogram of 45 grape cultivars based on similarity index from SSR data*

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Table 3. *Number of alleles per locus, He, Ho, and PI*

Marker	Number of alleles	He	Ho	PI
VVS2	10	0.85	0.84	0.83
VVMD5	9	0.83	0.87	0.80
VVMD7	7	0.67	0.60	0.62
VVMD27	9	0.73	0.58	0.70
VrZAG47	10	0.75	0.73	0.71
VrZAG62	7	0.81	0.78	0.78
VrZAG79	7	0.78	0.69	0.74
Mean	8.43	0.77	0.73	0.74

Table 4. *Number of alleles per locus reported by previous studies*

Number of genotypes	Number of microsatellite locus	Alleles range	Mean number of alleles	Reference
406	8	4-16	9.60	Borrego et al. (2001)
64	25	3-11	6.58	Crespan and Milani (2001)
41	11	4-11	8.00	Dangl et al. (2001)
62	9	4-16	9.60	Fatahi et al. (2003)
111	13	4-16	9.85	Ibáñez et al. (2003b)
176	6	9-13	11.00	Martín et al. (2003)
74	9	4-10	8.10	Hvarleva et al. (2004)
60	12	7-12	9.10	Akkak et al. (2005)
69	8	6-9	8.00	Costantini et al. (2005)
116	12	6-16	11.90	Vouillamoz et al. (2006)
94	9	5.01-8.57	12.78	Li et al. (2017)
69	17	1.94-2.0	2.0	Zequim Maia et al. (2018)
34	15	1-8	3.6	Dong et al. (2018)

Of the loci used for the analysis of 43 genotypes in our study, VVS2 and VrZAG47 were detected with the highest number of alleles (n = 10 each). In previous studies, VVS2 has also been reported to have the highest number of alleles by Lopes et al. (1999), Borrego et al. (2001), Lefort and Roubelakis-Angelakis (2001), Fatahi et al. (2003), Martín et al. (2003) and Núñez et al. (2004). This marker was followed by VVMD5 and VVMD27 (n = 9) and VVMD7, VrZAG62, and VrZAG79 (n = 7).

The PI values varied from 0.62 to 0.83 with a mean value of 0.74 (*Table 3*). The mean PI value was similar to the value reported by Ramezani et al. (2009) (0.77). The most informative locus was VVS2 (0.83) (*Table 3*). This finding was consistent with the finding reported by Li et al. (2017) that indicated that VVS2 showed the highest level of polymorphism with a value of 0.815.

The PI value per locus in our study was higher than 0.05, the value proposed by Sefc et al. (2001). This finding implicates that the microsatellite markers used in this study are highly polymorphic for genotypic analysis of grapevine. With the highest PI values, VVMD5 (0.80) and VVS2 (0.83) were the most informative markers in our study, also revealing maximum differentiation among the genotypes. In other words, these two markers showed the highest level of differentiation compared to other markers. In other

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studies, the most informative markers have been shown to be VVMD5 (Lefort and Roubelakis-Angelakis, 2001; Ibáñez et al., 2003b; Martín et al., 2003) VVS5 (Borrego et al., 2001), and VVMD14, VVMD28, and VVMD36 (Crespan and Milani, 2001). Meaningfully, VVMD5 has been shown to be an informative marker in numerous studies, as seen in our study.

The expected and observed heterozygosity in our study were 0.77 and 0.73, respectively (*Table 3*). Accordingly, the observed heterozygosity was lower than the expected heterozygosity, which could be attributed to the existence of null alleles. However, the absence of a significant difference between these two rates further confirms that our cultivars were heterozygous. Moreover, the observed heterozygosity in our study was lower than those reported by other studies including Sefc et al. (2000), Dangl et al. (2001), Aradhya et al. (2003), Fatahi et al. (2003), Costantini et al. (2005) and Vouillamoz et al. (2006).

Based on the proportion of shared alleles among the 45 genotypes characterized by 7 microsatellite loci, 15 synonyms and 8 homonyms were detected among the genotypes (*Table 5*).

The reference genotypes used in our study, Cabernet Sauvignon and Merlot, have also been studies by other researchers (Bowers et al., 1999; This et al., 2004). The researchers found similar differences to those of our study, between the two alleles in a single loci for each of the 7 loci.

Table 5. *Synonyms and homonyms detected in the genotypes of the samples collected from Diyarbakır that were characterized by 7 microsatellite markers*

Synonyms
D Vilki, *D* Abderi, *TD* Abderi, *TD* Abdullah
D Balcani, *D* Asuri, *D* İm Büyük
D Şaraplık, *D* Morek
D Tahannebi, *D* İstanbullu
D Avkenek, *TD* Vanki
D Şitu, *D* Kışgıldun
Homonyms
D Hatunparmağı black, *D* Hatunparmağı white
D Vanki, *TD* Vanki
D Tahannebi, *TD* Tahannebi
D Kızılbanki, *Ş* Kızılbanki

Conclusion

In conclusion, we obtained a very high allelic polymorphism among the genotypes that were expected to be different or between the genotypes that were supposed to have the same variety name. Moreover, 15 synonyms and 8 homonyms were detected among the 45 genotypes characterized by 7 microsatellite loci, based on the proportion of shared alleles among the genotypes. However, in the genotypes detected with homonyms, it was not clear as to which homonym represented the real name of each genotype. A total of 30 distinct cultivars were detected among the 45 genotypes characterized by 7 microsatellite markers, suggesting that Diyarbakır Province is rich in genetic diversity of grapevine and that the gene sources of these cultivars should be protected.

We consider that the differentiation in the names of homonymous cultivars could be attributed to erroneous naming of the cultivars or to the variations (e.g. clone, type) that

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have emerged within the same cultivar over time. Considering the naming of synonymous cultivars, it appears that a single genotype might have been erroneously given several different names.

Our results indicated that the microsatellite markers used in our study were highly suitable for the genetic identification of grape cultivars and the determination of synonyms and homonyms. Notably, VVS2 and VVMD5 were the most informative markers among the others and successfully differentiated the 45 genotypes.

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