

Genetic relationships among grapevine cultivars native to Croatia, Greece and Turkey

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Summary

Three sets of grapevine cultivars were analysed: (1) 6 Croatian cultivars from the island of Hvar, (2) 5 Greek cultivars from the island of Paros and (3) 9 Turkish cultivars from the region of Anatolia. These cultivars were assayed by molecular markers (RAPDs with 8 primers and SSRs on 8 loci) and analyzed in terms of genetic similarity. Cluster analysis based on Dice genetic similarity indices resulted in dendrograms using two types of data. The cultivar DNA profiles showed that there were no synonyms among the groups of cultivars tested. Cluster analysis did not point to any particular relationship among cultivars from different regions, although AMOVA analysis showed greater genetic similarity between Greek and Croatian cultivars in contrast to Turkish cultivars.

Key words: SSR, RAPD, genetic relatedness, cultivar introduction.

Introduction

Most contemporary grapevine (*Vitis vinifera* L.) cultivars are fairly old and of unknown genetic background. Grapevines have been vegetatively propagated in Croatia for centuries and the introduction and spread of cultivars in Croatia is thought to have occurred in the past. This hypothesis raises the chances of Croatia's present cultivars being genetically similar to the cultivars from the countries that have in the past settled or conquered the today's Croatian area.

According to various theories ancient Greeks from the island of Paros are said to have established one of their colonies at the site of today's town of Stari Grad on the island of Hvar 2400 years ago and named it Faros after their home island (SUIĆ 2003). Despite the lack of firm evidence about their precise origin, there are many archeological traces of the ancient Greeks' presence on the island of Hvar. The genotypes of a vegetatively propagated species, such as grapevine, could corroborate that.

The influence of Turkey (the Ottoman empire) on the entire Balkan peninsula was intense and long-lasting in the nearer past. Archeological and historical research in Anatolia in Turkey proves that this region was very important for the history of viticulture (GORNÝ 1996).

In this study a random sample of 9 cultivars from Turkey (the region of Anatolia), 5 most widely spread samples

from the island of Paros in Greece and 6 samples of native cultivars known to be grown only on the island of Hvar in Croatia were analyzed using Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) markers in order to determine the potential synonyms and genetic relatedness among them. The analysis of the microsatellite (SSR) profiles facilitates determining the relatedness between the cultivars and their origin (SEFC *et al.* 2000; PILJAC *et al.* 2002; MALETIĆ *et al.* 2004). This is due to the fact that the codominant markers SSRs have the potential of determining the "parent-offspring" relationships while RAPD markers can detect variation on clone level.

Material and Methods

Young leaves were taken from cultivars listed in Tab. 1. All samples were taken from standard vineyards, not the official collections, by ampelographers or by viticulturists. The samples from Paros (Greece) were collected at the beginning of the vegetation season (early June) in local vineyards and the affiliation of samples to the cultivar name was based upon the vineyard data and the features of young leaves. Cultivar samples from Croatia and Turkey were collected from vines that were positively identified in previous years.

Young leaves picked in the spring of 2003 were lyophilized and stored at -80 °C. The DNA extraction method (DOYLE and DOYLE 1990) was conducted using 2 % CTAB. The method was slightly modified by adding 6 % PVP to the extraction buffer. The DNA concentration was checked by band confrontation with λ DNA on 0.8 % agarose gels.

PCR amplification for RAPD analysis was carried out in 25 μ l of the reaction mix containing 10 ng of template DNA, PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.8), 0.2 μ M of primer, 0.1 mM of each dNTP, 1.5 mM MgCl₂, 1U of thermostable Taq polymerase (Sigma) and stabilized with 20 μ g BSA. RAPD primers were identical with those used previously by VOKURKA *et al.* (2003), the DNA was amplified in PTC-100 thermal cycler (MJ Research) with one step of 92 °C for 60 s, followed by 40 cycles of 60 s at 92 °C for denaturation, 60 s at 36 °C for annealing and 120 s at 72 °C for extension. The amplification products were separated in 1.2 % agarose gels at 120 V for 2.5 h.

Eight microsatellite loci were analysed: VVS2 (THOMAS and SCOTT 1993), VVMD5 (BOWERS *et al.* 1996), VVMD25,

Table 1

Basic descriptive characteristics for 9 Turkish, 6 Croatian and 5 Greek grapevine cultivars

Cultivar	Sampling location	Country	Utility	Color of skin	Berry shape	Bunch density	Total acids	Sugar content
Kışmış	Olur	TR	table grape	Green-yellow	reverse oval	dense	low	high
Hatun parmağı	Olur	TR	table grape	Green-yellow	cylyndrical	medium dense	low	high
Kabarcık	Olur	TR	table grape	Green-yellow	roundish	medium	low	high
Al üzüm	Erzincan	TR	table grape	Red	roundish	medium dense	low	high
Karaerik	Olur	TR	table grape	Dark red-violet	roundish	very dense	high	medium
Pirtik	Olur	TR	table grape	Green-yellow	wide oval	medium	low	high
At memesi	Olur	TR	table grape	Dark red-violet	oval	dense	high	medium
Beyaz üzüm	Olur	TR	table grape	Green-yellow	roundish	very dense	low	high
Kara	Olur	TR	table grape	Dark red	roundish	medium dense	low	medium
Drnekuša type I	Hvar	HR	wine grape	Dark red	roundish	medium	low	medium
Drnekuša type II	Hvar	HR	wine grape	Dark red light	roundish	medium	low	medium
Bogd. type I	Hvar	HR	wine grape	Green yellow	roundish	medium	low	medium
Bogd. type II	Hvar	HR	wine grape	Green yellow	roundish	medium	low	medium
Prč	Hvar	HR	wine grape	Green yellow	roundish	medium	low	high
Kuč	Hvar	HR	wine grape	Green yellow	roundish	medium	low	low
Aidani	Paros	GR	wine grape	Red	cylyndrical	-	-	-
Mantilaria	Paros	GR	wine grape	Dark red	roundish	-	-	-
Monemvasia	Paros	GR	wine grape	Green yellow	oval	-	-	-
Aetonychi	Paros	GR	wine grape	Red	roundish	-	-	-
Vaftra	Paros	GR	wine grape	Red	roundish	-	-	-

- data not available

VVMD27, VVMD28, VVMD32 (BOWERS *et al.* 1999), *ssrVrZAG62* and *ssrVrZAG83* (SEFC *et al.* 1999). The DNA was amplified in volumes of 25 μ l containing 10 ng of template DNA, PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.8), 0.2 mM of each, forward and reverse primer, 0.2 mM of each dNTP, 2.0 mM MgCl₂, 1U of thermostable Taq polymerase (Sigma) and stabilized with 20 μ g BSA.

Electrophoresis was done on EL-800 Precast Spreadex™ gels in 30mM TAE buffer using SEA 2000 chambers (Elchrom Scientific) at 55 °C and 92 V ranging in running time from 1.5 h for smaller fragments (150 bp) up to 3 h for longer fragments (260 bp). Gels were stained by SYBR® Gold (Molecular Probes) and photographed using Polaroid® film type 667.

Genetic distance calculations based on separate RAPD and SSR data were run as described by PEJIĆ *et al.* (1998) and computed using NTSYS-pc software (ROHLF 1990). AMOVA analysis (EXCOFFIER *et al.* 1992) was based on the procedure used in the similar study (BELAJ *et al.* 2002) and was done using the Arlequin software (SCHNEIDER *et al.* 2000).

Results and Discussion

The basic descriptive characteristics for all studied cultivars are given in the Tab. 1. Eight RAPD primers generated informative profiles with an average of 5 polymorphic bands per primer. Only well-defined bands were taken into account. Based on a total of 42 RAPD polymorphic fragments each cultivar showed a unique RAPD profile. Eight

SSR primers generated codominant profiles shown in Tab. 2. The dice genetic distances among all possible pairs of cultivars were calculated from both the RAPD and the SSR data using the UPGMA algorithm and NTSYS-pc software clustered dendrograms as depicted in Figs. 1 and 2.

Hierarchical analysis of molecular phenotypic diversity based on combination of the RAPD and the SSR data using AMOVA was performed to analyze the partitioning of molecular variation in the grapevine cultivars among and within countries (Croatia, Greece and Turkey) (Tab. 3). Most of the genetic diversity was attributable to the differences among the cultivars within countries (82.07 %), significant inter-population distance average (Φ -value) among zones ($\Phi_{st}=0.179$; $p < 0.001$) suggested the existence of phenotypic differentiation. Φ_{st} values between each pair of countries were mostly significant in all cases except for Greece and Croatia (Tab. 4), suggesting that the cultivars from these two countries are more closely related to each other. The dendrograms based on the Dice genetic similarities computed from both the RAPD and the SSR data also support the conclusion that the genetic differences of the Turkish cultivars are very high when compared to those from Greece and Croatia. The majority of Turkish cultivars forms specific clusters which are separate from the Croatian and the Greek cultivars, which did not form separate clusters (Figs. 1 and 2).

To test this result the SSR data from this research were joined with the SSR data from 87 additional Croatian cultivars (data not shown) and a larger dendrogram was constructed (data not shown). The Greek cultivars from this study were equally scattered in the dendrogram, while the Turkish sam-

Table 2

Genotypes at 8 SSR loci for 9 Turkish, 6 Croatian and 5 Greek grapevine cultivars

Cultivar	VVS2	VVMD5	VVMD25	VVMD27	VVMD28	VVMD32	VrZAG62	VrZAG83
1 Kişmiş	135 141	240 246	243 243	185 194	251 271	251 257	189 203	193 197
2 Hatun parmağı	135 141	240 246	243 243	185 194	251 271	251 257	189 203	193 197
3 Kabarcık	135 141	240 240	243 243	185 194	251 271	251 257	189 203	193 197
4 Al üzüm	133 141	236 246	243 243	185 194	261 271	273 273	201 203	191 193
5 Kara erik	141 151	234 234	259 259	185 194	247 247	257 257	205 205	197 197
6 Pirtik	143 143	236 246	243 245	185 194	271 271	273 273	195 x	191 x
7 At memesi	141 145	236 236	243 243	185 194	247 271	257 273	201 205	197 197
8 Beyaz üzüm	135 135	236 236	243 253	183 185	239 251	273 273	189 189	191 197
9 Kara üzüm	- -	- -	243 253	185 185	239 239	273 273	189 189	191 197
10 Drnekuša type I	133 145	228 240	245 259	179 194	251 261	257 273	189 191	193 197
11 Drnekuša type II	143 145	226 228	243 245	179 179	251 261	257 257	191 205	197 197
12 Bogdanuša type I	143 151	222 228	245 267	183 194	251 261	251 257	189 191	193 197
13 Bogdanuša type II	143 151	222 228	245 267	183 194	251 261	251 257	189 191	193 197
14 Prč	133 151	226 228	243 253	183 194	261 275	273 273	195 205	191 197
15 Kuč	133 145	- -	245 245	189 189	261 261	273 273	189 197	197 197
16 Aidani	133 135	240 240	245 247	179 194	139 247	257 257	189 205	193 197
17 Mantilaria	145 145	- -	247 259	179 179	257 261	265 265	197 203	191 203
18 Monemvasia	133 141	226 234	243 253	175 179	247 261	241 251	189 197	191 197
19 Aetonychi	133 145	234 x	243 249	179 185	251 251	273 273	189 189	193 197
20 Vaftra	- -	226 226	249 259	179 183	257 257	265 265	197 205	191 197

x = very faint bands (scored as missing data)

- = missing data.

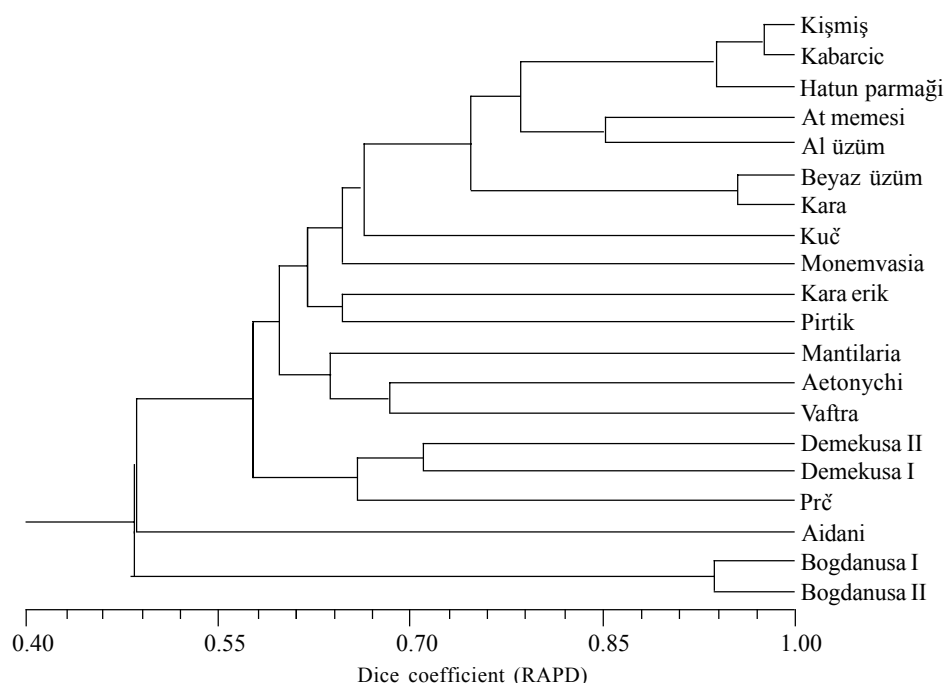


Fig. 1: Dendrogram based on Dice genetic similarities among 6 Croatian (HR), 5 Greek (GR) and 9 Turkish (TR) grapevine cultivars, computed from RAPD data generated by 8 primers.

ples were clearly separated in one individual cluster, supporting the results of the AMOVA analysis and the dendrograms shown in this study (Figs. 1 and 2).

The native vegetatively propagated varieties have the potential to be very old genotypes and, considering the

isolated position and the viticultural tradition of the Mediterranean islands, they might represent the genotypes that may be several centuries, even millenniums, old. The idea of this study was to provide the evidence of introducing wine grapes that might support the assumption that the island of

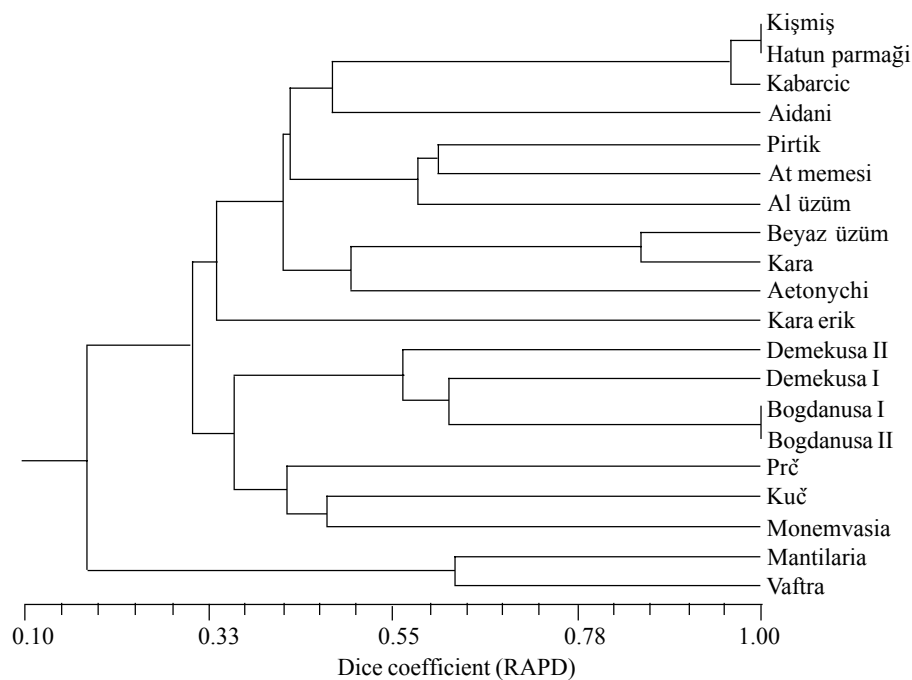


Fig. 2: Dendrogram based on Dice genetic similarities among 6 Croatian (HR), 5 Greek (GR) and 9 Turkish (TR) grapevine cultivars, computed from SSR data generated by 8 primers.

Table 3

AMOVA analysis for the partitioning of molecular variation among and within sets of grapevine cultivars from Croatia, Greece and Turkey

Source of variation	<i>df</i>	Variance components	% Total variance	Φ -Statistics	<i>p</i> -value
Among countries	2	2.943	17.93	0.179	<0.001
Within countries	17	13.471	82.07		

Table 4

Φ_{st} distance values among sets of cultivars from three countries (lower matrix diagonal), and corresponding *p* values (upper matrix diagonal)

Country	Turkey	Croatia	Greece
Turkey		0.000	0.000
Croatia	0.231		0.078
Greece	0.180	0.094	

Hvar was colonized by ancient Greeks. Our investigation did not show direct evidence of any introduction due to the fact that there was no synonym among the analyzed samples. Although the primary aim of this work was to analyze the relationships between three populations of the geographically separated cultivars, some interesting results showed up after further data analysis within the national populations. The 5 Greek samples used in this work were compared on 3 common SSR loci with the SSR data from the

Greek Vitis Database, www.biology.uoc.gr/gvd (LEFORT and ROUBELAKIS-ANGELAKIS 2000). Aetonychi and Aidani from our study do not match with those referred in the Greek Vitis Database. Cv. Aetonychi is mentioned to be a very old variety mentioned by Columelle and Plinius in the 1st century A.D. It also has plenty of synonyms and very likely homonyms. This variety was described and genotyped in the Greek Vitis Database but Paros was not referred to as the geographic area for this cultivar. Our sample of Aetonychi, which is probably a homonym, was taken from the village of Paroikia, and has the following properties: black berry, rather late ripening, aromatic, not very high quality, round berry with elongated tip, loose bunch.

Cv. Aidani from this study is probably another homonym because all samples of Aidani in the Greek Vitis Database are not genotyped yet (collection no. 8, originating from Santorini). The other 3 Greek samples match the database on 3 loci analyzed in this study. Although the comparison was made on 3 loci only, it is very likely that these three samples belong to the labeled cultivar names (Mantilaria, Monemvasia and Vaftra). Among the Croatian samples, it seems that the Bogdanuša type I and the Bogdanuša type II

are the clones of the same cultivar. They shared the same SSR profile but revealed polymorphism with two RAPD primers. The morphological differences support this hypothesis. The Drnekuša type I and the Drnekuša type II are quite different cultivars (different genotypes on 7 out of 8 loci) but share alleles on all analyzed microsatellite loci, indicating that they may be closely related. The RAPD results also support this hypothesis (Fig. 1).

Among the Turkish samples Kişmiş and Hatun parmağı have an identical SSR profile, while Kabarcik is almost identical. The difference is only on the VVMD5 locus where the allele 246 was detected in Hatun parmağı and in Kişmiş but was very faint (not scored) in Kabarcik. The RAPD profiles also show high relatedness between those samples but reject identical genotypes. A certain morphological similarity could be observed between these three genotypes of the table grape. At any rate, it is certain that there are three different but closely related genotypes, sampled from the same location (the village of Olur) that might be the clones of the same stock population derived a long time ago. It will be necessary to conduct more extensive ampelographic and DNA analysis to examine this hypothesis.

The cultivars At memesi and Al üzüm also seem to be related. They share alleles on 7 SSR loci out of 8, and the RAPD profiles support their relatedness. They are probably cultivars originating from the same gene pool. They are taken from the same location, and are considered to be autochthonous to that region.

A strong relatedness is found in Beyaz üzüm and Kara. These two cultivars have identical genotypes on 6 out of 8 SSR loci and share alleles on remaining two. The RAPD profiles confirm their relatedness. The morphological differences are obvious, Beyaz üzüm ("white grape") has white berries and Kara ("black") black ones. The leaf morphology differs too. One interesting hypothesis could be that Kara is the progeny of a self-fertilized Beyaz üzüm. This is supported by the SSR results where Kara is a homozygote on VVMD27 and VVMD28 loci, sharing the same alleles with Beyaz üzüm, which is a heterozygote on these loci (see Tab. 2). However, it would be necessary to carry out genotyping on more loci to test this hypothesis.

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References

- BELAJ, A.; ŠATOVIĆ, Z.; RALLO, L.; TRUJILLO, I.; 2002: Genetic diversity and relationships in olive (*Olea europea* L.) germplasm collections as determined by randomly amplified polymorphic DNA. *Theor. Appl. Genet.* **105**, 638-644.
- BOWERS, J. E.; DANGL, G. S.; MEREDITH, C. P.; 1999: Development and characterization of additional microsatellite DNA markers for grape. *Am. J. Enol. Vitic.* **50**, 243-246.
- BOWERS, J. E.; DANGL, G. S.; VIGNANI, R.; MEREDITH, C. P.; 1996: Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). *Genome* **39**, 628-633.
- DOYLE, J. J.; DOYLE, J. L.; 1990: Isolation of plant DNA from fresh tissue. *Focus* **12**, 13-15.
- EXCOFFIER, L.; SMOUSE, P. E.; QUATTRO, J. M.; 1992: Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction sites. *Genetics* **131**, 479-491.
- GORNY, R. L.; 1996: Viticulture in Ancient Anatolia. In: P. E. MCGOVERN, S. J. FLEMING, S. H. KATZ (Eds.): *The Origins and Ancient History of Wine*, 133-174, Gordon and Breach Publ., Philadelphia.
- LEFORT, F.; ROUBELAKIS-ANGELAKIS, K. A.; 2000: The Greek *Vitis* Database, a multimedia web-backed genetic database for germplasm management of *Vitis* resources in Greece (<http://biology.uoc.gr/gvd/>). *J. Wine Res.* **11**, 233-242.
- MALETIĆ, E.; PEJIĆ, I.; KAROGLAN-KONTIĆ, J.; PILJA, J.; DANGL, G. S.; VOKURKA, A.; LACOMBE, T.; MIROŠEVIĆ, N.; MEREDITH, C. P.; 2004: Zinfandel, Dobričić and Plavac mali - the genetic relationship among three cultivars of the Dalmatian coast in Croatia. *Am. J. Enol. Vitic.* (in press).
- PEJIĆ, I.; AJMONE-MASSAN, P.; MORGANTE, M.; KOZUMPLIK, V.; CASTIGLIONI, P.; TARAMINO, G.; MOTTO, M.; 1998: Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs. *Theor. Appl. Genet.* **97**, 1248-1255.
- PILJAC, J.; MALTEIĆ, E.; KONTIĆ, J. K.; DANGL, G. S.; PEJIĆ, I.; MIROŠEVIĆ, N.; MEREDITH, C. P.; 2002: The parentage of Pošip bijeli, a major white wine cultivar of Croatia. *Vitis* **41**, 83-87.
- ROHLF, F. J.; 1990: NTSYS. Numerical Taxonomy and Multivariate Analysis System, version 1.60, New York Exter Publishing Ltd.
- SCHNEIDER, S.; ROESSLI, D.; EXCOFFIER, L.; 2000: Arlequin ver. 2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
- SEFC, K. M.; LOPEZ, M. S.; LEFORT, F.; BOTTA, R.; ROUBELAKIS-ANGELAKIS, K. A.; IBÁÑEZ, J.; PEJIĆ, I.; WAGNER, H. W.; GLÖSSL, J.; STEINKELLNER, H.; 2000: Microsatellites variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geographic origin of cultivars. *Theor. Appl. Genet.* **100**, 498-505.
- SEFC, K. M.; REGNER, F.; TURETSCHKE, E.; GLÖSSL, J.; STEINKELLNER, H.; 1999: Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. *Genome* **42**, 367-373.
- SUIĆ, M.; 2003: *Antički grad na istocnom Jadranu*. 2nd ed., Golden Marketing, Zagreb.
- THOMAS, M. R.; SCOTT, N. S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). *Theor. Appl. Genet.* **86**, 985-990.
- VOKURKA, A.; MALETIĆ, E.; BENJAK, A.; KAROGLAN-KONTIĆ, J.; PEJIĆ, I.; 2003: Application of molecular markers for analysis of presumed synonyms and homonyms within Croatian grapevine cultivars. In: E. HAJDU, E. BORBÁS (Eds.): *ISHS 2003. Acta Hort.* **603**, 581-584.

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Erratum

In the paper

Genetic relationships among grapevine cultivars native to Croatia, Greece and Turkey

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the sampling locations of cultivars Al üzüm and Karaerik in Table 1 have been confused. Al üzüm has been sampled at Olur, whereas Karaerik has been sampled at Erzincan.

The participating institutes have been specified in the title, but author's names haven't been assigned to their institute. S. ERCISLI is staff of the Faculty of Agriculture, Ataturk University, Erzurum, Turkey. All other authors are members of the Faculty of Agriculture, University of Zagreb, Croatia.

The publishers apologize for this error.