GENETIC DIVERSITY AND RELATIONSHIPS OF INDIGENOUS AND NEWLY BRED BULGARIAN GRAPE CULTIVARS ASSESSED BY NUCLEAR AND CHLOROPLAST MARKERS

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Abstract

Aim: Assessment of the genetic diversity and relationships in a group of 31 Bulgarian grape accessions through microsatellite markers.

Methods and results: Thirty-one accessions, including 20 old rare local and 11 newly bred varieties were characterized through 13 nuclear and 5 chloroplast microsatellite loci. The genetic diversity (0.81±0.01) obtained for the investigated group of cultivars was comparable to those reported for other grapevine germplasms. The low PI value ($1.0x10^{-16}$) allowed proper genetic identification and determination of synonyms. Microsatellite analysis of the 31 accessions resulted in 26 unique genotypes and 2 groups of synonyms. Four cases of supposed synonymy with local Bulgarian and foreign cultivars were rejected. Three chlorotypes, B, C and D, were defined among the studied cultivars, with a prevalence of chlorotype C (62 %).

Conclusion: The high genetic diversity found in the set of old rare grapevines demonstrated their importance as a rich source of alleles for breeding. The pattern of chlorotype distribution observed among local varieties confirmed the previous results and supports the hypothesis of an Eastern origin of local Bulgarian cultivars.

Significance and impact of the study: The obtained results provide an important support for the preservation of grape biodiversity in Bulgaria as well as for the clarification of genetic relationships between local and foreign cultivars.

Key words: grapevine, microsatellites, genetic diversity, chlorotypes

Résumé

Objectif: La diversité et les relations génétiques ont été étudiées dans un groupe de 31 cépages bulgares par des marqueurs microsatellites.

Méthodes et résultats: Trente et un échantillons, dont 20 anciens rares locaux et 11 nouvelles variétés produites ont été caractérisés par 13 microsatellites nucléaires et 5 microsatellites chloroplastiques. La diversité génétique (0,81± 0,01) obtenue pour le groupe de cépages étudiés était comparable à celles rapportées dans la littérature chez la vigne. La faible valeur PI (1,0x10-16) a permis l'identification génétique appropriée et la détermination des synonymes. L'analyse des microsatellites de 31 échantillons a révélé 26 génotypes uniques et deux groupes de synonymes. Quatre cas de synonymie supposée avec des cépages locaux bulgares et étrangers ont été rejetés. Trois chlorotypes, B, C et D, ont été définis parmi les variétés étudiées avec une prévalence du chlorotype C (62 %).

Conclusion: La grande diversité génétique observée parmi des vignes anciennes et rares a prouvé leur importance comme source d'allèles pour l'amélioration de la sélection. Le modèle de distribution des chlorotypes observés chez les variétés locales a confirmé des résultats déjà observés et soutient l'hypothèse d'une origine orientale des cépages locaux bulgares.

Signification et impact de l'étude: Les résultats obtenus ont un impact important pour la préservation de la biodiversité des vignes en Bulgarie ainsi que pour la clarification des relations génétiques entre les cépages locaux et étrangers.

Mots clés: vigne, microsatellites, diversité génétique, chlorotypes

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INTRODUCTION

Grapevine is a crop of major economic importance in Bulgaria where viticulture and winemaking have been traditional since ancient times. Due to the economic and social changes set in Bulgaria during the last 20 years, the previous diversity of local cultivars grown in the industrial vineyards is nowadays replaced by the predominant cultivation of few commercial cultivars spread worldwide. According to the 2009 statistics of the Bulgarian Ministry of Agriculture, varieties such as Merlot, Cabernet-Sauvignon, Chardonnay, Syrah, Rkatsiteli and Muskat Ottonel presently occupy more than 50 % of the area allocated for growing wine grapes. At the same time the famous traditional Bulgarian wine cultivars Dimyat, Pamid, Misket Cherven, Mavrud and Shiroka Melnishka occupy only 28 % of this area. Many other Bulgarian cultivars of minor economic interest, which were traditionally grown in the past, were replaced by modern ones and are now almost extinct. This situation raised a concern related to the regional specificity of viticulture and winemaking and the prevention of the erosion of grapevine diversity in the country. Taking this into account, an effort has been made to address the molecular characterization of the major autochthonous and valuable new locally selected grapevine varieties (Hvarleva *et al*., 2004 ; Dzhambazova *et al*., 2009). A special attention should also be paid to the detection, characterization and conservation of rare, neglected and endangered grapevine cultivars as a potential source of valuable traits. Such varieties are usually not included in the National List of Cultivars. Some of them can still be found in isolated farms and old vineyards, mixed with other varieties. There are also a number of rare local grapevine varieties stored in the grapevine collection of the Institute of Viticulture and Enology, Pleven (Bulgaria). However, the historical records tracing the origin, spread and ampelographic characterization of a large part of these varieties are limited and related to several sources dating from the first half of last century. Molecular characterization together with the available ampelographic data will allow the genetic identitification of these varieties as well as the verification of synonyms, homonyms and clones among this group.

Nuclear microsatellite markers have been favored for molecular identification of cultivars due to their high polymorphism, reproducibility and co-dominant nature. The extended application of these markers to a large number of cultivars in many countries has allowed the precise genetic identification and comparison of grapevine varieties from different wine growing regions and germplasm collections. The accumulated vast volume of microsatellite data has enabled the detection of synonyms, homonyms and clones as well as a parentage analysis (Thomas *et al*., 1993 ; Bowers and Meredith, 1997 ; Bowers *et al*., 1999a ; Sefc *et al*., 1998 ; Crespan and Milani, 2001 ; Vouillamoz *et al*., 2006 ; De Mattia *et al*., 2007 ; Karatafl *et al*., 2007 ; Salmaso *et al*., 2008 ; Ibáñez *et al*., 2009 ; Laiadi *et al*., 2009 ; Cipriani *et al*., 2010 ; Riahi *et al*., 2010).

The application of chloroplast microsatellite markers, which are maternally inherited in grapevine (Arroyo-García *et al*., 2002), provides important information about the frequency and geographical distribution of chlorotypes in a given group of cultivars or populations of wild grapes (Grassi *et al*., 2003, 2006 ; Imazio *et al*., 2006 ; Arroyo-García *et al*., 2002, 2006 ; Salmaso *et al*., 2010) as well as defines the direction in the cross in parentage analysis (Vouillamoz *et al*., 2006 ; Salmaso *et al*., 2008 ; Ibáñez *et al*., 2009 ; Cipriani *et al*., 2010).

In this study we used nuclear and chloroplast microsatellite markers to characterize a set of 31 old rare local (ORL) and newly bred (NB) Bulgarian grapevine accessions from the grapevine collection of the Institute of Viticulture and Enology, Pleven, Bulgaria, in order to assess the genetic diversity and relationship among the investigated cultivars and to clarify synonyms and clones within the group of rare accessions.

MATERIALS AND METHODS

1. Plant material

Thirty grapevine (*Vitis* vinifera ssp. sativa) accessions maintained in the germplasm collection of the Institute of Viticulture and Enology (Pleven, Bulgaria) were characterized through microsatellite analysis (Table1). Nineteen accessions are ORL varieties and 11 are NB cultivars bred in Bulgaria during the second half of last century (Kirmidchi, 1927 ; Nedelchev, 1938, 1951 ; Kondarev *et al*., 1962 ; Katerov and Kostov, 1964). The old Bulgarian cultivar 'Pamid', previously characterized at 8 microsatellite loci (Hvarleva *et al*., 2004 ; Dzhambazova *et al*., 2009), was also included in the study. The cultivar Cabernet-Sauvignon maintained at the collection of the AgroBioInstitute (Sofia, Bulgaria) was used as reference to standardize the size of nuclear and chloroplast microsatellite alleles.

2. DNA extraction and microsatellite analysis

Genomic DNA was isolated from 100 mg frozen leaves following the protocol of Murray and Thompson (1980). Grape samples were genotyped at 13 nuclear and 5 chloroplast microsatellites. The SSRs (simple sequence repeats) used for the analysis of nuclear polymorphism included the set of 6 SSRs previously proposed as a standard set for cultivar identification (This *et al*., 2004) and used in the GENRES081 project (http://www.genres.de/index.htm) : VVS2 (Thomas and Scott, 1993),

Table 1. List of 31 accessions of *Vitis vinifera* ssp. sativa L.: name, chlorotype (Ha), berry color (B-white, N-black, RG-red or RS-pink), use (T-table, W-wine) and microsatellite profile at 13 nuclear loci (allele size is given in base pairs, NA- no amplification)

Accession name*	H[a]	color	use	UCH29		VVMD5		VVMD7		VVMD8		VVMD25		VVMD27		VVMD28		VVMD36		VVS2		VVS5		ZAG21		ZAG62		ZAG79	
New cultivars (NB)																													
Biljana	C	B	T	289	309	226	230	246	246	141	147	244	258	183	195	218	258	248	268	134	134	111	119	200	200	199	203	230	246
Bulgaria	C	B	T	207	309	230	234	244	246	141	143	252	258	179	195	244	268	252	262	134	148	149	149	200	200	185	191	254	254
Chaush x Bolgar	C	B	T	207	289	230	236	246	248	143	147	258	258	179	185	234	258	274	276	134	134	111	111	206	214	185	187	248	250
Cherven Septemvrijski	B	N	T	289	303	234	236	230	244	141	143	244	258	181	195	244	268	248	262	140	154	89	89	200	202	195	203	246	258
Dunavski Lazur	B	B	W	211	291	234	238	234	244	143	153	242	244	179	179	236	244	264	268	132	132	91	129	204	206	179	189	248	254
Maj	C	B	T	209	297	230	234	236	236	143	147	242	258	179	183	258	258	242	252	134	142	95	111	200	206	187	203	238	246
Maj 10	C	B	T	207	289	230	234	244	246	143	143	244	258	179	185	218	258	274	294	134	134	149	149	190	200	185	203	250	250
Plovdiv	D	B	T	211	291	234	236	246	246	143	157	244	252	179	181	244	268	252	262	148	154			200	206	185	191	254	258
Slavijanka	C	B	W	211	309	224	232	236	240	143	175	242	244	179	181	218	234	248	274	132	132	95	95	200	202	187	187	246	254
Srebrostrouj	B	B	W	209	309	230	238	248	248	153	169	258	270	179	181	234	236	238	252	132	140	91	129	190	200	193	199	248	254
Trakija	B	B	W	207	211	230	238	236	236	143	147	252	252	181	185	246	258	262	274	132	142	111	149	190	200	187	187	250	258
Rare old cultivars (ORL)																													
Bela Dinka	D	B	W	211	309	224	244	236	244	143	147	242	258	179	195	236	244	250	250	132	142	111	149	204	206	187	193	248	258
Gamza Varnenska	D	N	W	209	295	224	224	230	236	143	143	242	242	189	195	236	246	242	252	132	132	117	149	204	206	187	199	242	248
Keratzouda	C	B	W	211	211	236	236	236	236	141	143	242	252	179	189	236	260	264	286	132	142	111	125	200	212	187	187	250	250
Kokorko	B	B	W	207	295	236	244	236	246	141	159	252	258	187	189	248	262	262	262	142	142	111	111	200	212	187	193	242	250
Kostenurkovi Jajtca	D	RG	T	211	297	230	234	236	244	141	143	244	244	185	185	234	236	248	248	138	144	111	119	202	202	185	187	250	250
Mechtka	C	N	W	211	299	226	230	244	244	141	157	244	244	185	185	258	260	264	274	132	138	111	111	200	202	185	203	242	250
Pamid	C	RS	W	207	297	224	244	236	236	147	159	252	258	183	189	248	258	262	274	134	142	101	111	200	206	187	187	242	250
Pamid Bjal	C	B	W	207	297	224	244	236	236	147	159	252	258	183	189	248	258	262	274	134	142	101	111	200	206	187	187	242	250
Pamid Cher	C	N	W	207	297	224	244	236	236	147	159	252	258	183	189	248	258	262	274	134	142	101	111	200	206	187	187	242	250
Pamid Edar	C	RG	W	207	297	224	244	236	236	147	159	252	258	183	189	248	258	262	274	134	142	101	111	200	206	187	187	242	250
Pamid Siv	C	N	W	207	297	224	244	236	236	147	159	252	258	183	189	248	258	262	274	134	142	101	111	200	206	187	187	242	250
Parmak Tcherven	C	RG	T	207	211	230	244	236	244	141	157	244	252	181	185	236	258	252	264	140	140	149	149	202	214	185	187	250	258
Razakija Bjala	C	B	T	209	295	226	226	236	244	143	143	242	242	183	195	236	278	248	262	140	140	111	111	200	202	187	193	244	256
Razakija Cherna	B	N	T	211	309	234	234	244	246	143	157	244	252	179	185	234	244	262	268	136	142	149	149	190	206	187	199	246	246
Razakija Cherna s Tochitsi	C	N	T	211	297	224	234	236	244	143	143	244	258	179	195	248	270	252	262	136	142	119	149	200	206	187	203	250	250
Razakija Chervena	C	RG	T	209	295	226	226	236	244	143	143	242	242	183	195	236	278	248	262	140	140	111	111	200	202	187	193	244	256
Razakija Mirizliva	C	B	T	211	297	226	226	244	246	143	147	242	258	185	195	236	246	242	248	142	142	119	119	200	206	193	193	256	256
Razakija Pembjana	C	RS	T	209	209	234	244	236	246	141	157	242	258	181	185	234	244	262	286	134	144	119	149	204	206	187	199	242	258
Varnensko Bialo	C	B	W	211	211	226	230	240	244	141	147	244	244	179	195	258	260	262	262	144	144	119	119	206	206	199	203	250	256
Vinenka	B	B	W	209	209	232	236	236	246	147	157	242	258	179	195	246	258	262	262	132	142	101	111	202	204	187	203	236	242

*Accession names were spelled according to the VIVC (Vitis International Variety Catalogue)

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Table 2. Genetic diversity parameters found in the set of local cultivars: Na (number of alleles), Ne (number of effective alleles), Ho (observed heterozygosity), He (expected heterozygosity) and PI (probability of identity). Synonymous genotypes were excluded.

	Na cumulative	Na average	Ne cumulative	Ne average	Ho average	He average	PI cumulative
Set of 26 genotypes	115	8.85±0.59	71	5.45±0.35	0.77±0.03	0.81±0.01	$1.0x10^{-16}$
ORL - 15 genotypes	87	6.69±0.47	61	4.70±0.33	0.74±0.04	0.78±0.02	$3.8x10^{-15}$
NB - 11 genotypes	91	7.00±0.39	65	4.98±0.35	0.82±0.05	0.79±0.02	$1.0x10^{-15}$

ORL, old rare local cultivars; NB, newly bred cultivars

VVMD5, VVMD7, VVMD27 (Bowers *et al*., 1996, 1999b), ssrVrZAG62 and ssrVrZAG79 (Sefc *et al*., 1999). Seven additional nuclear loci were selected based on their high informative content: ssrVvUCH29 (Lefort *et al*., 2002), ssrVrZAG21 (Sefc *et al*., 1999), VVS5 (Thomas and Scott, 1993), VVMD8, VVMD25, VVMD28 and VVMD36 (Bowers *et al*., 1999b). The following chloroplast loci cpSSR3, cpSSR5, cpSSR10 (Weising and Gardner, 1999), ccSSR5 and ccSSR9 (Chung and Staub, 2003) were chosen for evaluation of chloroplast polymorphism. Amplification reactions and PCR product analysis were performed as described previously (Hvarleva *et al*., 2004; Dzhambazova *et al*., 2009).

The allele sizes of the studied accessions were standardized according to the corresponding values of Cabernet-Sauvignon published by Sefc *et al*. (2000) (for loci VVS2, VrZAG21, VrZAG62, VrZAG79, VVMD5, VVMD7 and VVMD27) or available on the VIVC website (Vitis International Variety Catalogue, http://www.vivc.de/index.php) for comparison of the microsatellite profiles of local accessions and cultivars published in this database.

3. Genetic diversity analysis

The calculation of allele frequency, expected (He) and observed (Ho) heterozygosity, and probability of identity (PI) was performed with GENALEX software (Peakall and Smouse, 2006). Genetic distances between grapevine genotypes were calculated as [-ln (proportion shared alleles)] using Microsat (Minch *et al*., 1997), and the obtained data were used for the construction of a dendrogram using the KITCH from PHILIP package (Felsenstein, 1989) and Treeview programs (Page, 1996).

RESULTS

Thirty-one accessions of local grape varieties were genotyped at 13 nuclear and 5 chloroplast microsatellite loci. The studied varieties formed two groups. A first group consisted of ORL cultivars and included 19 accessions collected from various vineyards in Bulgaria during the last century. The old Bulgarian variety 'Pamid', subject of industrial cultivation at present, was also included in this group. The second group consisted of NB cultivars and included 11 local cultivars bred in the country during the second half of last century. The analyzed microsatellite loci were amplified in all but one of the studied grapevine samples. The exception was locus VVS5, which did not amplify in one grape accession, cultivar 'Plovdiv', even after modifying the PCR conditions. The obtained genotypes are presented in Table 1.

1. Genetic diversity and genetic relationship among local varieties based on nuclear polymorphisms

The nuclear microsatellite analysis of 31 accessions resulted in the identification of 26 different microsatellite profiles. These 26 unique genotypes were used for the assessment of the genetic diversity within the whole set of investigated local varieties and within each ORL and NB group. The cumulative number of alleles for the 13 nuclear loci was 115, with a mean number of alleles per locus of 8.85±0.59 (Table 2). The number of effective alleles was found to be 71, with an average of 5.45±0.35, which is similar to those found for other groups of cultivars (Laiadi *et al*., 2009). The calculated mean value of genetic diversity (expected heterozygosity-He) was 0.81±0.01, which is comparable to the corresponding values reported for other sets of grapevine cultivars (Sefc *et al*., 2000; Hvarleva *et al*., 2004; Vouillamoz *et al*., 2006). The observed heterozygosity (Ho) was lower than the expected one (He), with a mean value of 0.77±0.03 over all loci examined (Table 2). The most informative marker for the studied group of cultivars was VVMD28, with 12 alleles, Ho 0.96 and PI 2.9x10-2. The estimated low value of cumulative PI for all nuclear loci ($1.0x10^{-16}$, Table 2) corresponded to high genetic diversity. The calculation of the genetic diversity parameters separately for the two subgroups (ORL and NB) showed no significant differences between them (Table 2). The obtained values of Ne were 4.70±0.33 for ORL and 4.98±0.35 for NB, while He was 0.78±0.02 for ORL and 0.79±0.02 for NB.

The data from the nuclear microsatellite analysis were further used to construct a dendrogram demonstrating the genetic relationships between the analyzed varieties (Figure 1). The 31 accessions were distributed in 10 groups (from A to J). The two cultivars 'Srebrostrouj' and 'Dunavski Lazur' were placed together in the most

divergent cluster A, which remained outside the clusters formed by the rest of the accessions. The dendrogram showed two subclusters of identical genotypes: the first, corresponding to the group of 5 accessions related to the cultivar 'Pamid' and located inside cluster J, and the second, including the old varieties 'Razakija Bjala' and 'Razakija Chervena' located inside cluster C. Three of the observed clusters (C, F and I) were comprised of only ORL varieties, while one cluster (A) included only NB varieties. The remaining 3 clusters (E, H and J) included both old and new cultivars. The old local varieties 'Pamid' and 'Kokorko' (cluster J) and 'Mechtka' and 'Kostenurkovi Jajtca' (cluster F) were found to be genetically related. The closest relationship was determined between the NB varieties 'Plovdiv'and 'Bulgaria' as well as 'Maj10' and 'Chaush x Bolgar' (cluster H), due to common parents in their pedigree.

2. Genetic diversity based on chloroplast polymorphisms

The genetic diversity, based on variation among the chloroplast genome of the studied grape varieties, was assessed through analysis of 5 chloroplast microsatellite loci previously used for characterization of wild and cultivated grape germplasms (Arroyo-Garcia *et al*., 2002, 2006 ; Dzhambazova *et al*., 2009 ; Laiadi *et al*., 2009 ; Ibáñez *et al*., 2009). The chlorotype of each accession was determined. Each of the 5 analyzed chloroplast loci was represented by 2 alleles, which were combined in 3 chlorotypes, B, C and D, according to Arroyo-García *et al*. (2002) (Table 3). Considering that the identical genotypes mentioned in the previous section also have identical chlorotypes, the frequencies of the 3 chlorotypes were calculated for the 26 unique genotypes. The results showed that the most abundant chlorotype is C (62 %), followed by B (23 %) and then D (15 %).

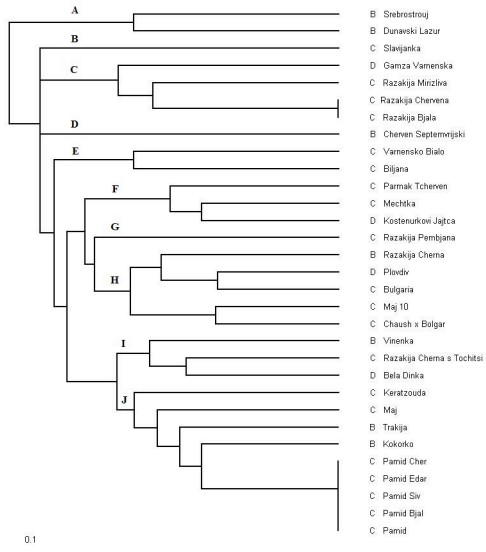

Figure 1. Dendrogram of 31 accessions of local grapevines based on the data of 13 nuclear microsatellite markers.
The varieties are grouped in 10 clusters (A-J).
The letters B, C and D in front of the name of each grape variety denotes the chlorotype of the sample.

DISCUSSION

1. Genetic diversity and relationships based on nuclear polymorphisms

The obtained high values of genetic diversity parameters (i.e., number of effective alleles, expected and observed heterozygosity) for the set of 26 genotypes investigated were comparable to those obtained for other grape germplasm pools (Sefc *et al*., 2000 ; Vouillamoz *et al*., 2006 ; Laiadi *et al*., 2009). The present study included the ORL group comprised of 20 accessions with unknown pedigree and the NB group comprised of 11 accessions of new varieties bred in Bulgaria during the second half of last century. The 11 analyzed NB varieties were generated by crosses between genetically divergent, both foreign and Bulgarian varieties, which is prerequisite for the high genetic diversity found in this group. The comparison of genetic diversity parameters calculated for the ORL and NB groups showed no significant differences between the 2 groups (Table 2). These results demonstrated the significance of the genetic pool of old rare varieties as a potential source of valuable alleles.

The cumulative PI for all loci was found to be very low, corresponding to $1.0x10^{-16}$, which allows proper genetic identification and determination of synonyms (Sefc *et al*., 2001). The nuclear microsatellite study of 31 accessions resulted in the identification of 26 unique DNA fingerprints and two groups of accessions with identical genotypes. Four accessions ('Pamid Siv', 'Pamid Cher', 'Pamid Bjal' and 'Pamid Edar') were collected from the Bulgarian vineyards in the last century and

Table 3. Chlorotypes, their frequency (%) in the set of investigated cultivars and allele sizes (bp) at 5 polymorphic chloroplast loci. Synonymous genotypes were excluded.

Chlorotype	cpSSR3	cpSSR5	cpSSR10	ccSSR5	ccSSR9	Frequency
B	106	105	115	255	165	23%
C	106	105	116	255	165	62%
D	107	104	115	254	165	15%

reported as intra-varietal diversity of the old variety 'Pamid'. They differ phenotypically from 'Pamid' in their berry color and size (Kirmidchi, 1927). 'Pamid Edar' was thought to have originated from 'Pamid' by polyploidization. Here the comparison of the microsatellite profiles of 5 accessions in the 'Pamid' group revealed that 'Pamid Bjal', 'Pamid Edar' 'Pamid Siv' and 'Pamid Cher' matched the old cultivar 'Pamid' at all 13 loci. Thus, they were considered sports, derived by somatic mutations of the cultivar 'Pamid', which were not distinguished with the markers used in this study. Generally, the level of polymorphism detected with microsatellite markers among grape clones is very low, although recently the clones of several varieties were successfully discriminated with a large number of SSR markers (Regner *et al*., 2000 ; Riaz *et al*., 2002 ; Moncada *et al*., 2006 ; Pelsy *et al*., 2010). The application of proper and large number of SSR markers is necessary for the discrimination of these 'Pamid'-related accessions. As far as the number of somatic variants of a cultivar could be accepted as a measure of its age and spread (This *et al*., 2006), the presence of these few clones confirmed the fact that 'Pamid' is an old cultivar that has been wide spread in the area of the Balkans during previous centuries.

Among the analyzed grapes there is another set of 6 accessions that were supposed to have a close relationship because their names are derivatives of 'Razakija' ('Razakija Bjala', 'Razakija Chervena', 'Razakija Cherna', 'Razakija Cherna s Tochitsi', 'Razakija Mirizliva' and 'Razakija Pembjana'). This group of accessions revealed 5 distinct allelic profiles. Two of them, 'Razakija Bjala' and 'Razakija Chervena', showed identical genotypes and therefore could be considered synonyms. However, since the two accessions differ in their berry color, they are most probably somatic variants of the same original genotype. The microsatellite profiles of the rest of the 'Razakija'-accessions ('Razakija Cherna', 'Razakija Cherna s Tochitsi', 'Razakija Mirizliva' and 'Razakija Pembjana') differ vastly from each other and from the profiles of the above mentioned two varieties, thus leading to the conclusion that they are genetically distinct cultivars. This divergence of the 'Razakija'-varieties was also demonstrated by their position in the dendrogram. The 6 'Razakija'-varieties are grouped in 4 distinct clusters according to their genetic relationship : 3 accessions in cluster C and 1 in clusters G, H and J. 'Razakija Mirizliva' was grouped next to the clones 'Razakija Bjala' and 'Razakija Chervena', which revealed their genetic relationship. 'Razakija Cherna s Tochitsi' was grouped together with the ORL variety 'Bela Dinka'.

Proceeding from the similarity of the names 'Razakija' (Bulgarian) and 'Razaki' (Greek) we compared the microsatellite profiles of Bulgarian 'Razakija'-accessions with those of 4 'Razaki' genotypes (corresponding to 6 cultivars) published in the Greek *Vitis* Database (http://gvd.biology.uoc.gr/gvd/contents/databases/index.htm), at 6 loci common to both studies. None of the Bulgarian 'Razakija'-varieties corresponded to any of the Greek 'Razaki' genotypes. The comparison of the microsatellite profiles of Bulgarian 'Razakija'-accessions and another cultivar with a similar name, 'Rosaki', known as one of the numerous synonyms of the popular variety 'Afus Ali' (Ibáñez *et al*., 2009 ; VIVC database), excluded the possibility of a common genetic origin. Moreover, the chlorotype of 'Afus Ali' is A (Ibáñez *et al*., 2009), while the 6 local 'Razakija'-varieties bear chlorotype C (5) and B (1). Thus, we can conclude that the 6 Bulgarian 'Razakija'-varieties are authentic local cultivars that are genetically distinct from 'Rosaki' and 'Razaki'-cultivars.

The microsatellite profile of accession 'Gamza Varnenska' was compared with that of the old cultivar 'Gamza' previously analyzed at 8 nuclear loci common with recent study (Hvarleva *et al*., 2004 and 2005). No identity was found between the DNA profiles of these two varieties. Both genotypes differed vastly, sharing only 6 alleles at 5 loci, and thus they were considered as distinct varieties.

According to the VIVC database the numerous synonyms of the popular cultivar 'Muscat blanc à petits grains' include 'Bela Dinka', a variety spread on the territory of Bulgaria and former Yugoslavia. The microsatellite profile of the Bulgarian accession 'Bela Dinka' was compared with that of 'Muscat blanc à petits grains' and its Bulgarian synonym 'Tamyanka' as reported in the VIVC database. The profile of the Bulgarian accession did not match the reference profile of either 'Muscat blanc à petits grains' published in the VIVC database at the six standard loci or 'Tamyanka' reported by Hvarleva *et al.* (2004) and Dzhambazova *et al.* (2009). The possibility for synonymy of varieties 'Muscat blanc à petits grains'/'Tamyanka' and the Bulgarian accession 'Bela Dinka' was excluded.

According to the dendrogram, the cultivars 'Kokorko' and 'Pamid' were genetically close. Comparison of the microsatellite profiles of these varieties showed that they shared 15 out of 26 alleles and at least 1 allele per locus, which indicated a possibility for parent-offspring relationship between them.

2. Genetic diversity based on chloroplast polymorphism

Chloroplast microsatellites have been extensively exploited for the evaluation of frequency and geographical distribution of chlorotypes among wild and cultivated grapevines (Arroyo-Garcia *et al*., 2002, 2006 ; Doulati Baneh *et al*., 2007 ; Dzhambazova *et al*., 2009 ; Ibáñez *et al*., 2009 ; Laiadi *et al*., 2009 ; Beridze *et al*., 2011).

Considering chloroplast DNA variation among the 26 unique genotypes, it was found that chlorotype C was the most abundant chlorotype with a frequency of 62 % (Table 3). As far as chlorotype C is reported to be a distinctive mark for the grapes that originated from the Near and Middle East (Arroyo-Garcia *et al*., 2002, 2006), we can conclude that a large part of the selected set of grapes has an Eastern origin.

Chlorotype B is considered ancestral due to its abundant presence in the other *Vitis* species (Arroyo-Garcia *et al*., 2002) and even distribution in the Eurasian region. It was found in the analyzed local accessions at quite high frequency of 23 % (Table 3) in comparison with the low frequency (8 %) found in most groups of grapes throughout Eurasia (Arroyo-Garcia *et al*., 2006). Considering the group of 15 native rare genotypes, 3 accessions (20 %) bear chlorotype B. The abundance of chlorotype B in the investigated set of old varieties could be casual, because the number of studied cultivars was low. The other possibility could be the speculation that some ancestral grapevines, bearing chlorotype B together with some adaptive traits, have been favored through natural or human selection. Due to the ancestral nature of chlorotype B, it could be assumed that it has appeared most early in the evolution of the *Vitis* genus and thus, the *Vitis* specimens bearing that chlorotype have accumulated enough mutations in the genome, some of them corresponding to valuable traits. Moreover, few studies reported that chloroplast genome itself could determine important traits such as cold tolerance (Chung *et al*., 2007) or disease resistance (Avni *et al*., 1992). However, such an interpretation should be made with caution, given that a low number of randomly sampled specimens were analyzed. More accessions with a local origin should be analyzed for better understanding of the pattern of distribution of this chlorotype among the local grapevine germplasms.

Chlorotype D was found in 15 % of the investigated accessions, thus supporting the previously observed frequency of this chlorotype (16 %) among Bulgarian native cultivars (Dzhambazova *et al*., 2009).

None of the local Bulgarian varieties was found to bear chlorotype A. This result is in agreement with the observed low frequency of chlorotype A on the Balkan Peninsula (Arroyo-Garcia *et al*., 2006) as well as with our previous results where only 1 of 19 local varieties, 'Bolgar' (synonym of 'Afus Ali'), had chlorotype A (Dzhambazova *et al*., 2009).

The pattern of chlorotype distribution obtained in this study (i.e., the prevalence of chlorotype C) as well as the coexistence of chlorotypes C and D within native Bulgarian germplasms are in agreement with our previous results (Dzhambazova *et al*., 2009). They support the proposed Eastern origin of native Bulgarian grapes as a result of intensive exchange of grape material during the past centuries between the Eastern Mediterranean and the Near/Middle East, where these two chlorotypes are common. This is in accordance with the history and location of Bulgaria on the crossroad between Europe and Asia. In ancient times the territory of present-day Bulgaria has been inhabited by different nations, and some of them, including Bulgarian, coming from the East, brought along their culture, tradition and living.

CONCLUSION

Here we presented the use of microsatellite markers for the characterization of genetic diversity of local varieties at the chloroplast and nuclear DNA level. The results obtained in this study allowed precise genetic identification of newly bred and rare, endangered grapevine cultivars and detection of synonyms and clones, thus providing an important support for the preservation of genetic diversity in the country. The high genetic diversity found in the set of ORL varieties demonstrates that they represent a rich source of alleles that can contribute to the improvement of breeding programs.

The assessment of chloroplast diversity within the ORL varieties confirmed the previously obtained distribution and frequency of chlorotypes in Bulgaria (i.e., prevalence of chlorotype C) that showed the Eastern origin of local grapevines.

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