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Assessment of Diversity in Grapevine Gene Pools from Romania and Republic of Moldova, Based on SSR Markers Analysis

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1. Introduction

In the last 15 years, inventory of the genetic diversity for agricultural purposes and environmental protection has become a constant preocupation of European Union in the biological research field.

Due to the economical importance of *Vitis vinifera* species, and to the long viticulture tradition of many countries, the European Union has developed international projects having as main objective improving the knowledge, conservation and sustainable use of *Vitis* genetic resources in Europe.

One of the first such initiatives was GENRES CT96 081 (European Network for Grapevine Genetic Resources Conservation and Characterization) project (started in March 1997, ended in February 2002), aiming to establish the European *Vitis* Database, with free access via Internet, in order to enhance the utilization of relevant and highly valuable germplasm in breeding. The *Vitis* Working Group which has been constituted at the end of the GENRES project, decided to start the establishment of an SSR-marker database as part of the European *Vitis* database (www.ecpgr.cgiar.org/workgroups/vitis/).

Another international project, financially supported by the Government of Luxembourg and coordinated by Biodiversity International, named „*Conservation and sustainable use of grapevine germplasm in Caucasus and Northern Black See region*", was developed during the years 2003-2008, having as main result a better conservation of germplasm collections in this region (Armenia, Azerbaijan, Georgia, Moldova, Russian Federation and Ukraine) which represent a very unique and rich source of grapevine genetic variation.

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Starting from 2010, a third important European project is in course (ending in 2014): the COST project named *„East-West collaboration for grapevine diversity exploration and mobilization of adaptive traits for breeding"*. In this project are involved 35 member states countries (Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, The Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey, United Kingdom, Serbia, Former Yugoslav Republic of Macedonia), one cooperating state Israel, and also reciprocal agreements with Australia, New Zealand, South Africa and Argentina. The main objective is to create a knowledge platform about European grapevine germplasm, essential for (1) the maintenance of genetic resources, (2) the discrimination and identification of grapevine varieties and (3) the availability and exchange of germplasm. These grapevine collections gathering autochthonous and unique varieties with valuable genetic- and phenotypical traits, are also essential for long-term conservation and sustainable use of this economically important species.

Romania and Republic of Moldova have a multimillenary tradition in grapevine cultivation and wine production. Thus, both ancient- and newly created grapevine varieties from these two countries are valuable gene resources which must be inventored for a complete genetic characterization, based on reliable molecular markers.

As a response to the European Union initiative for inventory and conservation of grapevine genetic resources, two research teams from Romania and Republic of Moldova have initiated a research direction aiming to establish a genetic profile – based on SSR markers analysis - for the grapevine varieties cultivated in these two countries, in order to make an inventory of them, and also to facilitate the registration of Romanian- and Moldavian originated cultivars in the European *Vitis* Database. These specific genetic profiles represent also a real passport that certifies their authenticity and represents a guarantee for further preservation of grapevine cultivars with economic value.

2. SSR markers for designing the genetic profile of grapevine cultivars

SSR (***s***imple ***s***equence ***r***epeats, or microsatellites) are arrays of short motifs, highly repeated, of 1 to 6 base pairs. These locus specific markers are characterized by hypervariability, abundance, high reproducibility, Mendelian inheritance and codominant nature. They are not affected by environmental factors such as soil, climate, cultivation methods or diseases (Scott et al., 2000; Meredith, 2001).

Therefore, microsatellites are the favourite type of DNA markers used to characterized the grapevine cultivars, their properties enabling a wide range of applications: cultivar identification and discrimination, parentage testing and pedigree reconstruction, genetic stability of all morphological-physiological features, yield capacities of each cultivar, management of germplasm collection (Thomas & Scott, 1993; Bowers et al., 1996; Thomas et al. 1998; Bowers et al., 1999; Sefc et al., 1999; Sefc et al., 2000; Sefc et al., 2001). Their relative abundance within the genome and being very easy to be detected by PCR reaction explain that at present, more than 600 grapevine microsatellite markers are available (Moncada et al., 2006), making possible refined genetic profiles of different varieties.

Analysis of microsatellite loci in different cultivars (genotypes) provides useful information about: (i) genetic data for each conserved genotype; (ii) taxonomy relatedness, geographic

origin and ecological aspects (genetic diversity); (iii) the distinctness between cultivars, uniformity of planting material belonging to the same genotype, and genetic stability of all morphological-physiological features and yield capacities of each cultivar.

The *Vitis* Working Group highly recommended to include in each SSR-marker genotyping in grapevine cultivars, six microsatellite loci which proved to have a high variability among different cultivars, making possible a good discrimination even between very close related ones. These SSR markers are: VrZAG62, VrZAG79, VVS2, and VVMD5, VVMD7, VVMD27. In our researches, we obtained good results with five of these SSR markers, along with other ten microsatellite loci.

From our work during the 7 past years, on grapevine cultivars genotyping based on SSR markers, for this book chapter we have made a selection of the most interesting results, to be presented. Some of these results have already been published (Gheţea et al., 2010, a; b), some others are in course to be published.

3. The grapevine cultivars for which genetic profiles have been obtained

The grapevine cultivars considered for the study are among the most economically important, and encountered in the vineyards, in the two countries. The Romanian grapevine cultivars are included in the Official Catalogue of Cultivated Plants from Romania, and are maintained in the stock collection of the National Institute for Research and Development for Biotechnologies in Horticulture (NIRDBH), Ştefăneşti-Argeş. The Moldavian grapevine cultivars are in the stock collection of the National Institute for Viticulture and Oenology, Chişinău.

In this paper, we present our results on 14 grapevine cultivars, 7 cultivars from each of the two stock collections.

3.1 The grapevine cultivars from Romanian stock collection

Although, in Romania, vineyards produce grapes of unsurpassed quantity and quality with autochthonous genotypes, these cultivars are known and appreciated only in few regions. Also, the new breeders' creations are planted only in local areas and are commercialized mostly on national market. As Romanian grapevine cultivars were not studied from the genetic point of view, very limited information is available about their origin and gene pool value. Such genetic data regarding both the ancient and new creations of grapevine varieties from Romania become essential for recognition and registration in the International Grapevine Genome database. Moreover, this must be the main reason to promote a sustained research activity for a complete evaluation of Romanian grapevine genetic resources. So, the first step was to establish a core colection and a research program with the following objectives:

a. to verify the genetic identity of the cultivars, their stability and integrity during producing and conservation;
b. to identify duplicates in the grapevine collection, to eliminate them from the collection and to guaranty the authenticity of the planting material;
c. to prove the distinctness of the ancient and new grapevine varieties, important requirement for characterization and registration of Romanian germplasm.

d. Expectations as results from these activities are: a) a complete view of the Romanian grapevine gene resources; b) improve knowledge of the patent of distribution of grapevine genetic diversity; c) the genetic characterization of the Romanian cultivars would be equally very useful for all research units, for the owners of genotypes, for the grapevine growers and wine makers.

The cultivars we are presenting are as follows: (Dobrei et al., 2005):

1. **Italian Riesling** – opinions concerning its origin are different: German from Rhine Valley, or Austrian from Styria region, or Italian origin.
2. **Sauvignon** – French origin; with large areas cultivation; extensively cultivated in Bordeaux region.
3. **Cabernet Sauvignon –** French origin, old cultivar obtained in Bordeaux region, by cross-breeding between *Cabernet Franc* and *Sauvignon Blanc*; high geographical dispersion.
4. **Tămâioasă Românească** – uncertain origin; very old cultivar, known in ancient Greece as "Anathelion moschaton", and also during in Roman Empire, as "Aspinae"; cultivated in Romanian vineyards from ancient times, it is considered as autochthonous cultivar.
5. **Fetească neagră** – ancient Romanian cultivar, obtained by empirical selection from *Vitis sylvestris* Gmel; originated on the Prut river, in Iasi region.
6. **Fetească albă** – considered as autochthonous cultivar, obtained by empirical selection, from *Fetească neagră* cultivar; extensively cultivated in Romanian vineyards.
7. **Fetească regală** – Romanian origin cultivar, it is supposed to be result of a natural hybridization obtained by cross-breeding between two autochthonous cultivars: *Fetească albă* and *Grasă de Cotnari*.

3.2 The grapevine cultivars from Moldavian stock collection

A constant preoccupation in Moldavian breeding programs in viticulture, during the last 40 years, was obtaining valuable grapevine varieties, including large bunches and berries, early maturation of fruits, high accumulation of sugar, seedless berries, resistance of unfavourable environmental factors (drought, harsh winter conditions, diseases and pests) (Savin et al., 2010).

The cultivars we are discussing here are as follows:

1. **Cabernet Sauvignon** – its origin is mentioned above (at 3.1. section).
2. **Fetească neagră** – Romanian origin (see above).
3. **Fetească albă** – Romanian origin; extensively cultivated in Moldavian vineyards (see previous section).
4. **Fetească regală** – Romanian origin (see previous section).
5. **Muscat timpuriu de Bucureşti** – table grapes with early ripening, Romanian origin; obtained by cross-breeding between Romanian cultivars *Coarnă albă* and *Regina Viilor*.
6. **Timpuriu de Cluj** – table grapes, Romanian origin; obtained by cross-breeding between two Romanian cultivars: *Crâmpoşie* and *Frumoasă de Ghioroc*.
7. **Apiren extratimpuriu** – seedless cultivar obtained at National Institute for Viticulture and Oenology, Chişinău, by cross-breeding between *Urojainyi* and *Kismis turkmenski* cultivars; valuable for early ripening, high fertility and resistance to harsh winter conditions.

4. Methodology

DNA was extracted from young leaves of different grapevine genotypes, according to the method reported by Vallejos et al. (1992). For genotyping the selected cultivars, primer pairs for 15 microsatellite loci were chosen, 5 of which being recommended as reference SSR markers by the *Vitis* Working Group (www.ecpgr.cgiar.org/workgroups/vitis/).

The forward primer of each pair has been marked with one of the four fluorochromes: 6-FAM, NED, VIC or PET. The liophylised primers have been obtained from Applied Biosystems, USA.

For the amplification reaction, a thermocycler (Bio-Rad) with Peltier system was used. Optimal primer annealing temperature, presented in Table 1, was chosen for each primer pair, according to the results obtained in the temperature gradient PCR.

The PCR mix have had the following composition: 5X Colorless GoTaq® Flexi Buffer (Promega) – 5µl; $MgCl_2$ – 0,75µl; dNTP mix, 10mM (Promega) – 0,5µl; primer 1 – 1µl; primer 2 – 1µl; DNA sample – 30-45 ng/µl; GoTaq® DNA Polymerase (Promega) – 0,25µl; ddH_2O – X µl (X was calculated for each sample, depending on the volume of DNA sample, to a final volume of 25µl).

The PCR reactions were performed on an GeneAmp PCR System 9700 thermocycler (Applied Biosystems), using for the annealing step, the optimal temperature established for each primer pair (51^0C, 52^0C, 54^0C or 56^0C) (see Table 1).

Locus	Allele size range (bp) cited in the literature	Primer	
		Sequence	T °$_{annealing}$
ssrVrZAG7[1]	106 – 158	(F) gtggtagtgggtgtgaacggagtgg (R) aacagcatgacatccacctcaacgg	56°C
ssrVrZAG12[1]	140 – 172	(F) ctgcaaataaatattaaaaaattcg (R) aaatcctcggtctctagccaaaagg	51°C
ssrVrZAG15[1]	163 – 193	(F) ggattttggctgtagttttgtgaag (R) atctcaagctgggctgtattacaat	54°C
ssrVrZAG62[1]	185 – 203	(F) ggtgaaatgggcaccgaacacacgc (R) ccatgtctctcctcagcttctcagc	54°C
ssrVrZAG79[1]	236 – 260	(F) agattgtggaggagggaacaaaccg (R) tgcccccattttcaaactcccttcc	54°C
VVS1[2]	160 – 205	(F) acaattggaaaccgcgcgtggag (R) cttctcaatgatatctaaaaccatg	54°C
VVS2[2]	129 – 155	(F) cagcccgtaaatgtatccatc (R) aaattcaaaattctaattcaactgg	54°C
VVS4[2]	167 – 187	(F) ccatcagtgataaaacctaatgcc (R) cccaccttgcccttagatgtta	56°C
VVMD6[3]	194 – 214	(F) atctctaaccctaaaaccat (R) ctgtctaagacgaagaaga	52°C
VVMD7[3]	233 – 263	(F) agagttgcggagaacaggat (R) cgaaccttcacacgcttgat	54°C
VVMD14[3]	222 – 250	(F) catgaaaaaatcaacataaaagggc (R) ttgttacccaaacacttcactaatgc	54°C

VVMD17[3]	212 – 236	(F) tgactcgccaaaatctgacg (R) cacacatatcatcaccacacgg	52°C
VVMD21[3]	243 – 266	(F) ggttgtcttatggagttgatgttgc (R) gcttcagtaaaaagggattgcg	52°C
VVMD27[3]	173 – 194	(F) gtaccagatctgaatacatccgtaagt (R) acgggtatagagcaaacggtgt	54°C
VVMD36[3]	244 – 315	(F) taaaataataatagggggacacggg (R) gcaactgtaaaggtaagacacagtcc	56°C

([1] Sefc et al., 1999; Nuclear SSR primers of the Centre for Applied Genetics, University of Agriculture, Vienna, Muthgasse 18, A-1190 Vienna, Austria)
([2] Thomas & Scott, 1993; Thomas et al., 1998; Nuclear SSR primers of the Division of Horticulture CSIRO, Adelaide, Australia)
([3] Bowers et al.,1996; Bowers et al.,1999; Nuclear SSR primers of the Department of Viticulture and Enology, University of California, Davis, USA)

Table 1. List of the analysed SSR loci

The PCR reaction steps were:

I -	95^0C → 4 minutes (initial denaturation step)	
	95^0C → 1 minute	x 35 cycles
II -	X^{0}C (X=51^{0}C, 52^{0}C, 54^{0}C or 56^{0}C) → 1 minute	
	72^0C → 1 minute	
III -	72^0C → 7 minutes (final elongation step)	

The efficiency of the amplification reaction was analysed in a 2% agarose gel, in TAE buffer, according to Ausubel et al. (1990). A 5µg/ml ethidium bromide solution was used for the analysis of amplicon bands in UV light.

The amplicon genotyping was performed at an ABI PRISM 3100 Genetic Analyzer, using ROX 500 as internal standard. The samples were analysed with the GeneMapper program.

5. Results and discussions

Genotyping method allowed us to determine the base pair number in each amplicon obtained for the 15 microsatellite loci. Different cultivars showed, at a certain SSR locus, a homozygotic (the presence of a single allelic variant), or a heterozygotic (two, and in some cases, even 3 allelic variants) condition (see Table 2 and Table 3).

In figure 1, an electropherogram obtained using the GeneMapper, on ABI PRISM 3100 Genetic Analyser, is presented (Gheţea et al., 2010, a).

Based on amplicon dimensions corresponding to the 15 SSR markers, a genetic profile was obtained for each of the analysed cultivars.

The **VrZAG7** locus showed a homozygous condition in all cultivars we have analysed. The allelic variants having the highest frequency in Romanian cultivars are those of 155bp and 157 base pairs (bp) (approximately 43%), while in the Moldavian ones, the most frequent (approximately 29%) is of 156bp (Table 4 and Table 5). Most of the detected alleles are within the size interval cited in the literature (see Table 1 for allelic size range); in 3 Moldavian provenience cultivars (*Fetească regală, Muscat Timpuriu de Bucureşti* and *Timpuriu*

de Cluj), 2 significantly shorter allelic variants (of 102/103, and 107 bp) have been observed. The 102/103 variant is outside the known allelic size range (Table 2 and Table 3). The high degree of homozygocity showed at this SSR locus is an indication that it could be associated with a coding region where genes for economically important traits are located (Oliveira et al., 2006).

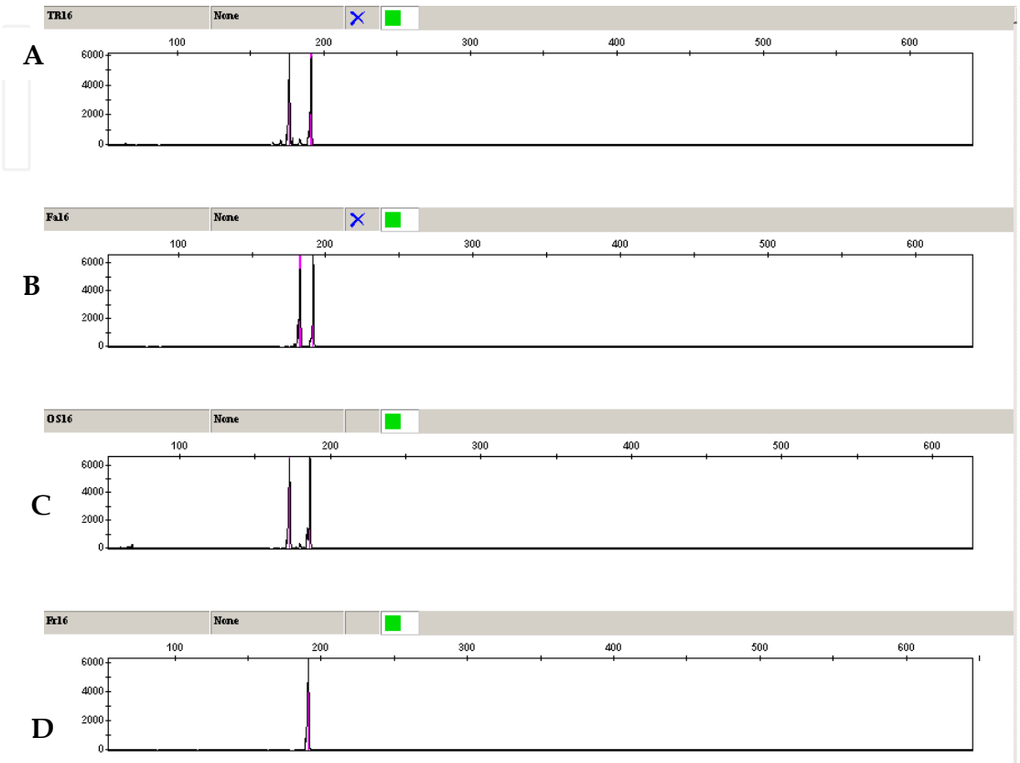

Fig. 1. Gene Mapper image for *Tămâioasă Românească* (A), *Fetească albă* (B), *Cabernet Sauvignon* (C) and *Fetească regală* (D) cultivars, at the VVMD27 microsatellite region. A heterozygotic condition appears for the first three cultivars, with significant diference (in base pair number) between the two allelic variants, while the fourth one is homozygous at this locus.

For the **VrZAG12** region, most of the analysed cultivars showed a homozygotic status. Only *Cabernet Sauvignon* and *Apiren extratimpuriu* from Republic of Moldova stock collection, and *Italian Riesling* from Romanian stock collection, are heterozygous. Significant differences appear in the same cultivar comming from different stock collections: *Fetească albă* Romania (R) – 150:150/Republic of Moldova (M) – 165:165; *Fetească regală* (R) – 170:170/(M) – 153:153; *Fetească neagră* (R) – 148:148/(M) – 166:166 (Table 2 and Table 3). One can conclude that this microsatellite locus could be placed in a coding region linked with genes for valuable traits. Although, a molecular mechanism (probably, during DNA replication) acting differently in the two gene pools (Romanian and/or Moldavian), by adding or remove a number of base pairs, created, in time, different allelic variants stabilized, at first, by self-pollination and later, by repeated artificial selection and vegetative multiplication. The most frequent allele at this SSR locus has 147/148bp, and is encountered in a homozygotic- or heterozygotic status, also in Romanian and Moldavian collection (Table 4 and Table 5). All the allelic variants we have found are within the allele size interval cited in the literature (Table 1).

The most frequent and well conserved allelic variant for the **VrZAG15** region, has 164 (163-165) bp (Table 4 and Table 5), and appears in a homozygotic status in 9 cultivars, and in a heterozygotic status, in other 4 cultivars (Table 2 and Table 3).

The **VrZAG62** locus was recommended by the *Vitis* Working Group as SSR marker due to high allelic variability observed in grapevine cultivars of different gene pools, in different countries. This high degree of variability is confirmed also by our results: in these 14 cultivars we discuss here, 5 allelic variants have been found in Romanian cultivars (the most frequent variant, of 198bp, approximately 36%), and 7 different alleles in Moldavian cultivars (the most frequent variant, of 190bp, approximately 50%) (Table 4 and Table 5). They are also in a homozygotic- or heterozygotic status (Table 2 and Table 3). In other Moldavian cultivars, we have obtained, at this locus, even 3 or 4 different amplicons, corresponding to a triallelic- or a tetraallelic condition (article in course to be published). This is not a singular SSR locus where multiallelic condition has been encountered, and this particular feature will be discussed later. The cultivars *Fetească albă, Fetească regală* and *Cabernet Sauvignon* have different allelic constitutions, depending on the Romanian- or Moldavian stock collection origin. Two allelic variants, of 205 and 206bp (slightly longer than the size interval cited in the literature), have been found in Moldavian cultivars *Fetească albă* and *Cabernet Sauvignon*. The SSR locus VrZAG62 was identified on the linkage group corresponding to chromosome 7 of the *Vitis vinifera* complement (Riaz et al., 2004; Lowe & Walker, 2006; Troggio et al., 2007; Vezzulli et al., 2008), probably in an non-coding region, which explains its high mutational rate (Oliveira et al., 2006).

Cultivar	Microsatellite loci														
	ZAG7	ZAG12	ZAG15	ZAG62	ZAG79	VVS1	VVS2	VVS4	VVMD 6	VVMD 7	VVMD 14	VVMD 17	VVMD 21	VVMD 27	VVMD 36
Fetească albă R	157	150	172 174	194	-	178 183	**128**	168	200 207	248 254	**216** 228	**210** 219	244	182 191	261 271
Fetească albă M	156	165	163	200 **206**	246 250	178	131 148	177	198 207	240 250	232	221	245 255	179	**240** 266
Fetească regală R	158	170	164 172	196	246	177	**128**	168	**187** 197	248 250	**216**	**209** 219	245	191	249 261
Fetească regală M	107	153	165 192	190 196	-	179 188	129	169 177	207	250 252	**219** 225	219	247 253	191	264 272
Fetească neagră R	157	148	162	198	246 254	176 186	139	167	**186**	240 256	**216** 228	**210** 219	**239** 244	176 186	249 259
Fetească neagră M	156	166	163	190 196	250 254	179 187	**126**	-	200 207	240	236	220	-	177 191	**236** **240**
Cabernet Sauvign. R	155	148	162 164	190 196	**228** 247	178	135 148	169 176	200	241	**218** 222	218	245 255	**172** 186	249 259
Cabernet Sauvign. M	157	147 156	164	190 **205**	248 258	170 178	**128** 141	176	**173** **187**	244	**218** 228	217 231	243	180 186	**103** **237** 262

Table 2. Allelic size (number of base pairs) at 15 microsatellite loci, in 4 grapevine varieties cultivated also in Romania and Republic of Moldova (see section 3.). ***R*** – Romania; ***M*** – Republic of Moldova. Bolded numbers – allelic variants shorter than the allele sizes cited in the literature (see Table 1). Dashes – no amplicon has been obtained.

Cultivar	Microsatellite loci														
	ZAG7	ZAG12	ZAG15	ZAG62	ZAG79	VVS1	VVS2	VVS4	VVMD 6	VVMD 7	VVMD 14	VVMD 17	VVMD 21	VVMD 27	VVMD 36
Italian Riesling R	157	147 140	164 174	198	**230**	180	130 148	168	200 207	248 258	**216**	219	243	182 186	249 259
Sauvignon R	155	148	164	190 196	**235**	178	**128** 148	169 176	**191** 200	241 258	**216**	220	246 253	182 191	267
Tămâioasă Românească R	155	150	164	188 198	248 252	176	**128**	169 176	199	235	**216**	219	244 263	176 191	**239** 259
Muscat Timpuriu de Buc. M	**102**	146	164	190	238 253	178 185	141	169 176	206	240	**217**	219	245 252	178	259
Timpuriu de Cluj M	**103**	146	164	190	237	178	148 153	176	206	240	**217**	220	246	176 182	260
Apiren extratimpuriu M	158	147 155	164	189 195	237 248	178	139 148	176	206 212	240 252	-	218 220	245 253	182	**103** **234** **236**

Table 3. Allelic size (number of base pairs) at 15 microsatellite loci, in 6 grapevine varieties cultivated in Romania or Republic of Moldova (see section 3.) ***R*** – Romania; ***M*** – Republic of Moldova. Bolded numbers – allelic variants shorter than the allele sizes cited in the literature (see Table 1). Dashes – no amplicon has been obtained.

The **VrZAG 79** locus was identified on the linkage group corresponding to chromosome 5 of the *Vitis vinifera* complement (Riaz et al., 2004; Troggio et al., 2007; Vezzulli et al., 2008). Like VrZAG62 locus, this region has a high variability of the number of base pairs, generating many allelic variants: in these 14 cultivars, we have found 8 different alleles also in Romanian- and in Moldavian group; the most frequent allelic variant in Romanian cultivars has 246bp (approximately 25%), and in Moldavian ones – 235bp – (approximately 25%) (Table 4 and Table 5); 3 allele forms – those of 228bp, 230bp and 235bp – are outside the allelic size range known in the literature (they are slightly shorter). Interesting, the alleles of 228bp and 230bp have been found in 3 non-Romanian origin cultivars: *Cabernet Sauvignon* (228:247), *Italian Riesling* (230:230) and *Sauvignon* (235:235), existent in Romanian stock collection (Table 2 and Table 3). This aspect indicates that the 3 cultivars were imported in the Romanian grapevine gene pool a long time ago, suffering discrete genetic modifications, under the influence of local molecular mechanisms.

For the **VVS1** microsatellite locus, homozygotic- and also heterozygotic cultivars have been found; the most frequent allelic variant was that of 178bp, in both Romanian and Moldavian group. The frequency of this allele in the 14 analysed cultivars is of 36% in Romanian group, and of 57% in Moldavian group (Table 4 and Table 5). At this locus, like in other SSR loci, some differences appear in the same cultivar provenant from different stock collections (Romanian / Moldavian): *Fetească albă* (R) – 178:183/(M) – 178:178; *Fetească regală* (R) – 177:177/(M) – 178:188; *Cabernet Sauvignon* (R) – 179:179/(M) – 170:178 (Table 2 and Table 3). All the allelic variants found in the 14 cultivars, are within the allelic size interval (Table 1).

The **VVS2** locus was identified on the linkage group corresponding to chromosome 11 of the *Vitis vinifera* complement (Riaz et al., 2004; Lowe & Walker, 2006; Troggio et al., 2007; Vezzulli et al., 2008). This locus was selected by the *Vitis* Working Group as SSR marker, due to its high allelic variability. In the 14 cultivars, we found 5 different alleles in Romanian group (most frequent being that of 128bp – 50%), and 8 allelic variants in Moldavian group (most frequent being those of 141bp and 148bp – 21,5% each of them) (Table 4 and Table 5); 2 allelic variants – those of 128bp, and 126bp – are shorter than the allelic size interval known for this locus (Table 1). The allele of 148bp is encountered both in Romanian- and Moldavian stock collection. The allelic variant of 128bp was found in an homozygotic condition in 3 Romanian cultivars: *Fetească albă*, *Fetească regală*, and *Tămâioasă Românească*. *Cabernet Sauvignon* from Moldavian collection has also the 128bp allele, but is heterozygous at this locus (128:141). 3 allelic variants, of 141bp, 129bp and 126bp were encountered only in Moldavian provenance cultivars *Muscat Timpuriu de Bucureşti* (141:141), *Cabernet Sauvignon* (128:141), *Fetească regală* (129:129), and *Fetească neagră* (126:126) (Table 2 and Table 3).

The **VVS4** locus was identified on the linkage group corresponding to chromosome 8 of the *Vitis vinifera* complement (Riaz et al., 2004; Troggio et al., 2007). This SSR region revealed a moderate allelic variability. In Romanian cultivars 4 allelic variants have been detected (with the most frequent, that of 168bp – 43%), while in the Moldavian group only 3 different alleles have been found (with the most frequent, having 176bp – 58%) (Table 4 and Table 5). The 168/169 allelic variants are well conserved in Romanian collection provenance cultivars, also in an homozygotic status (*Fetească albă, Fetească regală, Italian Riesling*), and also in an heterozygotic one (*Sauvignon* and *Cabernet Sauvignon* – 169:176, just like in *Tămâioasă Românească*). The 176bp allele is frequentlyt encountered in Moldavian collection cultivars: *Muscat Timpuriu de Bucureşti* (169:176), *Timpuriu de Cluj* (176:176), and *Apiren extratimpuriu* (176:176) (Table 2 and Table 3). Interesting, although *Muscat Timpuriu de Bucureşti* and *Timpuriu de Cluj* have Romanian origin, and *Apiren extratimpuriu* is a newly created cultivar, its parents having probably a Caucazian origin, the allelic variant of 176bp has a good penetrance and has been stabilized in the Moldavian grapevine gene pool.

The **VVMD6** locus was identified on the linkage group corresponding to chromosome 7 of the *Vitis vinifera* complement (Lowe et al., 2006; Riaz et al., 2004; Troggio et al., 2007; Vezzulli et al., 2008). At this SSR locus, in the 14 analysed cultivars, 7 allelic variants for Romanian- and also for Moldavian cultivars (Table 4 and Table 5) have been detected. The most frequent allelic variant in Romanian cultivars is that of 200bp (approximately 36%), and in Moldavian cultivars – that of 206bp (approximately 36%). Some of the alleles, shorter than the variants cited in the literature for this locus (Table 1), appear in both stock collections: *Fetească neagră* (R) – 186:186; *Fetească regală* (R) – 187:197; *Sauvignon* (R) – 191:200; *Cabernet Sauvignon* (M) – 173:187 (Table 2 and Table 3). The size and frequency of the alleles differ between the two stock collections.

The **VVMD7** locus was identified on the linkage group corresponding to chromosome 7 of the *Vitis vinifera* complement (Adam-Blondon et al., 2004; Lowe et al., 2006; Riaz et al., 2004; Troggio et al., 2007; Vezzulli et al., 2008). This SSR locus presents a high allelic variability, being selected by the *Vitis* Working Group among the recommended SSR markers. In Romanian cultivars, 8 allelic variants have been found, most frequent being those of 241bp and 248bp (approximately 21,5%) (Table 4 and Table 5). For Moldavian cultivars, only 4

different alleles have been found in the 7 analysed cultivars, with the 240bp allele presenting highest frequency (approximately 57%). 3 of the Moldavian cultivars are homozygous- and 2 are heterozygous for this allele (Table 2 and Table 3). In our previous researches (Gheţea et al., 2010, b; unpublished data) we have found a greater variability and also a relative high number of repetitions in sequenced amplicons of different allelic variants, which indicate that the microsatellite locus is placed in an non-coding region with high mutational rates (Oliveira et al., 2006).

ZAG7	**FA (%)**	**ZAG12**	**FA (%)**	**ZAG15**	**FA (%)**	**ZAG62**	**FA (%)**	**ZAG79**	**FA (%)**
155	**42,9**	140	7,1	162	21,4	188	7,1	228	8,3
157	**42,9**	147	7,1	**164**	**50,0**	190	14,3	230	16,8
158	14,2	**148**	**43,0**	172	14,3	194	14,3	235	16,8
		150	28,5	174	14,3	196	28,6	**246**	**24,9**
		170	14,3			**198**	**35,7**	247	8,3
								248	8,3
								252	8,3
								254	8,3

VVS1	***FA (%)***	***VVS2***	***FA (%)***	***VVS4***	***FA (%)***	***VVMD6***	***FA (%)***	***VVMD7***	***FA (%)***
176	21,4	**128**	**50,0**	167	14,3	186	14,3	235	14,3
177	14,3	130	7,1	**168**	**42,9**	187	7,1	240	7,1
178	**35,8**	135	7,1	169	21,4	191	7,1	**241**	**21,5**
180	14,3	139	14,3	176	21,4	197	7,1	**248**	**21,5**
183	7,1	148	21,5			199	14,3	250	7,1
186	7,1					**200**	**35,8**	254	7,1
						207	14,3	256	7,1
								258	14,3

VVMD 14	***FA (%)***	***VVMD 17***	***FA (%)***	***VVMD 21***	***FA (%)***	***VVMD 27***	***FA (%)***	***VVMD 36***	***FA (%)***
216	**71,5**	209	7,1	239	7,14	172	7,2	239	7,1
218	7,1	210	14,3	243	14,3	176	14,3	**249**	**28,6**
222	7,1	218	14,3	**244**	**28,6**	182	21,4	**259**	**28,6**
228	14,3	**219**	**50,0**	245	21,4	186	21,4	261	14,3
		220	14,3	246	7,14	**191**	**35,7**	267	14,3
				253	7,14			271	7,1
				255	7,14				
				263	7,14				

Table 4. Frequencies of the allelic variants found at each analysed microsatellite locus, in Romanian stock collection cultivars. Bolded numbers represent the most frequent allele/alleles.

The **VVMD14** locus was identified on the linkage group corresponding to chromosome 5 of the *Vitis vinifera* complement (Riaz et al., 2004). For the 14 analysed cultivars, 4 allelic variants (with the dominant allele of 216bp – approximately 71,5%) have been found in Romanian cultivars, and 7 allelic variants – in Moldavian ones (with the allele of 217bp having the highest frequency - 33%) (Table 4 and Table 5). In both cultivar groups appear shorter allelic variants (216bp, 217bp, 219bp), also in an homozygotic- and heterozygotic condition (Table 2 and Table 3). These shorter alleles could represent a particular feature for the grapevine gene pool from this East-European region.

The **VVMD17** locus was located on the linkage group corresponding to chromosome 18 of the *Vitis vinifera* complement (Adam-Blondon et al., 2004; Troggio et al., 2007; Vezzulli et al., 2008). A moderate variability was revealed by this microsatellite locus: 5 allelic variants in Romanian cultivars (the most frequent alelic variant is that of 219bp – 50%), and 6 variants in Moldavian cultivars (the allele of 220bp has the highest frequency – 35,5%) (Table 4 and Table 5). The relatively low number of allelic variants, and also short repetitive sequences (Gheţea et al., 2010, b) found at this SSR locus, indicate that VVMD17 is probably placed in a coding region of the genome, where the mutational rates are low (Oliveira et al., 2006). A shorter allelic variant than the size interval cited in the literature (Table 1), of 209/210bp, in a heterozygous condition, appears in 3 Romanian cultivars: *Fetească albă, Fetească regală* and *Fetească neagră*. One can say that all these 3 cultivars have the same genetic constitution at this locus – 209/210:219 (Table 2). In Moldavian cultivars, all the allelic variants fit in the allelic size interval, having a homozygous either heterozygous constitution (Table 2 and Table 3).

The **VVMD21** locus is placed on the linkage group corresponding to chromosome 6 of the *Vitis vinifera* complement (Adam-Blondon et al., 2004; Riaz et al., 2004; Lowe et al., 2006). Most of the analysed cultivars presented a heterozygous status for this locus, where we found 8 allelic variants in Romanian cultivars, and 7 different alleles in Moldavian cultivars. In Romanian group, the most frequent allele is that of 244bp (approximately 29%), while in Moldavian group, the highest frequency presented the 245bp allele (approximately 25%) (Table 4 and Table 5). Excepting the short allele of 239bp (found in *Fetească neagră* from Romania), all the other allelic variants fit in the known size interval (Table 1, Table 2 and Table 3).

The **VVMD27** locus is placed on the linkage group corresponding to chromosome 5 of the *Vitis vinifera* complement (Lowe K.M et al., 2006; Riaz et al., 2004; Troggio et al., 2007; Vezzulli et al., 2008). This microsatellite region is among the selected loci as SSR marker for grapevine genetic profiling, due to its high allelic variability revealed among different cultivars. The heterozygous constitution of most of the analysed cultivars indicate that this locus is a mutational dynamic one, placed most probably, in a non-coding region of the grapevine genome. In the 14 cultivars, we found 5 different allelic variants in Romanian group (with the most frequent allele – that of 191bp – approximately 36%), and 8 allelic variants in Moldavian group (here, two different alleles, one - the same as in Romanian cultivars – 182bp and 191bp – presented the highest frequency – approximately 21%) (Table 4 and Table 5). Excepting the *Cabernet Sauvignon* cultivar from Romania (which has an allele of 172bp – at the limit of the size interval), all other allelic variants are in the cited size range (Table 1, Table 2 and Table 3).

ZAG7	*FA (%)*	*ZAG12*	*FA (%)*	*ZAG15*	*FA (%)*	*ZAG62*	*FA (%)*	*ZAG79*	*FA (%)*
102	14,3	**146**	**28,6**	163	28,6	189	7,1	**237**	**25,1**
103	14,3	147	14,3	**164**	**57,2**	**190**	**50,2**	238	8,3
107	14,3	153	14,3	165	7,1	195	7,1	246	8,3
156	**28,6**	155	7,15	192	7,1	196	14,3	248	16,7
157	14,3	156	7,15			200	7,1	250	16,7
158	14,3	165	14,3			205	7,1	253	8,3
		166	14,3			206	7,1	254	8,3
								258	8,3

VVS1	*FA (%)*	*VVS2*	*FA (%)*	*VVS4*	*FA (%)*	*VVMD6*	*FA (%)*	*VVMD7*	*FA (%)*
170	7,1	126	14,3	169	24,9	173	7,2	**240**	**57,1**
178	**57,3**	128	7,1	**176**	**58,1**	187	7,2	244	14,3
179	14,3	129	14,3	177	17,0	198	7,2	250	14,3
185	7,1	131	7,1			200	7,2	252	14,3
187	7,1	139	7,1			**206**	**35,5**		
188	7,1	**141**	**21,5**			207	28,5		
		148	**21,5**			212	7,2		
		153	7,1						

VVMD 14	*FA (%)*	*VVMD 17*	*FA (%)*	*VVMD 21*	*FA (%)*	*VVMD 27*	*FA (%)*	*VVMD 36*	*FA (%)*
217	**33,2**	217	7,3	243	16,6	176	7,2	103	12,5
218	8,4	218	7,3	**245**	**25,0**	177	7,2	234	6,25
219	8,4	219	28,4	246	16,6	178	14,3	236	12,5
225	8,4	**220**	**35,5**	247	8,4	179	14,3	237	6,25
228	8,4	221	14,2	252	8,4	180	7,2	240	12,5
232	16,6	231	7,3	253	16,6	**182**	**21,3**	259	12,5
236	16,6			255	8,4	186	7,2	260	12,5
						191	**21,3**	262	6,25
								264	6,25
								266	6,25
								272	6,25

Table 5. Frequencies of the allelic variants found at each analysed microsatellite locus, in Moldavian stock collection cultivars. Bolded numbers represent the most frequent allele/alleles.

The **VVMD36** locus was located on the linkage group corresponding to chromosome 3 of the *Vitis vinifera* complement (Adam-Blondon et al., 2004; Riaz et al., 2004; Troggio et al., 2007; Vezzulli et al., 2008). This is a very interesting microsatellite locus, which revealed particular genetic constitutions, especially for Moldavian cultivars. There is a high genetic variability at this locus, a real mutational „hot spot", generating many allelic variants and also revealing multiallelic profiles in some cultivars. In our researches, we found an

important number of Moldavian cultivars having more than 2 different alleles at VVMD36 locus. Triallelic (Fig.2) and tetraallelic constitutions (Fig.3) are common in Moldavian grapevine gene pool. Here, we present 2 cultivars: *Cabernet Sauvignon* (103:237:262), and *Apiren extratimpuriu* (103:234:236) (Table 2 and Table 3).

Another phenomenon is also obvious at this SSR locus: a significant allele shortening tendency, an important number of base pairs being deleted. The allele of 103bp is more than a half smaller than the inferior limit of the allelic size interval known for this SSR locus (Table 1). Based on these results, we have presumed that in some microsatellite loci, gene duplication processes, followed by deletion or repeated deletions, take place.

These multiallelic profiles are interpreted by Moncada (Moncada et al., 2006) as a result of either a gene duplication phenomenon, or chimeras produced by mutation in meristematic layers L1 and L2. Such events could be considered as local evolutionary mechanisms that give, in time, particular features of a certain gene pool. Certainly, multiallelic profiles at SSR locus represent a characteristic feature for Moldavian grapevine germplasm collection.

At this locus, considering the 14 cultivars, we found 6 allelic variants in Romanian group (with 2 most frequent alleles, those of 249bp and 259bp – approximately 29% each), and 11 allelic variants in Moldavian cultivars (Table 4 and Table 5).

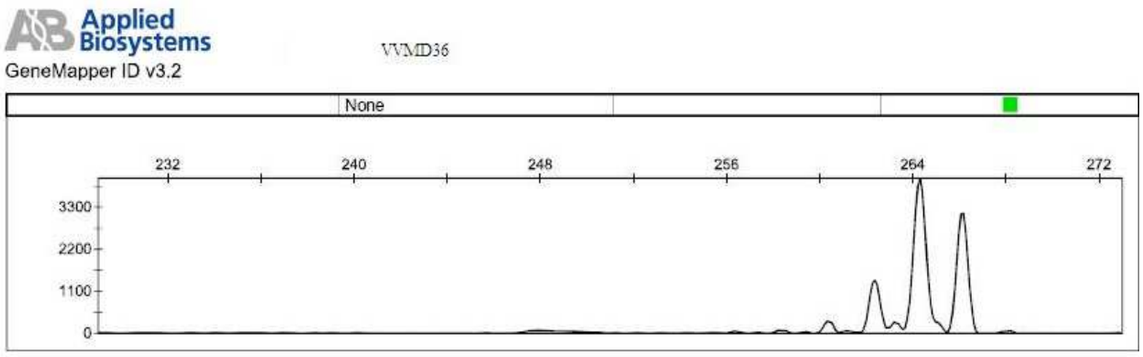

Fig. 2. Capillary electrophoresis showing a trialellic profile (262:264:266) at VVMD36 locus, for a cultivar from Moldavian stock collection (data not published)

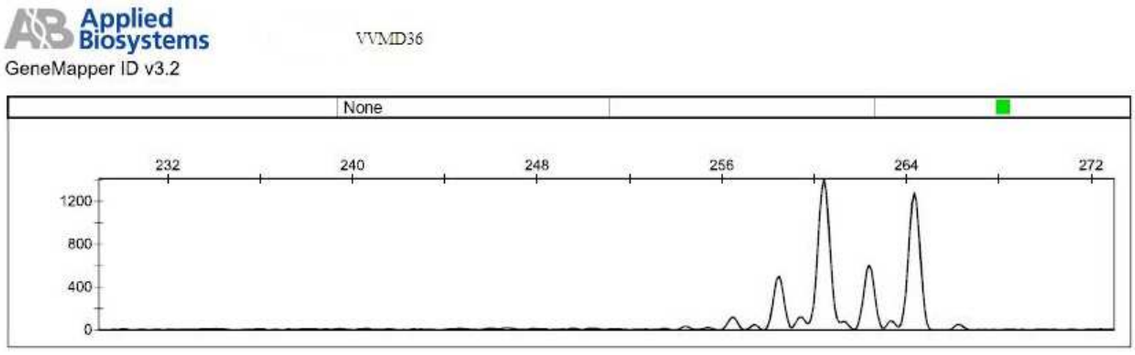

Fig. 3. Capillary electrophoresis showing a tetraalellic profile (258:260:262:264) at VVMD36 locus, for another cultivar from Moldavian stock collection (data not published)

Considering the two non-autochthonous cultivars – *Cabernet Sauvignon* and *Sauvignon* – we have compared genetic profiles obtained by us, for these two famous and geographical highly dispersed cultivars, to other citations from the literature. Table 6 and Table 7 summarize the results. Although we do not have the data for all the SSR loci we have considered in these tables, the comparison sustains the idea that discrete, molecular evolutive mechanisms are developing in every regional gene pool, giving, in time, unique particularities which make it an important and valuable genetic reservoir.

		ZAG62	ZAG79	VVS2	VVMD6	VVMD7
Romania	***Cabernet Sauvignon***	**190 196**	**228 247**	**135 148**	**200**	**241**
Republic of Moldova		**190 205**	**248 258**	**128 141**	**173 187**	**244**
Martin et al., 2003		187 193	245	136 150	-	237
Moncada et al., 2006		-	-	-	204 205	236

Table 6. Allelic variants (in base pair number) in 5 SSR loci, determined in *Cabernet Sauvignon* cultivar from Romanian- and Moldavian stock collections, compared to other citations for the same cultivar

		ZAG62	ZAG79	VVS2	VVMD6	VVMD7	VVMD27
Romania	***Sauvignon***	**190 196**	**235**	**128 148**	**191 200**	**241 258**	**182 191**
Santiago et al., 2007		187 193	243 249	150	-	241 255	185
Martin et al., 2003		187 193	243 249	-	-	241 255	-
Moncada et al., 2006		-	-	-	197 205 209	236 254	-

Table 7. Allelic variants (in base pair number) in 6 SSR loci, determined in *Sauvignon* cultivar from Romanian stock collection, compared to other citations for the same cultivar

6. Conclusion

The 15 microsatellite loci we have used, proved to be reliable molecular markers in assessing the genetic profile of the studied grapevine cultivars. A high number of microsatellite markers allow for a rigorous genetic characterization, a fine discrimination between

different cultivars and also pedigree analyses, in order to assess the origins, oldness and phylogenetic relationships between grapevine cultivars.

Some of these SSR loci showed high variability: for the cultivars from Romanian stock collection, the most variable loci have been VrZAG79, VVMD6, VVMD7, and VVMD21; for the cultivars from Moldavian stock collection, high numbers of allelic variants have been found in VrZAG12, VrZAG62, VrZAG79, VVS2, VVMD6, VVMD14, VVMD21, VVMD27 and VVMD36.

Besides a great allelic variability evidenced in these microsatellite loci, a detailed analysis of the genetic constitution of the grapevine cultivars, at each SSR locus, revealed also allelic variants well conserved inside a certain cultivar group (Romanian or Moldavian); these alleles are specific for that germplasm collection. There are also allelic forms conserved in both groups, proving, in some cases, the existence of a phylogenetic relationship between cultivars, or in other cases, being the consequence of the close vicinity and of a multimillenary tradition in grapevine cultivation, of Romania and Republic of Moldova.

Lower variability in some SSR loci (like VrZAG7, VVS4, VVMD17) can be explained by the fact that, very probably, these microsatellite regions are placed in coding regions of the genome, where the mutational rate is low. The conservation of certain allelic variants in these loci is stimulated also by the self-pollination or by repeated artificial selection and vegetative multiplication, if these SSR loci are associated with genes coding for economically important traits.

For Moldavian germplasm, the VVMD36 microsatellite locus proved to be very informative, revealing interesting multiallelic profiles. Significantly shorter allelic variants, and also triallelic loci, have been detected. The observation proves that these shorter alleles and the existence of 3 (or even 4) instead of 2 allelic forms at VVMD36 locus, are specific well conserved genetic features for the Moldavian grapevine gene pool. These results sustain the hypothesis of the existence of an evolutive event at this microsatellite locus: a gene duplication phenomenon followed by a deletion or repeated deletion events could explain this situation.

High genetic variability in a species is a sign of its health, and for an economically important species, it represents a valuable source for agricultural purposes. The final conclusion is that both – Romanian and Moldavian – germplasm collections are characterized by a high diversity, with specific allelic combinations which give unique and valuable features that must be known and inventored, enabling their long-term conservation and use in breeding programs.

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This book is about the novel aspects and future trends of the horticulture. The topics covered by this book are the effect of the climate and soil characteristics on the nitrogen balance, influence of fertilizers with prolongation effect, diversity in grapevine gene pools, growth and nutrient uptake for tomato plants, post-harvest quality, chemical composition and antioxidant activity, local botanical knowledge and agrobiodiversity, urban horticulture, use of the humectant agents in protected horticulture as well as post-harvest technologies of fresh horticulture produce. This book is a general reference work for students, professional horticulturalists and readers with interest in the subject.

How to reference

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