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SHORT COMUNICATION

Genetic variability revealed by sequencing analysis at two microsatellitic loci, in some grapevine cultivars from Romania and Republic of Moldavia

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Introduction

Initially used in human genetics researches, the microsatellite markers proved to be an efficient tool in plant- and animal genetics. This type of molecular markers offer relevant information for identifying the bioconservation units, and for evaluating the action of evolutive mechanisms in populations (e.g. gene flow and genetic drift) (M MORGANTE & al. [1]). Recent researches showed that the microsatellite frequency is high in plants, being significantly higher in the non-coding regions than in the coding regions of the genome (E.J. OLIVEIRA & al. [2]).

In the field of viticulture, microsatellites (SSRs – simple sequence repeats), are the molecular markers of choice, for cultivars and clones genetic profiling. Being high polymorphic, a minimum number of 6 microsatellitic loci would be sufficient for cultivar discrimination, but in the case of close-related varieties, a bigger number (optimal, 25) of SSR markers is necessary (K.M. SEFC & al. [3]).

Microsatellites are clasified, according to the type of repeat sequence, as perfect, imperfect, interrupted, and composite (E.J. OLIVEIRA & al. [2]).

The results we have obtained by sequencing amplicons from two SSR loci – VVMD7 and VVMD17 – explain the allelic variability observed by genotyping, at this microsatellitic loci, both in Romanian and Moldavian analysed cultivars.

Materials and methods

In Table 1, grapevine cultivars from Romania and Republic of Moldavia presented in this paper are listed. The Romanian grapevine cultivars are maintained in the stock collection of the National Institute for Research and Development for Biotechnologies in Horticulture, Ştefăneşti-Argeş. The Moldavian grapevine cultivars are in the stock collection of the National Institute for Viticulture and Oenology, Chişinău.

Table 1. The Romanian- and Moldavian grapevine cultivars sequenced at VVMD7 and VVMD17 microsatellitic loci

Romanian grapevine cultivars	Moldavian grapevine cultivars
Napoca	Prezentabil
Splendid	Basarabia
Fetească albă	Apiren alb
Fetească neagră	Apiren roz basarabean
Tămâioasă românească	Apiren negru de Grozeşti
Negru aromat	Romulus
	Călina

DNA was extracted from leaves of different genotypes of grapevine by two methods: according to the method reported by C.E. VALLEJOS & al. [4], and with the *MasterPure*^{TM} *Plant Leaf DNA Purification Kit* (Epicentre, USA), using the manufacturer's protocol.

The primer pairs for the two microsatellitic (SSR) sequenced loci (Table 2), were selected from the existing data bases (J.E. BOWERS & al. [5]; J.E. BOWERS & al. [6]). The VVMD7 locus is among the six SSR loci recommended by the *Vitis* Working Group [7].

Table 2. The sequenced SSR loci

Locus	Allele size range (bp) cited in the literature	Primer	
		Sequence	$T°_{annealing}$
VVMD7	233 – 263	(F) agagttgcggagaacaggat (R) cgaaccttcacacgcttgat	54°C
VVMD17	212 – 236	(F) tgactcgccaaaatctgacg (R) cacacatatcatcaccacacgg	52°C

The PCR reactions were performed on an GeneAmp 9700 thermocycler (Applied Biosystems). Annealing temperatures, presented in Table 2, were selected based on the results obtained in the temperature gradient PCR. The PCR mix was: PCR buffer (5x) (GoTaq, Promega) - 5µl ; $MgCl_2$ – 0,75µl ; dNTP 10mM (Promega) – 0,5µl ; primer 1/2 - 1µl, DNA sample - 30-45 ng/µl, Taq polymerase GoTaq (Promega) – 0,12µl, ddH_2O – X µl (X was calculated for each sample, depending on the volume of DNA sample, to a final volume of 25µl).

The amplification conditions were as follows:

I	95^0C → 4 minutes	x 35 cycles
II	95^0C → 1 minute X^0C (X=54^0C for VVMD7, and 52^0C for VVMD17) → 1 minute 72^0C → 1 minute	
III	72^0C → 7 minutes	

Amplicon purification has been done using the *Wizard® SV Gel and PCR Clean-Up System* kit (*Promega*), according to the manufacturer's protocol.

After spectrophotometric quantification of the amplicons (A_{260}/A_{280}), each amplicon was diluted to a final concentration of 2-3ng/µl.

The sequencing reaction was performed by Sanger enzymatic method, using the *BigDye Cycle Sequencing Kit v.3.1* (Applied Biosystems), on a Veriti termocycler (Applied Biosystems), according to the manufacturer's recommendations. The reagents are presented in Table 3.

Table 3. The reagents from the sequencing reaction mix

Reagent	Volume / sample
Water	3,5 µl
Buffer BigDye	2 µl
DMSO	0,5 µl
Primer (at a concentration of 4pmol / µl)	1 µl
BigDye	1 µl
Sample DNA	2 µl
Total volume	10 µl

The amplicons were purified with the *BigDye® Xterminator™ Purification Kit* (Applied Biosystems). The capillary electrophoresis was performed on the genetic analyzer *ABI PRISM 3130xl* (Applied Biosystems), using the standard protocol recommended by producer.

The obtained results were analysed with the *Sequencing Analysis v5.3.1* programme (Applied Biosystems). The dinucleotidic repetitions were counted.

Results and Discussions

The VVMD7 microsatellitic locus has been identified on the linkage group corresponding to chromosome 7 of the *Vitis vinifera* chromosomal complement (M. TROGGIO & al. [8]; S. VEZZULLI & al. [9]).

The sequence analysis of the amplicons obtained on this microsatellitic locus, in 6 Romanian- and 7 Moldavian cultivars, showed that the most frequent sequence repetition is represented by the dinucleotide GA (on one strand) / CT (on the other strand) (Table 4). Perfect- and imperfect repetitive sequences have been observed. The number of repetitions is relative high in both Romanian- and Moldavian analysed cultivars.

Table 4. Amplicon size (in number of base pairs, bp) and GA/CT dinucleotide repetitions number on strand 1/strand 2, at **VVMD7** SSR locus, for the studied Romanian and Moldavian cultivars

Romanian cultivars			**Moldavian cultivars**		
Cultivar	Amplicon / amplicons size (bp)	Repetitions number on str.1/str. 2	Cultivar	Amplicon / amplicons size	Repetitions number on str.1/str. 2
Napoca	-	21(GA) / 24(CT)	Prezentabil	98 / 98	20(GA) / 24(CT)
Splendid	-	19(GA) / 21(CT)	Basarabia	98 / 248 / 251	20(GA) / 22(CT)
Fetească albă	248 / 254	22(GA) / -	Apiren alb	247 / 252	20(GA) / 23(CT)
Fetească neagră	240 / 256	18(GA) / 22(CT)	Apiren roz basarabean	251 / 251	21(GA) / -
Tămâioasă românească	235 / 235	20(GA) / 20(CT)	Apiren negru de Grozeşti	98 / 239 / 248	19(GA) / 22(CT)
Negru aromat	243 / 243	18(GA) / 19(CT)	Romulus	98 / 98	18(GA) / 19(CT)
			Călina	98 / 98	17(GA) / 25(CT)

Genotyping at this microsatellitic locus has revealed 10 allelic variants in both groups of grapevine cultivars – from Romania and Republic of Moldavia (unpublished results). Due to the relative high number of sequence repetitions and of allelic variants, we presume that this microsatellitic locus is placed in a non-coding region of the *Vitis vinifera* genome. Petit (cited by E.J. OLIVEIRA & al. [2]) sustains that SSR loci having a bigger number of sequence repetitions, present higher rates of mutations. The consequence is the appearance of many allelic variants at such microsatellitic loci.

VVMD17 microsatellitic locus is placed on chromosome 18 of the *Vitis vinifera* chromosomal complement (M. TROGGIO & al. [8]; S. VEZZULLI & al. [9]).

For this locus, the amplicon sequencing revealed the presence of shorter repetitive sequences (Table 5) than in the case of the previous SSR locus. For VVMD17 locus, interrupted repetitive sequences, consisting of the dinucleotide pairs (GA on one strand / CT on the other strand), have been detected.

Table 5. Amplicon size and GA/CT dinucleotide repetitions number on strand 1/strand 2, at **VVMD17** SSR locus, for the studied Romanian and Moldavian cultivars

Romanian cultivars			**Moldavian cultivars**		
Cultivar	Amplicon / amplicons size (bp)	Repetitions number on str.1/str. 2	Cultivar	Amplicon / amplicons size (bp)	Repetitions number on str.1/str. 2
Napoca	-	- / 9(CT)	Prezentabil	217 / 219	9(GA) / 10(CT)
Splendid	-	9(GA) / 10(CT)	Basarabia	217 / 217	9(GA) / 9(CT)
Fetească albă	210 / 219	- / 10(CT)	Apiren alb	217 / 219	9(GA) / 10(CT)
Fetească neagră	210 / 219	- / 9(CT)	Apiren roz basarabean	217 / 219	9(GA) / 10(CT)
Tămâioasă românească	219 / 219	- / 10(CT)	Apiren negru de Grozești	217 / 219	9(GA) / 10(CT)
Negru aromat	208 / 218	- / 10(CT)	Romulus	209 / 219	8(GA) / 9(CT)
			Călina	217 / 219	- / 10(CT)

The number of allelic variants found at this locus was lower than in VVMD7 cultivars; (6 in Romanian cultivars; 8 in Moldavian cultivars) (unpublished results).

Shorter repetitive sequences and lower allelic variation indicate that VVMD17 microsatellitic locus is, probably, placed in a coding region of the genome.

Conclusions

The results we have obtained by sequencing the amplicons from two microsatellitic loci of the *Vitis vinifera* genome, sustain the ideea that molecular mechanisms responsible for inducing the variability at microsatellitic loci (by variation in number of dinucleotidic repetitions) are active both in Romanian- and Moldavian grapevine gene pools, generating allelic polymorphisms. The mutation rates are higher in the microsatellites placed in non-coding regions (like it seems to be the case of VVMD7 locus); the consequence is the presence of many allelic size variants for those microsatellitic loci.

The local molecular mechanisms acting in the two gene pools – from Romania and Republic of Moldavia – give particular characteristics which individualize these grapevine genofonds.

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