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Morphometric and molecular analysis of a pink-berried mutant within the population of grape cultivar 'Plavac mali'

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Summary

This study reports characteristics of pink-berried mutants found in the clone population of the Croatian red wine cultivar 'Plavac mali' on the basis of comparative ampelographic and DNA marker (SSR and *Gret1*) analysis. The pink-berried accession, also called 'Plavac mali sivi' (Croatian: sivi = English: grey or French: gris), along with the other 58 'Plavac mali' accessions of standard blue-black berry skin color, has been characterized for the first time using OIV descriptors and molecular markers. Using a set of 9 simple sequence repeat (SSR) markers, an identical SSR profile for all the analyzed accessions was revealed, indicating their monozygotic status. The analysis of *Gret1* insertion within the *VvMYBA1* locus revealed no DNA polymorphism responsible for the pink-berried phenotype. Surface color of the berry skin was measured with the CIELab technique using a reflectance spectrophotometer at full ripeness. The results of colorimetric variables (L*, a* and b*) suggest a significantly lower accumulation of anthocyanins in the pink-berried accession compared to the standard blue-black berries. The pink-berried accession shares all assessed morphological and genetic traits of 'Plavac mali', with the only difference being the color of the berry skin. This suggests that the pink-berried genotype is the result of a spontaneous mutation of a standard 'Plavac mali' genotype.

K e y w o r d s : clone, ampelography, anthocyanins, microsatellites, CIELab, *Gret1*.

Introduction

The process of vegetative propagation of grapevines, in most cases, produces genetically identical offspring of a parent vine. However, spontaneous mutation of regenerative cells can occur within populations of traditional cultivars, leading to irreversible morphological and physiological differences among vines. Over the years, vine growers and winemakers have been selecting and propagating specific mutants (more often called clones) with unique and superior traits. Usually, different clones of a variety share the majority of varietal characteristics, and their appearance and performance are not easily distinguishable. In some cases, however, such as the accumulation of anthocyanins in the berry skin, they are clearly visible and can significantly affect the quality of wine.

There is a wide range of berry colors in grapes from yellow-green to blue-black. Pigmentation of blue-black and red cultivars results from vacuolar accumulation of anthocyanins in the berry skin cells.

After many studies, it is widely accepted that SSR markers are the most suitable and reliable tool for identification of cultivars and detection of synonyms and homonyms among them (Maletić *et al.* 1999, This *et al.* 2004). At the same time they were not so efficient in clone differentiation (Sefc *et al*. 2009), even though some SSR loci have been shown to have a good potential for distinguishing clones within a cultivar (Riaz *et al.* 2002, Moncada *et al.* 2006, Pelsy *et al.* 2010).

One of the old examples of berry color variation is the 'Pinot' family (noir/gris/blanc). They share very similar morphological and genetic characteristics. The analysis of numerous 'Pinot' clones on a large number of SSR loci has discovered the triallelic state of some loci, which is attributed to chimerism (Hocquigny *et al.* 2004). They also concluded that 'Pinot meunier' arose from 'Pinot noir', indicating chimerism as an important source of intravarietal diversity in grape cultivars, which is in line with previous finding of Franks *et al.* (2002).

The occurrence of different skin colors from the original one for a particular cultivar is well known in international cultivars such as 'Cabernet Sauvignon' (bronze/white) or 'Grenache' (noir/gris/white) (Walker *et al*. 2006, This *et al.* 2006). Recently, pink- and green-berried mutants have been reported for 'Sangiovese' (Ramazzotti *et al.* 2008).

There are a number of other traits in grapevines that are changed by different types of mutation. These polymorphisms include very important agronomic traits such as earlier or later ripening, cluster density, and limited productivity (Wolpert 1996). Clonal selection procedures and certification have given rise to various superior individuals or "new cultivars", which differ from the population at least in one important characteristic. Clones usually retain the original cultivar name and acquire an identifying clone name. However, some of the berry color mutants are classified as new cultivars with regard to their quite different and new oenological value compared to the original cultivar.

'Plavac mali' is the major red wine cultivar in Croatia, grown in the coastal and insular area of central and south Dalmatia, and among the rare ones that have a determined

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parentage (Maletić *et al.* 2004). It has gained the greatest popularity among other native cultivars because of its good agronomic properties (low susceptibility to diseases, suitability for growing on slopes, and good fruit composition). Recently, a project of clonal selection of the cultivar 'Plavac mali' was started. One of the first steps was to collect all divergent phenotypes within the population and establish an *ex situ* collection that will enable reliable study of their morphological and genetic profiles (Zdunić *et al*. 2007). The pink-berried phenotype ('Plavac mali sivi' - PMS) showed apparent clonal variation within the population of 'Plavac mali'. Since 'Plavac mali' is suited to the unique Dalmatian viticultural climate (*terroir*), many wine growers from the region believe that a pink-berried mutant might keep its distinctive varietal characteristics, and could be very valuable for white wine production.

The very first notification of this 'Plavac mali' mutant was recorded by Jelaska (1960) who unfortunately never completed and published his research. The second attempt to evaluate this mutant accession was done by Mirošević (1988), who found a vine with a mutated shoot (bearing clusters expressing the pink-berried phenotype) on the island of Korčula in 1983. He succeeded to propagate the original vine into five grafts of PMS and five grafts of standard black-berried 'Plavac mali'. He came to the conclusion that PMS does not morphologically differ from the standard 'Plavac mali'. However, identification of the pink-berried mutant and its thorough comparison with other divergent genotypes of 'Plavac mali' has not been conducted so far. Therefore, the purpose of this study is (i) to identify the pink-berried mutant within the population of 'Plavac mali' using ampelographic analysis and SSR markers, and further (ii) to screen for presence of the *Gret1* retrotransposon at the *VvMYBA1* berry color locus among pink- and blue-black-berried accessions. The information presented in this study will help the preservation of biodiversity within a population of traditional cultivars and facilitate the process of clonal selection.

Material and Methods

Plant material: Plant material includes 59 accessions of 'Plavac mali', including a pink-berried mutant (PMS). These acessions were previously selected to represent the overall 'Plavac mali' phenotypic variation found across its growing area. The PMS originates from the island of Korčula and is a clonal offspring of PMS used in the previous study of Mirošević (1988). Each accession is represented by six vines derived from a clonally propagated single parent plant that was planted in 2005 on the experimental ground of the Institute for Adriatic Crops and Karst Reclamation in Split, Croatia, where they were grown using the same viticultural practices (Zdunić *et al.* 2007). Ampelographic and genetic evaluation of accessions in *ex situ* collection began in 2007.

Ampelographic characterization: In the period between the fruit setting and veraison, a representative sample of 10 fully grown leaves was taken from the middle third of a shoot for each genotype. Qualitative descriptors recommended in a project Genres081 (http://www.genres.de/vitis.htm) were recorded (OIV codes: 051, 053, 067, 068, 070, 072, 074, 075, 076, 079, 080, 081, 083, 084 and 087). Leaves were photographed with a digital camera (Olympus, model SP-500UZ), and Superampelo computer program (Soldavini *et al.* 2009) was used to measure the leaf variables which were used to construct the scheme of the average leaf.

At full ripeness, characteristics of the grape and the berries were registered (OIV codes: 208, 223 and 225).

Berry skin color measurements: Apart from the visual classification of the berry skin color according to the OIV descriptor (OIV-225), the differences between the PMS and standard 'Plavac mali' phenotype were quantified using the CIELab technique (Carreno *et al.* 1995). The color of the PMS and 11 blue-black phenotypes (ob036, ob071, ob078, ob095, ob097, ob202, ob219, ob228, ob259, ob262 and ob286) that showed the greatest variability by visual evaluation were compared.

In the period of full ripeness of grapes, 100 berries were collected from different parts of clusters from each of six vines for each genotype. To avoid irregularities in the measurement of color due to dust and/or the remains of pesticides, the grapes were carefully cleaned with a cotton cloth. The color was measured with a colorimeter (CR-400, Konica Minolta, Japan) in combination with a computer program SpectraMagic NX Lite, ver. 2.0. The colorimeter was calibrated with a standard calibration plate before use.

According to the CIELab scale, L* values define lightness (0 = black to 100 = white), a* defines redness-greeness (+a = red to –a = green), while b* defines yellow-blue value (+b = yellow to –b = blue). The chroma value (C) was calculated as $C = [(a^{*2} + b^{*2})]^{0.5}$ and the hue (h) value as h = arctang b*/a*. Based on these variables, the color index for red grapes (CIRG) was calculated according to Carreno *et al.* (1995).

SSR analysis: Young leaf samples from 59 accessions were collected from the *ex situ* collection vineyard of the Institute for Adriatic Crops and Karst Reclamation in Split, Croatia. In order to establish the standard SSR genotype of 'Plavac mali' and to identify the pink-berried genotype, nine microsatellites (SSRs): VVS2 (Thomas and Scott 1993); VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32 (Bowers *et al.* 1999), VrZAG-62 and VrZAG-79 (Sefc *et al.* 1999) were screened. These SSR loci were proposed as a standard set for cultivar identification by the consortium of GrapeGen06 project (http://www1.montpellier.inra.fr/grapegen06/accueil.php).

Total genomic DNA was extracted with Qiagen DNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol.

PCR amplification was conducted in the Veriti™ Thermal Cycler (Applied Biosystems, Foster City, California, USA) using fluorescent labelled primers. All PCR amplifications were done as described in Vilanova *et al.* (2009). Amplified products were size-separated by capillary electrophoresis performed on an ABI 3130 Genetic Analyzer (Applied Biosystems) together with GeneScan 500-LIZ size standard. The data were analyzed using the software

package GeneMapper 4.0 (Applied Biosystems) and algoritham "*2nd Order Least Square*".

The analysis of *Gret1* retrotransposon: In order to check for the presence of the *Gret1* retrotransposon in the standard and pink-berried mutant of 'Plavac mali', the *VvMYBA1* locus was genotyped using three published primers: a, b and c (Kobayashi *et al.* 2004). 20 ng of genomic DNA was used as a template in a 20-µL reaction with 2 µL of 10x PCR buffer (including 15 mM $MgCl_2$), 1 µL of 2 mM dNTP mix, 0.2 µL of Taq polymerase (GoTaq®, 5 U/µL, Promega, Madison, USA) and 2 µL of 2 µM primers (combinations: *ac* or *bc*). The cycling conditions were as follows: 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s and 72 °C for 2 min. The products were separated by electrophoresis using 1 % (w/v) agarose gel and stained with ethidium bromide.

Statistical analysis: The differences between mean values of colorimetric variables were analyzed using the analysis of variance (ANOVA) and cluster analysis (Ward's method). Statistical analysis was done using Statistica 8.0 software (StatSoft, Inc., USA).

Results

Ampelographic characteristics: Qualitative ampelographic characteristics of leaf and grape were defined for the standard and pink-berried phenotype of 'Plavac mali' (Tab. 1). They share the same qualitative characteristics, and the only difference is visible in the OIV code for the color of berry skin, blue-black for the standard, and pink for the PMS. The colour of a young leaf is green with the medium intensity of prostrate hairs between the veins on the lower side. Fully grown leaves have pentagonal blades and five lobes with clearly visible upper and lower lateral sinuses. The main veins on the lower side of the leaf show only slight anthocyanin coloring, only around the petiole. Goffering or blistering of blades was weak, and the profile of the leaf was revolute. Teeth of the leaves were rectilinear. The sinus of the petiole was open, U-shaped, and inside the petiole sinus teeth can often be detected, while at the base of the upper wide sinuses this is not the case. The density of prostrate hairs between the main veins on the lower side of the leaf was medium, while on the main veins it was weak. The clusters of grapes were conical, and very often have one wing. The density of the clusters varied significantly among the investigated accessions of 'Plavac mali', from very dense to very loose (data not shown). The PMS accession had clusters of medium density. Berries were of round shape.

Fig. 1 shows typical leaf morphology of standard and pink-berried phenotypes of 'Plavac mali', and includes the main variables on which the scheme was based.

Color characteristics: Mean values of colorimetric variables of 11 accessions with standard blue-black and one with pink skin colour are shown in Tab. 3. Significant differences were determined ($p < 0.05$) between the mean values of PMS and other accessions in all colorimet-

Table 1

Ampelographic characteristics of standard (PM) and pink-berried (PMS) phenotypes of 'Plavac mali'. Standard phenotype of 'Plavac mali' was obtained on the basis of 58 blue-black berried accessions in the ex situ collection vineyard of the Institute for Adriatic Crops and Karst Reclamation, Split, Croatia

OIV code	Characteristic	PM	PMS
OIV-225	Berry: color of skin	6.0 (blue-black)	2.0 (rose)
OIV-208	Bunch: shape	2.0 (narrow conical)	
OIV-223	Berry: shape	2.0 (roundish)	
OIV-051	Young leaf: colour of the upper side (leaf 4)	1.0 (green)	
OIV-053	Young leaf: density of prostrate hairs between veins at the lower side of leaf	5.0 (medium)	
OIV-067	Mature leaf: shape of blade	3.0 (pentagonal)	
OIV-068	Mature leaf: number of lobes	3.0 (five)	
OIV-070	Mature leaf: anthocyanin coloration of the main veins on the upper side of the blade	2.0 (petiole point red)	
OIV-072	Mature leaf: goffering of blade	3.0 (weak)	
OIV-074	Mature leaf: profile	4.0 (revolute)	
OIV-075	Mature leaf: blistering of upper side	3.0 (weak)	
OIV-076	Mature leaf: shape of teeth	2.0 (rectilinear)	
OIV-079	Mature leaf: degree of petiole sinus opening	4.0 (slightly overlapping)	
OIV-080	Mature leaf: shape of base of petiole sinus	1.0 (U-shaped)	
OIV-081-1	Mature leaf: presence of teeth in the petiole sinus	2.0 (occurrence of 1 or 2 teeth)	
OIV-081-2	Mature leaf: petiole sinus limited by veins	3.0 (both sides)	
OIV-083-2	Mature leaf: presence of teeth at the base of the upper leaf sinuses	1.0 (none)	
OIV-084	Mature leaf: density of prostrate hairs between the main veins (lower side)	5.0 (medium)	
OIV-087	Mature leaf: density of erect hairs on the main veins (lower side)	3.0 (weak)	

Table 2

Statistical parameters of angle (°) variables calculated from the mature leaves measured on 59 accessions of 'Plavac mali'

OIV code	Variables	Mean	Min	Max	SD	CV (%)
607	Angle between N1 and N2 measured at the first ramification	47.5	44.1	51.2	1.8	3.8
608	Angle between N2 and N3 measured at the first ramification	46.7	40.5	58.3	3.4	7.2
609	Angle between N3 and N4	58.6	49.9	65.7	3.6	6.2
610	Angle between N3 and tangent between petiole point and the tooth tip of N5	51.7	45.5	60.4	3.1	6.0

Means, Minimum (Min), Maximum (Max), standard deviation (SD) and coefficient of variation (CV).

ric variables except lightness (L*). PMS had significantly more red (a* = 5.20) and less blue (b* = 3.72) than the other accessions. The color index for red grapes (CIRG) was significantly lower for the PMS (4.17) compared to all other accessions (from 6.86 to 8.31). Also, a significant variability among the accessions with blue-black color was found in all colorimetric variables.

Microsatellite analysis: All 59 accessions showed identical genetic profiles in 9 tested microsatellite loci, including the PMS. All the loci showed heterozygosity, except VVMD27, which showed only one allele, indicating the homozygous state of this locus, or null allele locus. There was no triallelic state observed at any of the analyzed loci.

Gret1 locus: In order to determine the *Gret1* genotype at the *VvMYBA1* locus, possibly responsible for the establishment of the pink-berried phenotype, we applied the published *a*, *b* and *c* primer set of Kobayashi *et al.* (2004). Our results show that PMS has one copy of *Gret1* retrotransposon inserted in just one *VvMYBA1* allele, which is the case for the standard clone 'Plavac mali' as well (Fig. 3). Therefore we can conclude that both PMS and 'Plavac mali' exhibit a heterozygous allelic state in respect to the *Gret1* insertion, having one "red" allele and one "white" allele. This result showed that PMS shares its heterozygous *Gret1* genotype at the *VvMYBA1* locus with cultivars like 'Cabernet Sauvignon' and 'Pinot noir' (Walker *et al.* 2006), which is an important prerequisite for generation of periclinal chimeras.

Discussion

Ampelographic characteristics: The parent vine of PMS on which the mutation occurred has not been preserved and sufficiently documented, as was the case with the 'Sangiovese' mutant (Ramazzotti *et al.* 2008). Moreover, its presence in different studies (Jelaska 1960, Mirošević 1988) leads to the conclusion that this type of mutation is naturally occuring in some frequency. Also, genetic stability of PMS phenotype can be confirmed with accurate transfer of this mutation through generations of clonal offspring.

Typical morphology of a leaf of PMS does not differ from the standard ‘Plavac mali’. Reconstructed mean leaves (Fig. 1) clearly show identical pentagonal shapes of

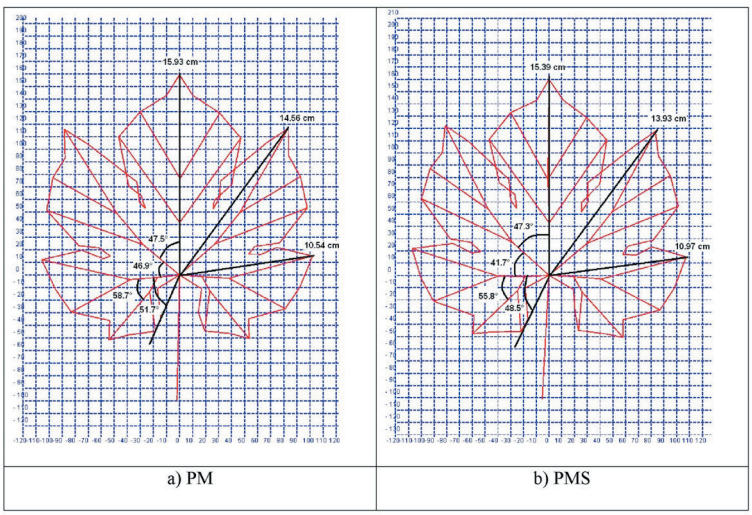

Fig. 1: Reconstructed mean leaves of a) standard (PM) and b) pink-berried (PMS) phenotype of 'Plavac mali' following the method of Soldavini *et al.* (2009). Standard phenotype of 'Plavac mali' is represented by 58 clonal lines.

Table 3

Mean and standard deviation (SD) values of the colorimetric variables (lightness, L*; red-green, a*; yellow-blue, b*; chroma, C; hue angle, h°; color index for red grapes, CIRG) for twelve accessions of 'Plavac mali' analyzed in 2010

Clone	L*			a*			b*			C			h			CIRG		
	Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
ob036	28.59	2.75	b[1]	1.22	0.81	fg	-2.88	1.13	f	3.30	0.90	b	-64.30	20.07	ef	7.71	0.63	i
ob071	25.07	0.93	e	1.55	0.81	d	-0.06	0.49	g	1.64	0.80	g	-3.12	19.71	g	6.86	0.65	h
ob078	25.10	1.01	e	1.27	0.45	cf	-0.09	0.53	g	1.35	0.51	e	-2.83	18.67	g	6.91	0.62	gh
ob095	28.00	2.81	d	1.00	0.75	eg	-2.17	1.11	e	2.62	0.79	f	-61.41	23.92	e	7.91	0.61	de
ob097	26.00	1.09	c	1.21	0.53	fg	-0.61	0.61	b	1.49	0.51	eg	-26.33	24.50	b	7.50	0.79	f
ob202	25.21	2.09	e	1.90	1.44	b	-0.99	1.00	d	2.43	1.32	f	-31.56	26.48	bc	7.69	0.94	fi
ob219	24.90	1.38	e	1.51	0.57	d	-0.22	0.65	g	1.65	0.59	g	-7.55	21.03	g	7.06	0.63	g
ob228	28.02	3.06	d	0.79	0.78	e	-2.62	1.17	f	2.90	1.00	c	-69.67	19.88	f	8.15	0.78	bc
ob259	24.76	1.47	e	1.47	0.65	cd	-1.12	0.74	d	1.95	0.76	d	-35.80	19.27	c	8.10	0.68	cd
ob262	26.39	1.71	c	0.82	0.37	e	-1.57	0.96	c	1.92	0.71	d	-55.26	24.71	d	8.31	0.62	b
ob286	27.35	2.65	a	1.18	0.69	fg	-2.01	1.37	e	2.57	1.08	f	-52.75	26.49	d	7.81	0.63	ei
pink-berried	28.42	1.29	bd	5.20	1.34	a	3.72	1.30	a	6.50	1.46	a	35.37	10.36	a	4.17	0.46	a

[1]Means followed by the same letter are not significantly different by LSD test, $p < 0.05$

the leaf blade with only slightly more open sinus of the petiole in PMS. Qualitative descriptors of leaves are also identical, and they cannot be used as a mean discrimination from the standard 'Plavac mali'. Morphology of leaves and shoots have been used in many ampelographic studies as a reliable and complementary method to SSR markers for identification and differentation of cultivars (Detweiler *et al.* 2000, Ortiz *et al.* 2004, Santiago *et al.* 2005), and sometimes for cultivars derived from the same clonal source (Reisch *et al.* 1993). There is little evidence for such differences on a clonal level. This is in line with the assumption that PMS is very likely a consequence of a single gene mutation without visible pleiotropic effects.

A significant variability among 'Plavac mali' accessions was detected using the variables of the main vein angles on the leaves. The size of the angles is considered one of the highly stable parameters for differentiation of cultivars (Tomažić and Korošec-Koruza 2003). In this study, taking into account that the studied genotypes represent the clonal offspring of one cultivar, the variability of the main angles was unexpectedly quite high. A possible reason for this variability, apart from genetic factors, could be viral infections, since they can significantly change the morphology of leaves (Walter and Martelli 1998). However, looking for a possible discrepancy of PMS genotype in regard to differences in vein angles, cluster analysis (data not shown) confirmed PMS was quite average in regard to variation of this trait. Generally, we observed that differences in angles did not have a significant influence on the leaf morphology, and their stability and efficiency in detection of intravarietal variability should be investigated further through several vegetative seasons.

The main difference between PMS and other accesions of 'Plavac mali' was in the surface color of the berry skin. The color was quantified using CIELab parameters, and visual differences were confirmed, with the lowest CIRG for PMS (4.17). The standard blue-black accessions varied from 6.86 for ob071 to 8.13 for ob262, which indicates significantly different content of anthocyanins, taking into

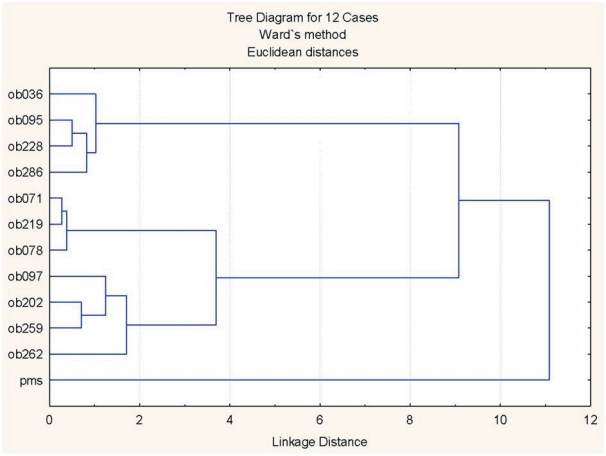

Fig. 2: Dendrogram for twelve accessions of 'Plavac mali' obtained by cluster analysis of the CIELab parameters and CIRG colour index using Ward's method.

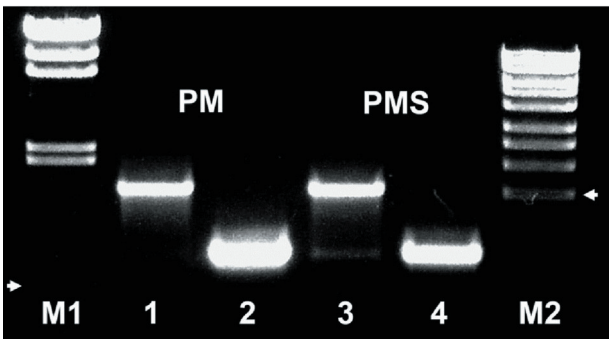

Fig. 3: *Gret1*-specific amplicons at the *VvMYBA1* locus of 'Plavac mali' (PM) and 'Plavac mali sivi' (PMS). Lanes 1 and 3: *ac* primer combination ("white" allele ~ 1.7 kb); lanes 2 and 4: *bc* primer combination ("red" allele ~ 0.85 kb); M1: lambda *Hind* III marker (arrow indicates the position of the 564-bp fragment), M2: 1500-10 000 bp DNA ladder (arrow indicates the 1500-bp fragment).

consideration a strong correlation between CIRG and the content of anthocyanins analyzed using standard chemical methods (Carreno *et al.* 1996, Rolle and Guidoni 2007). While the very different level of content of anthocyanins among PMS and standard 'Plavac mali' was expected (considering the very obvious difference in berry skin color), the observed level of anthocyanins among other referenced 11 standard genotypes became quite intriguing (Fig. 2). These differences, if confirmed to be genetically stable, might be of great importance for future clonal selection of 'Plavac mali'. This fact has been observed previously in grapes of 'Tempranillo' and 'Barbera' clones (Revilla *et al.* 2009, Ferrandino and Guidoni 2010).

Molecular differences: The SSR analysis confirmed the uniform genotype of 'Plavac mali' and the monozygotic status of all accessions. Among nine analyzed loci, there were two (VVS2 and VVMD32) out of five that had strong polymorphism and were recommended for the detection of clones within a cultivar (Pelsy *et al.* 2010); but in this research they did not show any difference among the tested accessions of 'Plavac mali'. The uniform SSR genotype at six SSR loci was also obtained in the study of berry color mutation for 'Garnacha Blanca', 'Garnacha Gris' and 'Garnacha Negra' (Ortiz *et al.* 2004), and generally low level of polymorphism at clone level by Moncada *et al.* (2006). Along with many other similar studies, our study confirms SSR markers as being very good for validation of cultivar/variety status, but not so efficient for clone differentiation.

Considering the particular mutation responsible for berry skin color, a more direct way is the analysis of genes responsible for the synthesis of anthocyanins. However, in this study, we performed the preliminary analysis of one berry color-related locus (*VvMYBA1*) in respect to the presence of the *Gret1* retrotransposon. We showed the heterozygous state of the *Gret1* insertion in both 'Plavac mali' and PMS. Walker *et al.* (2006) have demonstrated that the deletion of a large region that includes both genes (*VvMYBA1* and *VvMYBA2*) is a genetic basis that caused the appearance of pink (Malian) and white (Shalistin) color of berries in 'Cabernet Sauvignon'’. The authors proposed a similar mechanism, involving a smaller deletion, to explain the appearance of pink ('Pinot gris') and white ('Pinot blanc') color in 'Pinot'. It seems that the mechanism of genetic changes in the clones with changed berry color is fairly complex. Since 'Plavac mali' is heterozygous for the *VvMYBA1* locus, similar to 'Cabernet Sauvignon' and 'Pinot noir', it could be that PMS arises by an analogous mechanism: inactivation/deletion of the remaining functional *VvMYBA1* allele in the L2 cell layer. However, this hypothesis should be further tested experimentally, including the *VvMYBA2* locus as well (Walker *et al*. 2007).

The results of this study prove PMS as being genetically stable and a quite distinctive mutant of the cultivar 'Plavac mali'. The type of mutation that caused the PMS phenotype and its locus are still unknown. SSR profiling, ampelographical description, and morphometry made so far provide sufficient data necessary for adding 'Plavac mali sivi' (PMS) on the official Croatian cultivar list.

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