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Source / Izvornik: **GENETIKA - BELGRADE, 2019, 51, 1061 - 1073**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.2298/GENSR1903061I

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:151:886594

UDC 575.
https://doi.org/10.2298/GENSR19030061I
Original scientific paper

GENETIC, MORPHOLOGICAL AND CHEMICAL CHARACTERISATION OF THE GRAPE VARIETY 'PROBUS' (*Vitis vinifera* L.)

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Ivanišević D., M. Kalajdžić, G. Di Gaspero, M. Drenjančević, N. Korać, F. Schwander, U. Braun, G. Barać, S. Foria (2019): *Genetic, morphological and chemical characterisation of the grape variety 'Probus' (Vitis vinifera L.).*- Genetika, Vol 51, No.3, 1061-1073.
In the middle of the 20th century, varieties of *Vitis vinifera* locally grown in the Balkans were crossed with varieties from Western Europe with the aim of improving wine quality. The variety 'Probus' originated from the crossing of 'Kadarka' and 'Cabernet Sauvignon' and was released in 1983. The acreage of 'Probus' has recently increased in Serbia, along with the reputation of its wine. A detailed characterisation of this variety is therefore desirable. Traits with high heritability such as phenology, leaf morphology, anthocyanin profile, and microsatellite DNA were compared between 'Probus' and its parents were compared. 'Probus' and 'Cabernet Sauvignon' showed a synchronized phenology and highly similar leaf shape. The anthocyanin composition of 'Probus' wines was more similar to 'Cabernet Sauvignon' than to 'Kadarka', due to high anthocyanin content and high relative abundance of tri-substituted monoglucosides and acetylated conjugates,

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which are usually associated with deeper and more durable colour during the ageing of red wines. For total anthocyanin content, 'Probus' performed even better than 'Cabernet Sauvignon' in the environmental conditions of Northern Serbia. 'Probus' has compact bunches and large berries typical of the high-yielding 'Kadarka'. DNA analysis confirmed the alleged parentage of 'Probus' and revealed the high heterozygosity of its genome, as result of the cross between distantly related parents. These results confirm that 'Probus' is a promising variety for the production of quality red wines in Southeastern Europe.

Keywords: ampelography, anthocyanins, grapevine microsatellite markers, *Vitis vinifera*, 'Probus'

INTRODUCTION

Local grape varieties are an important component of viticulture in historical wine countries. In the domestic or regional market, the consumers' taste is usually stereotyped around local varietal wines and wine districts are renowned mostly for their flagship varieties, as it happens for Prokupac in Serbia and for Vranac in Montenegro. Contrary to globally spread varieties, also known as international varieties, local grapes are usually more suited to local growing conditions. The use of autochtonous varieties is popular in the Balkans, although they were partially replaced by the introduction of international varieties, because their wines allow wineries to increase more easily their export sales. The wines obtained from local varieties in the Balkans hardly achieve the same level of quality, and among-years consistency, as those obtained from Western varieties under the same growing conditions.

To bridge this gap, Serbian breeders have crossed in the middle of 20th century Balkan wine grapes with Western Europe varieties to develop new cultivars characterized by local heritage and improved enological characteristics. As a result of a cross between 'Kadarka', which is a typical high-yielding large-berried variety of the Southern Balkans, and 'Cabernet Sauvignon', which is a small-berried variety that originated from North of the Pyrenees, breeders have selected the variety 'Probus' (VIVC variety number 9719), which was released for cultivation in 1983. This variety was named after Marcus Aurelius Probus (232-282), the Roman emperor who introduced viticulture to the Roman province of Pannonia, today corresponding to Hungarian, Croatian and Northern Serbian plains. As it usually happens to newly bred wine grapes, 'Probus' has remained confined in germplasm collections for more than 25 years, before breaking into the market. 'Probus' grapes are more similar in morphology to 'Kadarka' than to 'Cabernet Sauvignon' (CINDRIĆ *et al.*, 2000). In spite of this, 'Probus' wines bear more resemblance to the nobility of 'Cabernet Sauvignon' than to 'Kadarka'. 'Probus' is now regarded by Serbian growers as an interesting alternative to growing international varieties for the production of quality red wines and the domestic acreage of cultivation is rapidly increasing. Therefore, further work on the characterisation of 'Probus' is desirable.

Ampelography is used for the description of grape varieties. The mature leaf is an ideal organ for varietal identification (GALET, 1979). Traditional ampelography was cumbersome, relying on manual measurements and/or arbitrary assignment to classes, based on visual comparison with standard varieties. Today, digital imaging software assists evaluators in taking coordinates of the leaf relevant points for biometric descriptions and scanning rapidly many leaves. This makes ampelography less dependent on the evaluators' scores, less affected by biological variation within and between plants, and ultimately more precise. Ampelographic identification is irreplaceable for growers, nurserymen, inspectors, and gene bank managers to

recognize a grape variety in commercial vineyards, nurseries and germplasm collections, avoiding gross errors during the spread of the propagating material. However, ampelography requires the support of DNA fingerprinting for a conclusive proof of true-to-typeness and parentage, in forensic and scientific analyses (SANTIAGO *et al.*, 2005).

Similar to leaf shape, the anthocyanin profile also has high heritability and low environmental variation. In the past, the composition of anthocyanin monoglucosides in grape juice and red wine was used as a chemical marker. More recently, the ratio between acetylated to p-coumaroylated anthocyanin conjugates has been used for assessing the varietal origin of wines (BURNS *et al.*, 2002). The concentration of anthocyanin diglucosides is a marker for grapes and wines of non-vinifera origin. Anthocyanin composition determines the gradation of red colour in wines and their potential for ageing. Being highly dependent on the grapevine genotype and little pliable by agronomic and enological practices (HE *et al.*, 2012), a detailed knowledge of anthocyanin profile in a new variety is important to growers for guiding their planting decisions.

Phenology is another phenotypic trait under a strong genetic control. The duration of the period between blooming and véraison – a French word for indicating the onset of ripening in viticulture – is controlled by a single major locus (ZYPRIAN *et al.*, 2016). This interval is approximately 60 days long in most grape varieties, but it is significantly shortened by 10-15 days in a group of varieties with the commonality of sharing familial relatedness with 'Pinot Noir'. A shorter blooming-to-veraison interval is associated with anticipated commercial maturity. In warmer climates of Southern Europe, early-ripening varieties force growers to anticipate grape harvest in hot days of middle summer.

The aim of this work was to characterise the variety 'Probus' for ampelographic traits, DNA profile, key phenological stages, and anthocyanin content. These characteristics against to the parental cultivars 'Cabernet Sauvignon' and 'Kadarka' were compared.

MATERIALS AND METHODS

The vines used for this research were grown in Sremski Karlovci, at the experimental station of the University of Novi Sad, Faculty of Agriculture (45º10´ N, 20º10´ E), in the season 2015. Vines of 'Probus', 'Cabernet Sauvignon' and 'Kadarka' were trained to a modified Guyot training system, with an average of 14 buds per vine. Row and vine spacing were 2.8 m (between rows) × 1.6 m (between adjacent vines in a row). Rows had a NE-SW orientation. Fermentation was carried out in three repetitions for each variety. Grapes were destemmed and crushed. Pomace was added 15 mg L^{-1} SO_2 and inoculated with *Saccharomyces cerevisiae* (Uvaferm BDX). Fermentations were performed in 5 L glass fermenters at a temperature of 25°C. Pomace was mixed twice a day. After seven days of fermentation and maceration, must was extracted using a mechanical press. Wines were finally added 20 mg L^{-1} SO_2 and aged in anoxic conditions. Wine samples were taken for analyses nine months after the end of fermentation.

Phenological observations

Three key phenological stages were scored on the basis of the BBCH-scale: BBCH-07 corresponding to the beginning of budburst, which is the date when green shoot tips become visible; BBCH-60 corresponding to the beginning of flowering, which is the date when first flower hoods detach from the receptacle; and BBCH-80 corresponding to the beginning of veraison, which is the date when the first green berries turn red.

Ampelometric analysis

Digital photos of 20 representative leaves per variety were taken using a digital camera and processed by the computer program SuperAmpelo, a software for ampelographic descriptions and measurements (SOLDAVINI *et al.,* 2009). For each variety, SuperAmpelo calculated and plotted an average leaf image. SuperAmpelo was also used for computing the index of similarity (%). Leaf descriptors were expressed according to the standards of the International Organization of Vine and Wine (ANONYMOUS, 1983).

High-performance liquid chromatography (HPLC) of anthocyanins

Wine samples stored at -20°C prior to HPLC analysis. Wines were centrifuged for 3 min. The supernatant was transferred into HPLC-vials. Sample preparation was performed according to the OIV-MA-AS315-11 protocol (ANONYMOUS, 2007). Anthocyanins were injected into an Agilent 1100/1200 series HPLC system equipped with an Agilent photodiode array detector (DAD). The separation was performed on a reversed phase column LiChrospher 100 RP 18 (5 µm) in LiChroCart 250-4 (MERCK) with a guard column LiChroCart 4 mm RP 18 (MERCK), at a temperature of 20°C. The following HPLC-grade solvents were used: water/formic acid/acetonitrile (87:10:3, v:v:v) as solvent A, and water/formic acid/acetonitrile (40:10:50, v:v:v) as solvent B. Elution was performed at a flow rate of 0.4 mL/min, using a gradient elution starting with 6% (B), increasing to 30% (B) at 15 min, increasing to 50% (B) at 30 min, increasing to 60% (B) at 35 min, and decreased to 6% (B) at 41 min. The detection wavelength was 520 nm. Anthocyanin compounds were identified by comparing the retention time with available standards or the spectral characteristics with data from literature (BURNS *et al.*, 2002; RYAN and REVILLA, 2003; RADOVANOVIĆ and RADOVANOVIĆ, 2010). Quantification was made by means of a calibration curve ($R^2 = 1$), obtained by injecting the standard malvidin-3-monoglucoside chloride in a serial dilution. Data were expressed as malvidin-3-glucoside equivalents (mg/L). All analyses were performed in three repetition and results were expressed as mean values. The significance of differences between means was tested by analysis of variance and a Least Significant Difference (LSD) test.

Data were validated in terms of limits of detection and quantification, linearity, repeatability and reproducibility. The limit of detection (LOD) and the limit of quantification (LOQ) were determined at signal/noise ratios of 3 and 10, respectively, and estimated for seven anthocyanins using purchased standards. The seven anthocyanins showed linearity with $R^2 >$ 0.999 in the range of 0.05 – 50 mg/L for Cyanidin-3,5-*O*-diglucoside, 0,05-100mg/l for Cyanidin-3-*O*-glucoside and 0,05- 500mg/l for Delphinidin-3-*O*-glucoside, Malvidin-3,5-*O*-diglucoside, Petunidin-3-*O*-glucoside, Peonidin-3-*O*-glucoside and Malvidin-3-*O*-glucoside. Intraday repeatability and interday reproducibility were determined using an acidic ethanol-water extract (EtOH/H2O/HCl, 70:29:1, v:v:v) of grape skins from the cultivar 'Pinot Noir' (VIVC variety number 9279). Repeatability and reproducibility were calculated by relative standard deviations (RSD) of five anthocyanin monoglucosides (Delphinidin, Cyanidin, Petunidin, Peonidin, and Malvidin). For intraday repeatability the extract was injected into the HPLC system eight times within 24 h. Intraday variation was evaluated on five consecutive days.

Genotyping

Genomic DNA was extracted from leaf samples using a CTAB-based method (http://www.opsdiagnostics.com/notes/protocols/ctab_protocol_for_plants.htm). PCR reactions

were carried out in a volume of 10 μL containing 200 μM of each dNTP, 0.2 μM of each primer, 10 ng of genomic DNA, and 0.5 U of HotMaster Taq polymerase (Eppendorf). A total of 16 SSR primers were used for genotyping: VrZAG62, VrZag79 (SEFC *et al.*, 1999), VVMD5, VVMD7, VVMD21, VVMD27, VVMD32 (BOWERS *et al.*, 1996), VVS2 (SCOTT *et al.*, 2000), VVIB01, VVIH54, VVIN16, VVIN73, VVIP31, VVIQ52 (MERDINOGLU *et al.*, 2005) VMC4F3-1 (DI GASPERO *et al.*, 2000) and VMC1B11 (NCBI database, https://www.ncbi.nlm.nih.gov/nucleotide/BV681754). Forward primers were labelled with a fluorescent dye. The PCR reactions were carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems), with the following thermal profile: 95°C for 2 min, followed by 10 touch down cycles at 94°C for 20 s, 55°C (– 0.5°C/cycle) for 20 s, 65°C for 40 s, followed by 25 cycles at 94°C for 20 s, 50°C for 20 s, 65°C for 40 s, and a final elongation of 30 min at 65°C. PCR products were diluted in 100 μL dH_2O. Then 2 μl (diluted PCR products) were added to 0.2 μL LIZ 500 size standard and 7.98 Hi-Di Formamide (Applied Biosystems) and separated by capillary electrophoresis using an ABIPrism 3730xl DNA analyzer (Applied Biosystems). Alleles were called and sized using GeneMapper Software v4.0 (Applied Biosystems), with supervised user annotation of the identified peaks.

RESULTS

Phenology

The dates of key phenological stages are presented in Table 1. Bud burst was coincident in 'Probus' and both parents. 'Probus' and 'Cabernet Sauvignon' required a shorter period of shoot growth from budburst to the beginning of flowering (37 days), compared to 'Kadarka' (40 days). The beginning of véraison differed among varieties. Véraison was first observed 60 days after flowering in 'Cabernet Sauvignon', 61 days after flowering in 'Probus', and 66 days after flowering in 'Kadarka'.

Table 1. Beginning of the phenological stages of 'Probus', 'Cabernet Sauvignon' and 'Kadarka' in 2015.

Variety	Budburst	Flowering	Veraison
Probus	19.04.	26.05.	26.07.
Cabernet Sauvignon	19.04.	26.05.	25.07.
Kadarka	19.04.	29.05.	31.07.

Leaf shape

The average leaf of 'Probus', as a result of the computation by the software SuperAmpelo (Figure 1), was more similar to 'Cabernet Sauvignon' (similarity index of 93.4%) than to the average leaf of 'Kadarka' (similarity index of 82.7%). The length between the petiole sinus and the upper leaf sinus (OIV code 605) and the length between the petiole sinus and the lower leaf sinus (OIV code 606) were shorter in 'Probus' and 'Cabernet Sauvignon' compared to 'Kadarka'. A comprehensive list of OIV codes with descriptions, values and notes is given in Table 2.

Anthocyanin content and profile

Eighteen anthocyanins in the wines of 'Probus', 'Cabernet Sauvignon' and 'Kadarka' were detected, 14 of which were present in all wines. 'Probus' wines had significantly higher content

of total anthocyanins (133.3 mg/L ± 0.54) than 'Cabernet Sauvignon' wines (83.48 mg/L ± 0.50) and Kadarka wines (54.06 mg/L ± 0.10). 'Probus' showed the highest concentration for all detected anthocyanins, except for five compounds for which 'Probus' was not significantly higher than 'Kadarka' but it was significantly higher than 'Cabernet Sauvignon' (Table 3).

Table 2. OIV codes for the average leaves of 'Probus', 'Cabernet Sauvignon' and 'Kadarka'; different letters in the superscript in the same row indicate statistically significant means ($p < 0.05$); Notes according to OIV are given in the brackets.

Codes	Description	Probus	Cabernet S.	Kadarka
OIV601	Length of vein N1 (mm)	135.9 ± 17.7 ^{a} (medium)	121.1 ± 12.1 ^{b} (medium)	125.0 ± 20.7 ^{b} (medium)
OIV602	Length of vein N2 (mm)	121.2 ± 15.3 ^{a} (long)	105.7 ± 10.0 ^{b} (medium)	107.8 ± 13.8 ^{b} (medium)
OIV603	Length of vein N3 (mm)	83.4 ± 11.1 ^{a} (medium)	79.4 ± 8.6 ^{a} (medium)	78.0 ± 9.5 ^{a} (medium)
OIV604	Length of vein N4 (mm)	50.5 ± 8.9 ^{a} (very long)	49.0 ± 6.5 ^{a} (very long)	50.6 ± 6.6 ^{a} (very long)
OIV605	Length petiole's to upper sinus (mm)	47.1 ± 6.7 ^{a} (short)	43.4 ± 6.8 ^{a} (short)	65.8 ± 12.3 ^{b} (medium)
OIV606	Length petiole's to lower sinus (mm)	46.1 ± 7.3 ^{a} (short)	41.6 ± 5.8 ^{a} (short)	58.8 ± 8.7 ^{b} (medium)
OIV607	Angle between N1 and N2 (°)	54.1 ± 3.4 ^{a} (medium)	57.5 ± 4.9 ^{b} (large)	61.9 ± 3.2 ^{c} (large)
OIV608	Angle between N2 and N3 (°)	45.9 ± 5.9 ^{a} (medium)	48.1 ± 4.8 ^{a} (medium)	54.1 ± 6.5 ^{b} (medium)
OIV609	Angle between N3 and N4 (°)	64.2 ± 8.1 ^{a} (large)	60.8 ± 5.3 ^{a} (large)	63.5 ± 5.7 ^{a} (large)
OIV612	Length of teeth N2 (mm)	13.4 ± 2.9 ^{a} (medium)	12.4 ± 1.8 ^{ab} (medium)	11.0 ± 2.3 ^{b} (short)
OIV613	Width of teeth N2 (mm)	18.0 ± 4.6 ^{ab} (wide)	16.0 ± 2.3 ^{a} (wide)	19.3 ± 3.0 ^{b} (wide)
OIV614	Length of teeth N4 (mm)	10.1 ± 2.9 ^{a} (short)	10.2 ± 1.5 ^{a} (short)	9.9 ± 1.9 ^{a} (short)
OIV615	Width of teeth N4 (mm)	15.5 ± 3.3 ^{a} (medium)	15.6 ± 2.2 ^{a} (medium)	15.2 ± 3.1 ^{a} (medium)

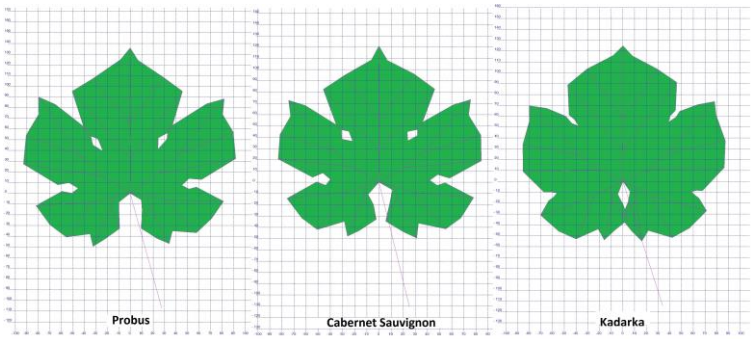

Figure 1. The computed average leaf of 'Probus', 'Cabernet Sauvignon', and 'Kadarka'.

In all three varieties, 3-*O*-glucosides were the most abundant type of anthocyanins. Malvidin-3-*O*-glucoside was the predominant anthocyanin, accounting for 51.8% of all anthocyanins in 'Kadarka', 54.8% in 'Probus', and 57.6% in 'Cabernet Sauvignon' wine. Malvidin-3-*O*-acetylglucoside was the second most abundant compound in the wines of 'Probus'

and 'Cabernet Sauvignon', whereas substantially lower amounts were measured in 'Kadarka' wines.

The profile of anthocyanin monoglucosides in 'Probus' showed higher similarity with 'Cabernet Sauvignon' than with 'Kadarka'. 'Probus' wines were dissimilar from 'Kadarka' in having a detectable level of Cyanidin-3-*O*-glucoside and higher relative content of Malvidin-3-*O*-glucoside (Figure 2A). Anthocyanin monoglucosides are usually classified in two groups based on the number of substitutions on the B-ring. The relative abundance of tri-substituted monoglucosides as percentage of Malvidin + Petunidin + Delphinidin 3-*O*-glucosides out of all monoglucosides was expressed (Figure 2B). The relative abundance of tri-substituted monoglucosides was highest in 'Cabernet Sauvignon' (95.8%), lowest in 'Kadarka' (90.3%) and intermediate in 'Probus' (94.6%).

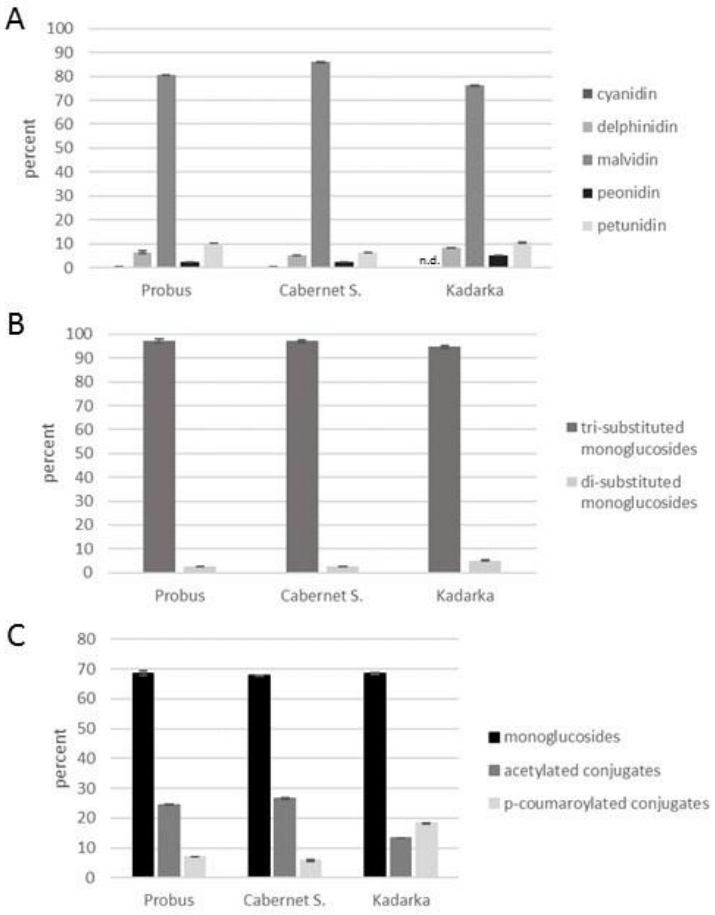

Figure 2. Anthocyanin composition. (A) Percentage of different 3-O-glucosides. (B) Relative abundance of tri-substituted anthocyanins among 3-O-glucosides. (C) Relative abundance of conjugated and non-conjugated anthocyanins.

All three varieties showed similar relative abundances of conjugated versus non-conjugated anthocyanins. However, within conjugated anthocyanins, the relative abundance of acetylated and p-coumaroylated anthocyanins differed significantly. The profile of anthocyanin conjugates was expressed as percentage of acetylated or p-coumaroylated anthocyanins out of all anthocyanins (Figure 2C). Acetylated anthocyanins were much higher in 'Cabernet Sauvignon' and 'Probus' wines, accounting for 26.5% and 24.4% of total anthocyanins respectively, than in 'Kadarka' (13.3%). As a result of this, 'Probus' and 'Cabernet Sauvignon' showed a ratio of acetylated to p-coumaroylated anthocyanins higher than three (3.4 and 4.6, respectively). 'Kadarka' showed the lowest ratio (0.7).

Table 3. Anthocyanins and their concentration (mg/L) in red wines of 'Probus', 'Cabernet Sauvignon' and 'Kadarka'; the results are expressed as means ± SD (standard deviation); different letters in the superscript in the same row indicate statistically significant means ($p < 0.05$).

Anthocyanin	Probus	Cabernet S.	Kadarka
Delphinidin-3,5-*O*-diglucoside	0.63 ± 0.02 [a]	0.44 ± 0.08 [b]	0.42 ± 0.04 [c]
Cyanidin-3,5-*O*-diglucoside	0.72 ± 0.02 [a]	0.41 ± 0.03 [b]	-
Delphinidin-3-*O*-glucoside + Petunidin-3,5-*O*-diglucoside	5.80 ± 0.53 [a]	2.90 ± 0.01 [b]	3.02 ± 0.07 [b]
Cyanidin-3-*O*-glucoside + Peonidin-3,5-*O*-diglucoside	0.49 ±0.04 [a]	0.24 ± 0.02 [b]	-
Petunidin-3-*O*-glucoside	9.20 ± 0.05 [a]	3.45 ± 0.04 [c]	3.85 ± 0.08 [b]
Peonidin-3-*O*-glucoside	2.00 ± 0.02 [a]	1.25 ± 0.00 [b]	1.88 ± 0.13 [a]
Malvidin-3-*O*-glucoside	72.97 ± 0.06 [a]	48.10 ± 0.19 [b]	27.99 ± 0.13 [c]
Delphinidin-3-*O*-acetylglucoside	2.39 ± 0.09 [a]	0.98 ± 0.15 [b]	0.51 ± 0.08 [c]
Cyanidin-3-*O*-acetylglucoside	2.50 ± 0.01 [a]	0.88 ± 0.05 [c]	1.62 ± 0.00 [b]
Petunidin-3-*O*-acetylglucoside	2.49 ± 0.01 [a]	0.85 ± 0.02 [b]	0.34 + 0.00 [c]
Delphinidin-3-*O*-coumaroylglucoside	0.55 ± 0.03 [a]	-	0.92 ± 0.00 [a]
Peonidin-3-*O*-acetylglucoside	1.41 ± 0.01 [a]	0.66 ± 0.01 [b]	0.37 ± 0.00 [c]
Malvidin-3-*O*-acetylglucoside	23.35 ± 0.05 [b]	18.53 ± 0.02 [c]	4.29 ± 0.01 [a]
Cyanidin-3-*O*-coumaroylglucoside	-	-	0.38 +0.01
Petunidin-3-*O*-coumaroylglucoside	0.73 ± 0.01 [a]	0.30 ± 0.01 [b]	1.21 ±0.01 [c]
Malvidin-3-*O*-(cis)coumaroylglucoside	0.59 ± 0.01[b]	0.27 ± 0.00 [c]	0.78 ± 0.00 [a]
Peonidin-3-*O*-coumaroylglucoside	0.83 ± 0.01 [a]	0.45 ± 0.13 [b]	0.95 ± 0.01 [a]
Malvidin-3-*O*-(trans)coumaroylglucoside	6.63 ± 0.03 [a]	3.72 ± 0.29 [c]	5.53 ± 0.09 [b]
Total anthocyanins	**133.3 ± 0.54 [a]**	**83.48 ± 0.50 [b]**	**54.06 ± 0.10 [c]**

Traces of anthocyanin 3,5-*O*-diglucosides (Delphinidin, Peonidin, Petunidin and Cyanidin) were detected in all varieties, accounting for 1% of total anthocyanins in 'Probus' and 'Cabernet Sauvignon', and 0.8% in 'Kadarka' (Table 2).

Genotyping

The DNA fingerprint of 'Probus' was compatible with an origin via hybridization of 'Cabernet Sauvignon' and 'Kadarka' (Table 4.). 'Probus' showed matching alleles with the alleged parents at all 16 SSR loci tested, confirming that the parent-offspring trio is genuine. 'Probus' was heterozygous at 15 out of 16 tested loci, while both parents showed lower levels of heterozygosity. We did not detect any Mendelian inconsistence in 'Probus' that could have led us to mistake hemizygosity for homozygosity in the parents.

Table 4. Inheritance of alleles from 'Cabernet Sauvignon' and 'Kadarka' to 'Probus'. For markers with a single peak, parental genotypes were assumed to be homozygous.

	VrZag62		VrZag79		VVMD27		VVMD5		VVMD7		VVS2	
Probus	193	203	243	245	186	191	223	237	237	252	130	150
Cabernet S.	187	193	243	-	173	186	228	237	237	-	136	150
Kadarka	187	203	245	247	183	191	223	-	245	252	130	132
	VMC4F3-1		VVIB01		VVIQ52		VVMD21		VMC1B11		VVIH54	
Probus	166	172	289	293	78	78	246	255	182	184	168	180
Cabernet S.	172	178	289	289	78	84	246	255	184	-	166	180
Kadarka	-	166	293	293	78	80	246	-	182	184	164	168
	VVIN16		VVIN73		VVIP31		VVMD32					
Probus	150	152	255	267	187	189	238	269				
Cabernet S.	152	152	263	267	189	189	238	-				
Kadarka	150	-	255	261	177	187	269	-				

DISCUSSION

DNA profiling confirmed the parentage of the newly bred variety 'Probus'. While this result was expected on the basis of the pedigree declared by the breeders, the molecular proof is not always confirmatory. In grapevine, the parentage test has frequently disproved the pedigree of varieties that were the result of intentional breeding (LACOMBE *et al.*, 2013). In most cases, the inconsistency between the biological parents and the parental combination the breeder intended to hybridize was due to the paternal variety and it was explained by ineffective measures to prevent pollen drift during artificial hybridization. This was fortunately not the case for 'Probus', which did not show Mendelian inconsistencies at DNA markers and also resembled phenotypically both biological parents.

'Probus' was alternatively more similar to either 'Cabernet Sauvignon' or 'Kadarka', depending on the trait concerned. For instance, 'Cabernet Sauvignon' donated to 'Probus' some morphological peculiarities of the leaf shape, especially the deep leaf lobes, which give the visual impression of holes punched in the leaf lamina (GALET, 1979). 'Kadarka' donated to 'Probus' some morphological peculiarities of the bunch (CINDRIĆ *et al.*, 2000). The seasonal timing of the growth cycle in 'Probus' was intermediate with respect to the parents. In spring, bud burst occurred in 'Probus' as late as in the parents, which is a positive characteristic for reducing the risk of exposure of young shoots to late frost. In line with its parents, 'Probus' is a late-ripening variety due a long blooming-to-veraison interval and is therefore suitable for warmer climates of Southern Europe with lengthy growing seasons.

For other traits, 'Probus' showed transgressive segregation. The improvement of enological characteristics surpassed the expectation of the breeders. Total anthocyanin concentration in 'Probus' is higher with respect to 'Kadarka', but is also higher with respect to 'Cabernet Sauvignon', the parent used for improving this trait. The anthocyanin content in the parental varieties observed under our experimental conditions is consistent with those previously reported for 'Cabernet Sauvignon' by OTTENDER *et al.* (2004) and for 'Kadarka' by NIKFARDJAM and PICKERING (2008). 'Probus' wines were also characterized by very high anthocyanin content under other growing conditions (CVEJIĆ *et al.*, 2016). It was not surprising to observe

transgressive segregation in a grapevine hybrid. Our molecular analysis showed that 'Probus' is highly heterozygous at the 16 genomic loci tested, higher than either parent. 'Probus' has originated from the cross of two genetically diverse varieties, one belonging to the convarietas *occidentalis* (Negr), the other belonging to the convarietas *balcanica* (Negr). The combination in a diploid individual of divergent alleles, which evolved in geographically distant populations, may explain superior phenotypes observed in the offspring.

'Probus' is a variety that deserves high regard for the production of red wines, due to distinguishing qualities of its anthocyanin profile. 'Probus' wines are similar to 'Cabernet Sauvignon' for the high percentage of tri-substituted anthocyanins, which confer deeper colour and are less sensitive to oxidation during wine ageing (SQUADRITO *et al.*, 2010). 'Probus' and 'Cabernet Sauvignon' are both enriched in acetylated conjugates at the expense of p-coumaroylated conjugates, compared to 'Kadarka'. BURNS *et al.* (2002) noticed that a ratio of acetylated versus p-coumaroylated anthocyanins higher than three is a peculiarity of 'Cabernet Sauvignon' wines. SQUADRITO *et al.* (2010) reported that the decline in concentration during ageing and after malolactic fermentation is faster for p-coumaroylated anthocyanins than for acetylated anthocyanins. The same authors hypothesized a higher stability of acetylated anthocyanins towards enzymatic hydrolytic reactions. GÓMEZ-PLAZA *et al*. (2008) investigated the differences in anthocyanin profiles in the progeny of a cross between 'Cabernet Sauvignon' and 'Monastrell'. They found that anthocyanin profiles were intermediate between the parental varieties, but more similar to 'Cabernet Sauvignon' than to 'Monastrell'. This confirms that anthocyanin profile is predominantly controlled by genetic factors and suggests that 'Cabernet Sauvignon' is a donor of favorable alleles.

We also detected traces of anthocyanin 3,5-*O*-diglucosides in all wines, despite the pure *vinifera* genome of all the varieties concerned. The amount of 3,5-*O*-diglucosides found in 'Probus' are in line with those found in 'Cabernet Sauvignon' or 'Kadarka'. We detected 3,5-*O*-diglucosides of cyanidin and delphinidin but not of malvidin, although malvidin was by far the most abundant 3-*O*-glucoside. These observations are consistent with previous reports in *V. vinifera* (HRAZDINA *et al.*, 1970; BALDI *et al.*, 1995; XING *et al.*, 2014;).

In this study, the experimental wines were produced by applying a standardized procedure of vinification and anthocyanins were analyzed at a single stage of wine ageing, approximately nine months after the end of alcoholic fermentation. GONSALES NEVES *et al.* (2013) found that wine composition in individual anthocyanins was consistent with grape skin composition, regardless the vinification process and the stage of wine ageing. However, the concentration of total anthocyanins in wines declines with ageing. RISTIĆ *et al.* (2007) reported a decrease of more than 50% within the first eight months after vinification. According to RIBÉREAU-GAYON *et al.* (2005), anthocyanin profiles slightly change within ageing due to different stability towards oxidation and enzymatic degradation or propensity to polymerize with other flavonoids. Further studies will be required to monitor the long-term evolution of colour and anthocyanin profiles of 'Probus' wines aged in wood barrels or in oxygen-excluding containers.

CONCLUSION

Ampelographic, DNA and chemical data for the varietal identification and characterization of 'Probus' were provided in the study. The parentage test confirmed that the two varieties used for the crossing are indeed the biological parents of 'Probus'. According to leaf shape and phenology, 'Probus' is more similar to 'Cabernet Sauvignon' than to 'Kadarka'. For

yield-related traits, 'Probus' has bunch compactness and berry size more similar to 'Kadarka' than to 'Cabernet Sauvignon', but the anthocyanin profile is more similar to 'Cabernet Sauvignon' than to 'Kadarka'. The high relative abundance of tri-substituted monoglucosides and the high ratio of acetylated to p-coumaroylated conjugates are positive factors for colour intensity and stability. For some characteristics, including total anthocyanin content, 'Probus' performed even better than its noble parent 'Cabernet Sauvignon'. Therefore, these results confirmed that 'Probus' is a promising variety for the production of quality red wines in Southern Europe, in particular in Balkan wine regions.

Received, February 27th, 2019
Accepted May 18th, 2019

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GENETIČKA, MORFOLOŠKA I HEMIJSKA KARAKTERIZACIJA CRNE VINSKE SORTE 'PROBUS' (*Vitis vinifera* L.)

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Izvod

Sredinom XX veka, lokalne sorte vinove loze, gajene na prostoru Balkana, ukrštene su sa sortama poreklom iz Zapadne Evrope sa ciljem unapređenja kvaliteta vina. Kao rezultat ukrštanja 'Kadarke' i 'Kaberne sovinjona' dobijena je sorta 'Probus', koja je priznata 1983. godine, a odlikuje se kvalitetom i raste joj reputacija u Srbiji. Usled toga došlo je do potrebe da se uradi detaljna karakterizacija sorte 'Probus'. U radu su upoređene osobine koje se odlikuju velikom heritabilnošću – fenologija, oblik lista, antocijaninski profil i mikrosateliti sorte 'Probus' i njenih roditelja. Sorte 'Probus' i 'Kaberne sovinjon' su pokazale sinhronizovanu fenologiju i sličan oblik lista. Kvalitativni i kvantitativni sadržaj antocijana u sorte 'Probus'je bio sličniji 'Kaberne sovinjonu' nego 'Kadarki' usled visokog sadržaja ukupnih antocijana i visok sadržaj acilovanih i trisubstituisanih monoglukozida izražen u procentima. DNA analiza je potvrdila da su 'Kadarka' i 'Kaberne sovinjon' roditelji 'Probusa'. Ustanovljena je izražena heterozigotnost genoma sorte 'Probus' što je posledica ukrštanja geografski udaljenih roditelja. Rezultati potvrđuju da je sorta 'Probus' pogodna za dobijanje kvalitetnog crvenog vina u uslovima Jugoistočne Evrope.

Primljeno 27.II 2019.
Odobreno 18. X 2019.