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Anthocyanin profile of wild grape *Vitis vinifera* in the eastern Adriatic region

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ABSTRACT

Wild grapevine (*Vitis vinifera* L., subsp. *sylvestris*) was found recently along the rivers in the karst landscape of the eastern Adriatic region, which includes Croatia and Bosnia and Herzegovina. The wild grapevine is almost extinct and grows in a restricted habitat in Europe. The anthocyanin profile of red grape berries from naturally growing populations of female wild var. *sylvestris* were characterized in two consecutive years and compared to the domesticated cultivars 'Plavac mali', 'Merlot' and 'Xinomavro'. Five anthocyanidin-3-monoglucosides and their corresponding five 3-(6-*O*-acetyl) and five 3-(6-*O*-coumaryl) derivatives were identified and quantified using HPLC-DAD. Most wild grape samples had both acylated and non-acylated anthocyanin monoglucosides, although two individuals lacked acylated forms. DpGl were a greater percentage of the total in wild grape than in 'Plavac mali', 'Merlot' or 'Xinomavro'. Principal component analysis showed differentiation of *sylvestris* from *vinifera* by anthocyanin profile. The anthocyanin profiles of *sylvestris* genotypes from the eastern Adriatic region, here presented for the first time, suggest genetic diversity within *sylvestris* in anthocyanin synthesis.

1. Introduction

The anthocyanins determine the quality of red grape juice and wine and are an important trait for breeding programs. The colour of red grape berries depends on the concentrations and proportions of different anthocyanins accumulated in the skin. The proportions of individual anthocyanins are primarily determined by genotype and anthocyanin profiles can be used to discriminate red grapes and wines produced from different cultivars (Eder et al., 1994; Pomar et al., 2005; Nogales-Bueno et al., 2015; Pisano et al., 2015). The genetic control of anthocyanin biosynthesis is very complex, involving many different enzymes catalyzing each reaction (Boss et al., 1996).

The anthocyanin composition varies significantly among different species of the genus *Vitis* (Liang et al., 2008). Anthocyanins found in *Vitis vinifera* L. include malvidin, cyanidin, delphinidin, peonidin and petunidin 3-monoglycosides with their corresponding acetyl, *p*-coumaroyl and caffeoyl derivates, known as acylated anthocyanins. The absence of acylated anthocyanins is a very rare genetic character in *Vitis vinifera* L., found in the Pinot family of cultivars: 'Pinot noir' and its coloured mutants, 'Pinot gris', 'Pinot tete de negre' and 'Pinot meunier', produce no acylated forms. This genetic trait appears in several cultivars grown in the Rhine basin that are genetically closely related to 'Pinot noir', like 'Blauer Arbst' and 'Deckrtot' (Wulf and Nagel, 1978; Mattivi et al., 2006). Only two red cultivars from Southern Italy, 'Gaglioppo' and 'Tintilia', which produce berries with slightly coloured skins, lack acylated anthocyanins. Some grey and rosé cultivars like 'Muscat rouge de madèré', which are usually mutants of white cultivars, also lack acylated anthocyanins (Mattivi et al., 2006).

Non-*Vitis vinifera* grape species, widely used as rootstocks and sometimes cultivated for human consumption, contain 3,5-diglucosides and pelargonidin-derived anthocyanins. These are also found in hybrid cultivars like 'Concord', obtained by crossing *V. vinifera* and native American species like *V. labrusca* or *V. rupestris* (Wang et al., 2003; Liang et al., 2008).

The *Vitis* ssp. includes uncultivated wild species that represent a valuable source of genes for grapevine breeders focused not only on stable yields and high-quality grapes and wines, but also on good resistance of new cultivars to fungal diseases (Sun et al., 2016; Ruocco et al., 2017).

The color of grape was an important attribute during its domestication, directly affecting visual preference for fruits. Therefore, the anthocyanin profile of different varieties and species has been very intriguing for many grape researchers (Mattivi et al., 2006; Picariello et al., 2014). Different varieties with blue-black, deep dark (teinturier varieties) or distinctive red grape berry color have been developed. In grapevine breeding programs, *Vitis vinifera* was not always considered a good source of color, while some American *Vitis* species showed excellent potential for color enhancement. However, the role of American *Vitis* species in breeding programs has been limited due to their unpleasant impact on aroma (foxy aroma) (Sun et al., 2011; Narduzzi

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et al., 2015).

The wild *Vitis vinifera* L. subspecies *sylvestris* (Gmelin) Hegi is the sole wild grapevine existing in Europe and the ancestor of all cultivated *V. vinifera* varieties (Levadoux, 1956). *V. sylvestris* is an endangered or rare European plant and appears on the red list published by the International Union for Conservation of Nature (IUCN, 1997; Ocete et al., 2011, 2014).

Wild germplasm resources have not been widely explored, leaving the potential of these wild species to improve domesticated grape quality unknown (Revilla et al., 2010). Recently, the first research on the anthocyanin composition of wild grape accessions in a germplasm collection in Spain provided a characteristic fingerprint for several genotypes that was very similar to 'Pinot noir' and 'Gaglioppo' (Revilla et al., 2010; 2012). Some genotypes lacked acylated anthocyanins, although no domesticated Spanish red grape cultivar lacks acylated anthocyanins (Revilla et al., 2012).

The objectives of this study were: (i) to characterize the anthocyanins in wild *sylvestris* grape sampled from four naturally growing populations found recently in Croatia and Bosnia and Herzegovina and (ii) to evaluate whether the anthocyanin composition differs from that of cultivated grape. Individuals from the domesticated cultivars 'Merlot', 'Xinomavro' and 'Plavac mali', planted in the grapevine germplasm collection at the Institute for Adriatic Crops and Karst Reclamation in Split, (Dalmatia) Croatia, were selected for a reference. This is the first data on the anthocyanin composition of *V. sylvestris* populations growing in the eastern Adriatic region along the Mediterranean.

2. Materials and methods

2.1. Reagents and standards

Malvidin-3-*O*-glucoside chloride was obtained from Extrasynthese (Genay, France). LC–MS grade methanol and perchloric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was used for the chromatography. All chemicals and reagents were AR or HPLC grade.

2.2. Plant material

Grapes from 11 previously identified (Zdunić et al., 2017) female individuals of *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi were collected from natural populations in Croatia and Bosnia and Herzegovina in 2014 and 2015 at their usual ripening time in this region, the end of September (Table 1). Because these grape samples were collected from vines growing on natural sites where ecology strongly affected the crop, it was not possible to collect grapes from all individuals in both years. Almost all clusters from wild individuals (~1.000 g/vine) were collected. Three replicate samples of 100 berries from different cluster positions were randomly selected.

Cultivated grape berries of *Vitis vinifera* L. cvs. 'Plavac mali', 'Merlot' and 'Xinomavro' were collected from the germplasm collection at the Institute for Adriatic Crops and Karst Reclamation in Croatia at technological maturity in both years. Three replicate samples of 100 berries from different vines and positions were collected from each cultivar. The grape berries were taken to the laboratory immediately after harvest and peeled. The skins were frozen at −70 °C and lyophilized.

2.3. Extraction of anthocyanins from grape skin

The freeze-dried skin samples were ground into powder using an electric grinder. Five hundred mg powder was extracted with 10 mL acidified methanol (methanol/water/perchloric acid 80/15/5, v/v/v) in a cooled ultrasonic bath based on a modified method (Will and Dietrich, 2013). Up to three successive extractions were performed if colour remained in the powder. The liquid extract and powdered grape skin were separated by centrifugation at 4000 rpm for 15 min. For each sample, the extracts were combined in a volumetric flask and brought up to 50 mL with extraction solution. The extracts were analyzed within three to four hours.

2.4. HPLC-DAD analysis of anthocyanins

The analysis of anthocyanins used a Varian HPLC system (Varian, Inc., Harbour City, CA, USA) consisting of a Star 9010 pump, a Rheodyne 7125 syringe-loading sample injector, a 500-LC module for a column oven, and a ProStar 330 Photodiode Array Detector and were controlled using the Star Chromatography workstation, version 5. Separation was carried out using a Kinetex C18 core-shell column (150 × 4.6 mm) filled with five-μm particles and furnished with a SecurityGuard ULTRA Cartridge UHPLC C18 for 4.6 mm ID column (Phenomenex, Torrance, CA, USA), both thermostated at 35 °C. Anthocyanins were identified and quantified as described in other publications (Vanzo et al., 2008; Fredotović et al., 2017).

The samples and standards were filtered through a 0.45 μm membrane syringe filter prior to analysis. Anthocyanins were identified by retention time and the UV-DAD spectra of each peak and quantified at 520 nm using a calibration curve made with malvidin-3-*O*-glucoside chloride (Extrasynthese, Genay, France). The resulting concentrations were expressed as mg/100 g dry weight and converted to percentages.

2.5. Statistical analysis

Statistically significant differences between the anthocyanin fingerprint data of wild and cultivated samples were determined using one-way analysis of variance (ANOVA) in each year using STATISTICA 10 software (StatSoft Inc., Tulsa, OK, USA). Differences tested by

Table 1
Wild and cultivatedgrapevines in Croatia and Bosnia and Herzegovina and their year(s) of sampling.

Genotype	Species	Site	Country of collecting/cultivation	Year
G1	*V. vinifera* subsp. *sylvestris*	Gizdavac	Croatia	2014, 2015
IJK 90	*V. vinifera* subsp. *sylvestris*	Paklenica	Croatia	2014
IJK 92	*V. vinifera* subsp. *sylvestris*	Paklenica	Croatia	2014
IJK 96	*V. vinifera* subsp. *sylvestris*	Paklenica	Croatia	2014
PAK1	*V. vinifera* subsp. *sylvestris*	Paklenica	Croatia	2015
NE14	*V. vinifera* subsp. *sylvestris*	Neretva	Bosnia and Herzegovina	2014
NE17	*V. vinifera* subsp. *sylvestris*	Neretva	Bosnia and Herzegovina	2014, 2015
NE18	*V. vinifera* subsp. *sylvestris*	Neretva	Bosnia and Herzegovina	2014
NE19	*V. vinifera* subsp. *sylvestris*	Neretva	Bosnia and Herzegovina	2015
IM3	*V. vinifera* subsp. *sylvestris*	Modro jezero	Croatia	2015
IM10	*V. vinifera* subsp. *sylvestris*	Modro jezero	Croatia	2015
'Plavac mali'	*V. vinifera* subs. *sativa*	IAC collection	Croatia	2014, 2015
'Xinomavro'	*V. vinifera* subs. *sativa*	IAC collection	Croatia	2014, 2015
'Merlot'	*V. vinifera* subs. *sativa*	IAC collection	Croatia	2014, 2015