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Comparison of DNA methylation landscape between Czech and Armenian vineyards show their unique character and increased diversity

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Abstract: Grapevine is a worldwide crop and it is also subject to global trade in wine, berries and grape vine plants. Various countries, including the countries of the European Union, emphasize the role of product origin designation and suitable methods are sought, able to capture distinct origins. One of the biological matrices that can theoretically be driven by individual vineyards' conditions represents DNA methylation. Despite this interesting hypothesis, there is a lack of respective information. The aim of this work is to examine whether DNA methylation can be used to relate a sample to a given vineyard and to access a relationship between a DNA methylation pattern and different geographical origin of analysed samples. For this purpose, DNA methylation landscapes of samples from completely different climatic conditions presented by the Czech Republic (Central Europe) and Armenia (Southern Caucasus) were compared. Results of the Methylation Sensitive Amplified Polymorphism method confirm uniqueness of DNA methylation landscape for individual vineyards. Factually, DNA methylation diversity within vineyards of Merlot and Pinot Noir cultivars represent only 16% and 14% of the overall diversity registered for individual cultivars. On the contrary, different geographical location of the Czech and Armenian vineyards was identified as the strongest factor affecting diversity in DNA methylation landscapes (79.9% and 70.7% for Merlot and Pinot Noir plants, respectively).

Keywords: authentication; grapevine cultivar; geographical origin; plant adaptation; epigenetic changes; methylation sensitive amplified polymorphism (MSAP)

Grapevine is a worldwide crop and it is also subject to global trade in wine, berries and grape vine nurslings. In this regard, the European Union protects agricultural products as strictly linked to the area of origin by means of designations of origin. For example, protected designation of origin (PDO) is usually used for wines of high economic value. The pressure to control origin of wines and grapes is further exacerbated by the fact that there are approx. 10 000 known vine cultivars, but only as few as 13 of them represent 66% of worldwide wine production (OIV 2017).

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Given the need to authenticate an origin of wine production, suitable methods are being sought for that would enable capturing of distinct geographical origins or even specific vineyards. Until now several methods have been described which differ by their principle and suitability for different purposes (confirmation of cultivar, vineyard, terroir, region, continent etc.). Regarding cultivar identification, the most successful strategy currently available are methods based on usage of DNA markers, namely simple sequence repeats (SSR) (This et al. 2004; Moravcová et al. 2006). The main advantage of SSR markers is that there exists a worldwide accessible database of SSR fingerprints covering thousands of grapevine cultivars (The European Vitis Database). Moreover, selected SSR markers are respected by International Organisation of Vine and Wine (OIV) as official descriptors frequently used for effective cultivar identification (Baránková et al. 2020).

For authentication of the terroir or a given vineyard outputs, methods analysing microbiome typical for respective location (Jara et al. 2016; Vitulo et al. 2019), stable isotopic ratio of the wine as the 87Sr/86Sr ratio (Braschi et al. 2018; Sighinolfi et al. 2018) or Ethanol(D/H) (Römisch et al. 2009) represent some of the successful strategies. However, both these analyses are costly, require highly specialised operators and needs the specific equipment. Another group of techniques utilised within food product authentication are chemometric approaches where the analytical target can be trace elements analysis (Capron et al. 2007) or volatile composition (Green et al. 2011). Currently, usage of spectroscopic techniques, such as near infrared (NIR), mid infrared (MIR) and Raman spectroscopy, is on the increase (Chandra et al. 2017; Teixeira dos Santos et al. 2017).

The biological matrix that can change due to different environmental conditions or cultural practise used in a given vineyard theoretically represent also DNA methylation. It belongs to a group of epigenetic tools by which a plant can alter its gene expression, most often in a respective promoter region (Li et al. 2012). Well described is also a linkage of DNA methylation with other epigenetic factor such as chromatin acetylation or activation of transposable elements. There already exist many examples where a plant responds to various conditions, frequently stressful ones, by altering its DNA methylation. (Baránek et al. 2015; Lämke & Bäurle 2017).

Reasonability of DNA methylation as a tool for geographical tracing is also supported by the fact that despite the same cultivars or even clones are used of throughout the world, different grape quality is observed in different locations (Berna et al. 2009; Green et al. 2011). These differences must be driven by different expression of the respective genes. And just epigenetic principles as DNA methylation and chromatin modification, together with standard gene regulatory mechanisms on the RNA or protein level, are the factors that control setting of currently expressed genes. Despite the hypothesis being obvious, only one paper has been published until now dealing with DNA methylation and its changes based on different wine growing regions (Xie et al. 2017). The aim of this work is to contribute to the discussion on whether DNA methylation can be used to confirm affiliation of samples to a given vineyard. Simultaneously, we assayed whether DNA methylation pattern can be affected by significantly different climates of two geographically distant areas. For this purpose, samples from 2 000 km distant vineyards with completely different climatic conditions were analysed, namely Merlot and Pinot Noir samples from the Czech Republic (Central Europe) and from Armenia (interface of Europe and Asia). DNA methylation landscape of the samples was verified by methylation sensitive amplified polymorphism (MSAP) method.

MATERIAL AND METHODS

Description of the vineyards and climatic conditions in the Czech Republic and Armenia. Vineyards with Merlot and Pinot Noir cultivars were selected for the analysis because of their worldwide use. Two neighbouring vineyards Sedlec u Mikulova and Lednice in the Czech Republic and vineyard of "Karas Wine" company ("Tierras de Armenia" CJSC) near Baghramyan village, Armenia were selected for both cultivars. To control plant age as a hypothetical factor influencing DNA methylation landscape, vineyards of the same age (8 years) were augmented by one vineyard of the age of two years in the Czech Republic. This age composition of the analysed vineyards (8 years + 8 years + 2 years) was preserved for both the analysed cultivars (for details see Table 1).

Climatic conditions of both countries included in the experiment are significantly different. The climate throughout the Czech Republic is mild, transient between oceanic and continental, with a typical alternation of four seasons (in the same way as throughout Central Europe). The climate of Armenia is completely different and affected by the fact that

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Table 1. Details of the vineyards from which the methylation sensitive amplified polymorphism (MSAP) analysis samples were taken

Variety/clone	Abbreviation	Location	Planted	Elevation (m a.s.l.)	GPS
Merlot/181	Me-CZ-2	Sedlec	2016	225	48.7978269N, 16.6822581E
Merlot/French unknown	Me-CZ-8	Lednice	2010	195	48.7898792N, 16.7974319E
Merlot/343	Me-AR-8	Baghramyan	2010	1 060	40.11723478N, 43.8708457E
Pinot noir/115	PN-CZ-2	Sedlec	2016	225	48.7978269N, 16.6822581E
Pinot noir/PO-20	PN-CZ-8	Lednice	2010	195	48.7898792N, 16.7974319E
Pinot noir/389	PN-AR-8	Baghramyan	2010	1 060	40.17234478N, 43.8708457E

Armenia lies on the southern slopes of Lesser Caucasus with more than 90% of its territory situated over 900 m a.s.l. Average climatic conditions of both studied territories are compared within Figure 1, where hotter summers and colder winters are evident for Armenia. Also, the distribution of precipitation in individual months is significantly different, with the maximum of precipitation in Armenia recorded during winter months and vice versa in the summer months in the Czech Republic. There is also more sunshine in Armenia (an average of 3 527 h/year) as compared to the Czech Republic (an average of 1 787 h/year) (data obtained from climatological station of Czech Hydrometeorological Institute in Lednice and Armenian State Hydrometeorological and Monitoring Service for Baghramyan territory).

Plant material and sampling of the tissue for DNA isolation. Six vines from each vineyard were selected in 2018 for sampling with taking care to exclude vines adjacent to missing vines, end of row vines and border rows to prevent differences in competition effects between the plants. The rows were also chosen in such way as to represent the entire vineyard area. Leaf samples (the first fully expanded leaf at a bud burst, E-L 7) (Coombe 1995) were collected from three nodes per plant and pooled into a single sample per plant. After sampling the leaves were briskly transported to the laboratory in a portable refrigerator where they were immediately frozen by liquid nitrogen and homogenized using pestle and mortar. DNA was isolated by DNeasy Plant Mini Kit (Qiagen, Netherlands) in accordance with the manufacturer's protocol.

Confirmation of cultivar authenticity by means of SSR analysis. Cultivar authenticity of all samples was confirmed by SSR analysis of 9 microsatellite loci used worldwide for cultivar identification purposes (This et al. 2004). SSR analysis was performed according to protocol (Baránková et al. 2020).

MSAP analysis. The extracted DNA (70 ng for each combination of endonucleases) was used as a template for MSAP reaction. The rest of the procedure was carried out in according to protocol (Baránek et al. 2010). Shortly: Isoschizomers *Hpa*II/*Msp*I (New England Biolab, USA) recognizing 5'CCGG, but different by susceptibility to inner and outer cytosine methylation were used for DNA restriction, both in

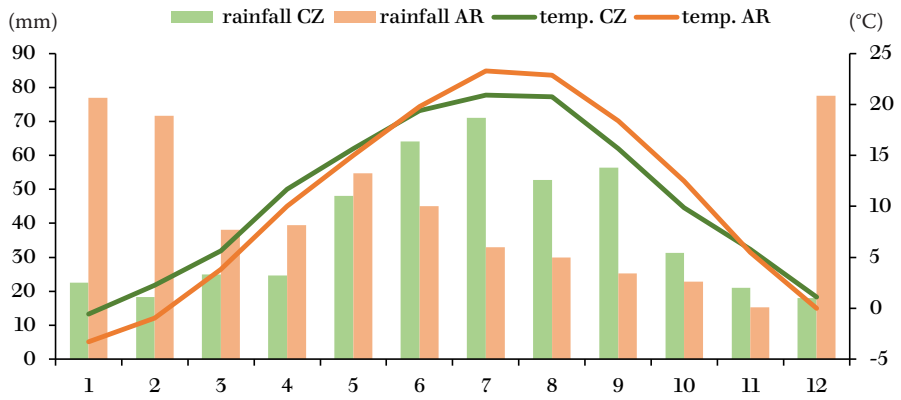

Figure 1. Monthly averages of precipitation and temperatures recorded in respective locations of the Czech and Armenian vineyards (2009–2018)

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Table 2. Data on the values of internal variability in individual vineyards

Abbreviation/location	Average coefficient of similarity within samples from the same location	Average number of polymorphic products within samples from the same location
Me-CZ-2/Sedlec u Mikulova	0.9936	2.9
Me-CZ-8/Lednice	0.9963	1.7
Me-AR-8/Baghramyan	0.9423	21.2
PN-CZ-2/Sedlec u Mikulova	1.0000	0
PN-CZ-8/Lednice	0.9968	1.4
PN-AR-8/Baghramyan	0.9895	4.6

combination with *Eco*RI restriction enzyme (New England Biolab). Three differently labelled primers derived from an *Eco*RI restriction site (*Eco*RI-ACA (FAM), *Eco*RI-ACT (JOE), *Eco*RI-ACC (NED)) were used for a selective amplification, each in a combination with a primer derived from *Hpa*II/*Msp*I restriction site (*Hpa*II/*Msp*I-TCAA, *Hpa*II/*Msp*I-TCGC, *Hpa*II/*Msp*I-AGCT). A total of 9 primer combinations were analysed. The amplification products together with a size standard 500 ROX (Applied Biosystems, USA) were separated in POP 4 polymer medium (Applied Biosystems) in the capillary of ABI PRISM 310 genetic analyzer (Applied Biosystems). GeneScan software (Applied Biosystems) was used to evaluate the presence or absence of individual MSAP amplicons across the samples. Distribution of MSAP amplicons within the individual samples (i.e., presence *vs*. absence of a given DNA fragment) was translated into a presence/absence data matrix and typed into a computer file as a binary matrix. Subsequently, MSAP data originating from digestion by *Msp*I and *Hpa*II were put together for each variant and used as a base for calculation of their mutual epigenetic similarity using the Nei and Li/Dice algorithm (Nei & Li 1979). Genetic similarity/dissimilarity coefficients were computed using the UPGMA method; corresponding dendrograms were generated using MEGA6 software (Kumar et al. 2018). The obtained binary matrices were also used to compute PCoA in order to visualize the main tendencies in molecular differentiation between the samples and PhiPT distances to determine the genetic differentiation between the vineyards, both using GenAlEx software (Peakall & Smouse 2006, 2012).

RESULTS

Confirmation of cultivar authenticity by means of SSR analysis. SSR analysis was performed to confirm the affiliation of all analysed genotypes to Merlot or Pinot Noir cultivar. For complete list of analysed genotypes see Table 1. SSR analysis unequivocally confirmed an authenticity of all samples to their respective cultivars. In fact, all samples showed SSR profiles identical with standards for Merlot and Pinot Noir cultivar in the worldwide database of SSR profiles (http://www.eu-vitis.de/index.php) used for the purpose of identification of vine cultivars. Obtained results are presented in Table S1 in Electronic Supplementary Material (ESM).

Similarity of DNA methylation patterns between analysed samples. For each genotype, the presence or absence of 274 amplicons in the Pinot Noir cultivar and 303 amplicons in the Merlot cultivar were evaluated. A higher variability was observed in Merlot, where the similarity of individual samples ranged from 0.79 to 1, with only two genotypes having identical profiles. Similarity within the Pinot cultivar was very high, ranging from 0.94 to 1. The numbers of polymorphic products registered between individual samples are available in Supplementary file (Tables S2 and S3 in ESM). Examples of polymorphic products within the generated electrophoregrams are then presented as Figures S1 and S2 in ESM.

Results of the MSAP analysis as a reflection of a different DNA methylation landscape established in individual samples are presented in form of dendrograms (Figure 2). Unequivocal distribution to the clusters assembled on the base of their affiliation to the respective vineyard is clearly evident for all 6 analysed group of genotypes. When comparing samples taken from the same vineyard, samples from Armenia showed a higher internal variability for both cultivars (i.e., Merlot and Pinot Noir samples), details in Table 2. Similarly, samples from Armenia formed clusters distinct from samples originating from the Czech Republic repeatedly for both cultivars.

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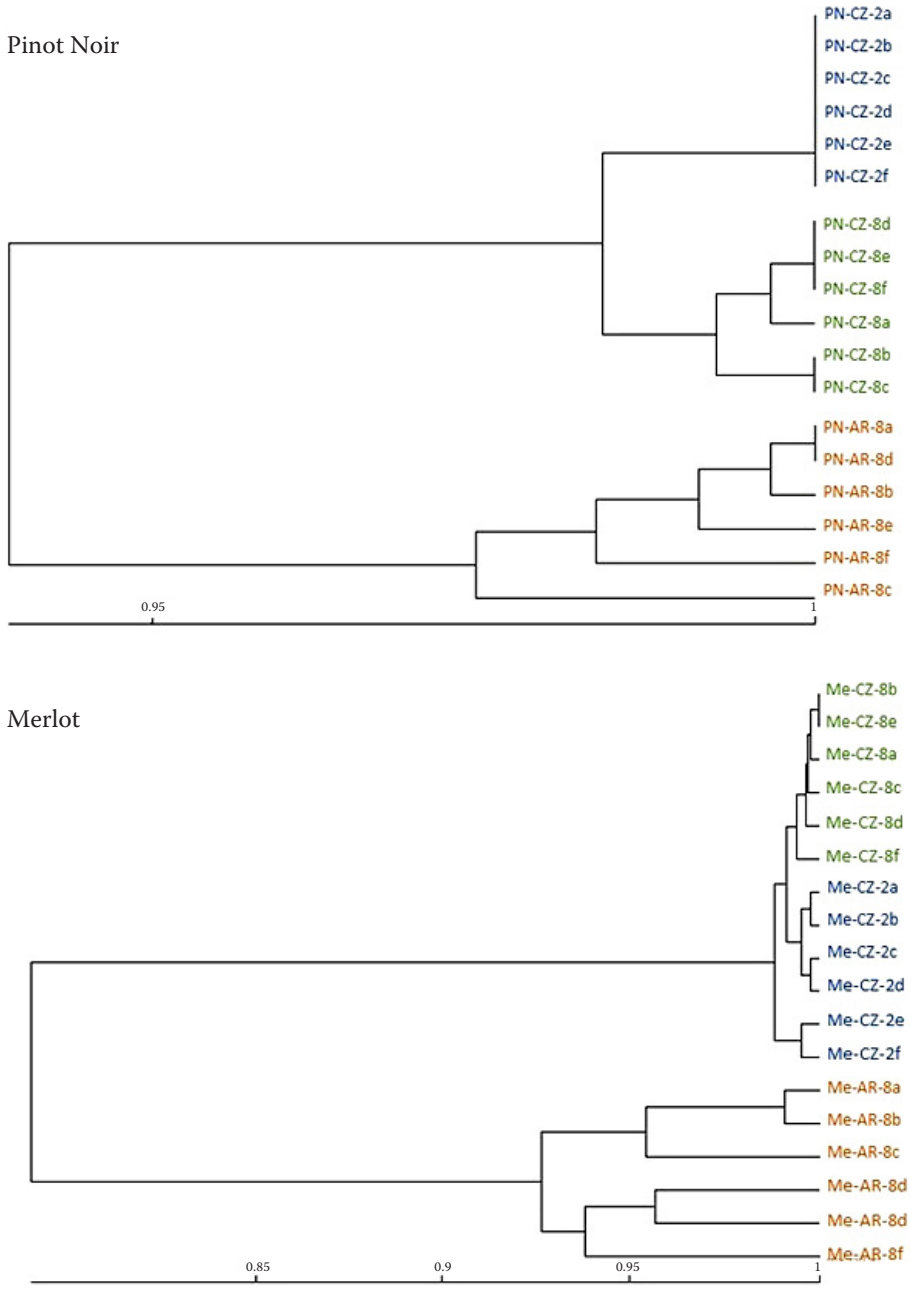

Figure 2. Dendrograms created based on calculated mutual coefficients of similarity
PN – Pinot Noir samples; Me – Merlot samples; PN/Me-CZ-2a-f – samples from a two years old vineyard in the Czech Republic; PN/Me-CZ-8a-f – samples from an eight years old vineyard in the Czech Republic; PN/Me-Ar-8a-f – samples from eight years old Armenian vineyard

Characterization of the main factors in molecular differentiation between samples. Visualization of main trends in the molecular variance of the MSAP profiles by using PCoA indicates that the majority of variance (79.9% and 70.7% for Merlot and Pinot Noir plants, respectively) is associated with the different geographical origin of the Czech and the Armenian samples (Figure 3). The second coordinate explains the 6.5% and 14.9% of the variability registered within Merlot and Pinot Noir respectively, but no controlling factor is apparent here.

Regarding internal variability within vineyards, analysis of PhiPT distances determines the average molecular variance within one vineyard to 14% for

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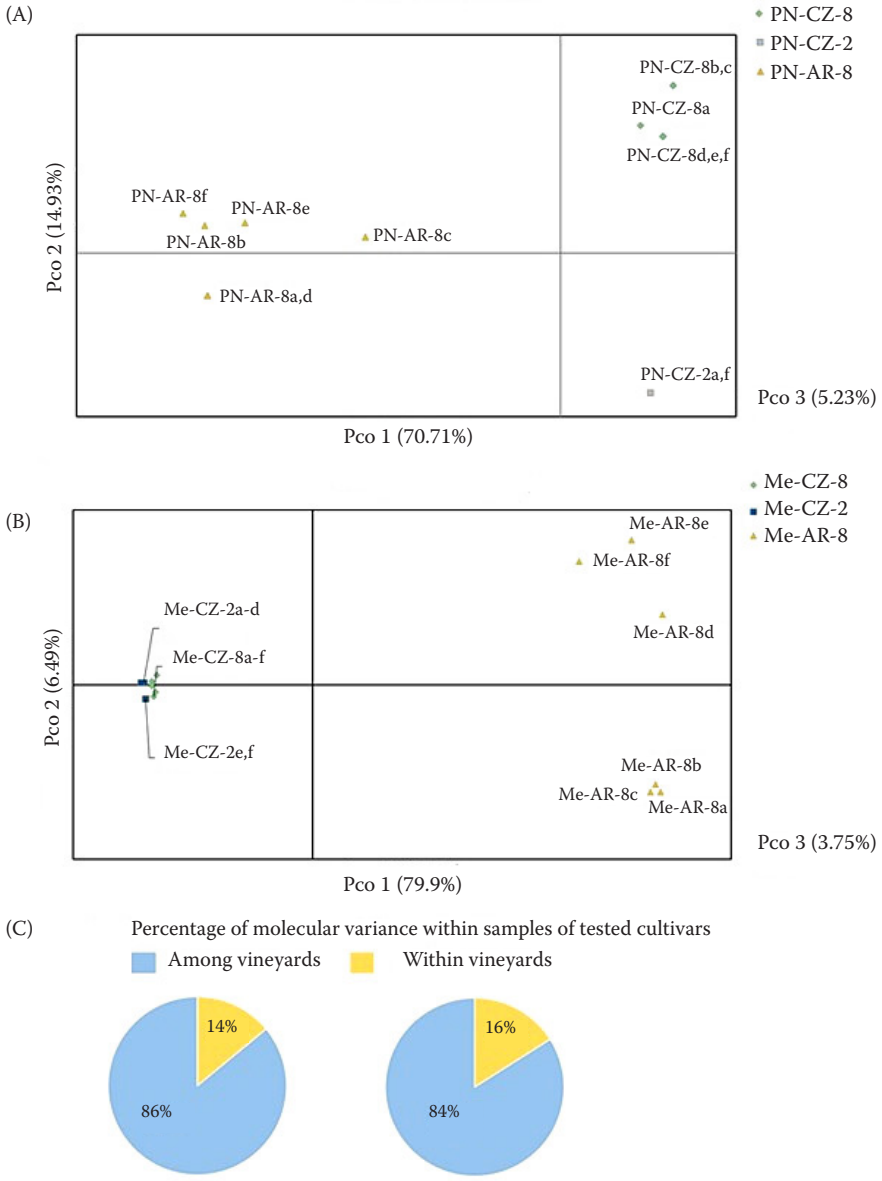

Figure 3. Characterisation of the registered molecular variance on methylation sensitive amplified polymorphism (MSAP) profiles using Principal coordinates analysis (PcoA) and PhiPT computing: (A) results of PcoA analysis as computed within the Pinot Noir samples group, (B) results of PcoA analysis as computed within the Merlot samples group, (C) percentage of molecular variance within and between vineyards obtained by PhiPT distances analysis

PN/Me-CZ-2a-f – samples from a two years old vineyard in the Czech Republic; PN/Me-CZ-8a-f – samples from an eight years old vineyard in the Czech Republic; PN/Me-Ar-8a-f – samples from eight years old Armenian vineyard

the group of Pinot Noir and 16% for the group of Merlot samples (Figure 3).

DISCUSSION

The results obtained confirmed appropriateness of a DNA methylation landscape as a suitable biological matrix to determine an origin of analysed samples at a vineyard level. We also proved that the observed differences are significantly influenced by distinct geographical origin of controlled vineyards. The findings thus confirm the main conclusions of a so far pivotal publication applying DNA methylation to differentiate vineyards (Xie et al. 2017),

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but also open up some other questions that are discussed below.

Use of DNA methylation profiles for identification of samples at a vineyard level. From the generated dendrograms it is evident that the most distinguishing factor determining the manner how the samples cluster is their affiliation to individual vineyards. This state is also confirmed by an analysis of factors guiding the variation. It indicates that the variability within the vineyards represent only 14% (Pinot Noir group) and 16% (Merlot group) from the overall level of variability registered for individual cultivars. Despite these rather unambiguous results it should be noted that there are some other factors and premises discussed below that may theoretically affect the results. Thus, before each use of DNA methylation profiling for sample authentication purposes they also needs to be considered.

One of such factors is certainly the influence of the clone of the cultivar. Clone differentiation in vegetatively propagated crops is generally quite complicated. Standard DNA markers such as SSRs are not suitable here due to low intravarietal polymorphism (Imazio et al. 2002). Somewhat better results are shown when applying high amplicon throughput methods such as AFLP (Anhalt et al. 2011). Even more promising approaches are those where the target for amplification is a conserved part of the transposable element (Baránek et al. 2012). MSAPs were also used for the purpose of clonal distinction (Ocaña et al. 2013), where 37 out of 40 analysed clones of Pinot Noir were discriminated by MSAP. Another theoretical factor affecting DNA methylation profiles is the age of the plant, where the level of DNA methylation increases with increasing age of the plant (Valledor et al. 2007; Dubrovina & Kiselev 2016). However, both these problematic factors (role of the clone and the plant age) are usually overridden by the standard process of vineyards establishment, where grape vine plants of a given clone are usually planted within a given year. There are some other still unanswered questions such as the possible influence of used cultural practices or climatic conditions in a given year on currently established DNA methylation landscape. But taken together, presented results suggest that the specific conditions of each plantation impose DNA methylation patterns specific for each vineyard. Similar results were previously shown also for other cultivated crops by Guarino et al. (2015) or by Lira-Medeiros et al. (2010) even in wild plant populations.

Influence of different geographical origin of samples on their DNA methylation landscape. The analysis of molecular variance on MSAP profiles indicates that the most of the observed variability is associated with different geographical locations of the Czech and Armenian vineyards (79.9% and 70.7% for the Merlot and the Pinot Noir plants groups respectively). Such observation that the terroir and different locations of the analysed samples affect DNA methylation patterns is in accordance with the pivotal study published on this topic so far (Imazio et al. 2002). In comparison with this publication, we managed to compile variants from significantly different conditions than one valley (Xie et al. 2017). Our analyzes also deal with the issue of vineyard age, but the effect on DNA methylation profiles has not been demonstrated.

The impact of different geographical origin on DNA methylation variability is more understandable when we compare meteorological conditions of the Czech Republic and Armenia. We see (Figure 1) that vines in Armenia are exposed to significantly higher temperatures during the growing season. They also have to manage with significantly less precipitation, and there is almost two times more sunshine as compared to the Czech Republic. Thus, the phenomenon of epigenetic priming as an adaptive strategy by which plants modify their behavior in different environmental conditions probably plays role here. And just DNA methylation is a major constitutional strategy in plant adaptation process (Garg et al. 2015; Viggiano & Concetta de Pinto 2017; Münzbergová et al. 2019; Bednarek et al. 2020), what makes observed differences in DNA methylation profiles of vines originating from the Czech Republic and Armenia understandable.

To conclude, we are facing the situation where despite the worldwide use of the same grapevine cultivars, there are significant differences in the final product. Consequently, there must be physiological mechanisms creating these differences driven by the different expression of respective genes. Except standard gene regulatory mechanisms on the RNA or protein level there is also DNA methylation that significantly contribute to set of molecular mechanisms driving gene expression. This makes them suitable matrices capable of determining the differences between different vineyards or regions. The present work confirms the uniqueness of DNA methylation profiles in samples originating from the same vineyard. At the same time, it demonstrates

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that considerably different climate conditions of the individual vineyards contribute significantly to the recorded DNA methylation variability. However, there remain some uncertainties discussed above that still need to be resolved in subsequent topical research. Whether the DNA methylation landscape will represent a successful strategy will be more obvious after additional follow-up studies are carried out, comparing more factors (different tissues, growing seasons) and more geographical locations.

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