Croatian white grape variety Maraština: First taste of its indigenous mycobiota

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Abstract

The indigenous vineyard mycobiota contribute both to wine quality and vineyard sanitary status. Wines made from same grape variety but from different geographical locations are appreciated for their diversity. Because no information on indigenous mycobiota of Croatian grapevines is available, the aim of the present study was to start filling this knowledge gap by characterizing the indigenous mycobiota of Maraština variety. The use of metataxonomic approach has enabled the identification of 25 different fungal genera present on Maraština grape berries collected from 11 vineyards located within the Croatian coastal winegrowing region of Dalmatia (Northern Dalmatia, Dalmatian hinterland, Central and Southern Dalmatia). The substantial regional and local scale differences in their distribution were observed~~., thus supporting the concept of microbial terroir.~~ Overall, *Aureobasidium* was the dominant genus followed by *Cladosporium* and *Metschnikowia*. *Botrytis* and *Plenodomus* were associated with the vineyards located in Central Dalmatia, whereas *Pichia* was associated with Northern Dalmatia vineyards. The largest abundance of *Buckleyzyma*, *Cladosporium*, *Eremothecium*, *Fusarium*, *Papiliotrema*, and *Rhodotorula* was observed in Dalmatian hinterland. Moreover, data suggested that climate conditions and soil type partially influenced the distribution of fungal communities. The local-scale differences emerged also for the physicochemical characteristics of fresh musts. The high malic acid content supported the development of *Metschnikowia*, and inhibited *Fusarium* growth, whereas a positive correlation between *Erysiphe* and pH values was observed. *Sporobolomyces* and *Cystobasidium* were negatively associated with high glucose concentration. The revealing of Maraština indigenous mycobiota provided information on the members of fungal community negatively influencing the grapevine sanitary status as well as those which could be employed in disease biocontrol. ~~The presence of autochthonous yeasts belonging to genera *Hanseniaspora*, *Metschnikowia*, *Lachancea*, *Pichia* and *Hyphopichia* could confer possible improvements to sensory characteristics of wine.~~

Keywords: Maraština, indigenous mycobiota, ~~microbial terroir,~~ metataxonomic approach, grapevine, Dalmatia, *Aureobasidium*

1. Introduction

Vitis vinifera L., native to southern Europe and western Asia, as well as other *Vitis* L. species are grown worldwide mostly for wine production (Pancher et al., 2012). Despite the fermentation of wine being strictly correlated to the conversion of sugar into ethanol, it is a complex procedure that starts in the vineyard and ends with the consumption (Bokulich et al., 2014). The indigenous vineyard mycobiota, including yeasts and other fungal communities, contribute both to wine quality and vineyard sanitary status. Yeast colonizing grape berries produce various compounds that can exert positive or even detrimental effects on the wine quality and aroma complexity (Capozzi et al., 2015). The grape berry surface is dominated by non-*Saccharomyces* yeasts, including basidiomycetous oxidative species from the genera *Filobasidium*, *Cryptococcus* and *Rhodotorula*; ascomycetous oxidative or weakly fermentative species from the genera *Aureobasidium* (yeast-like fungus), *Hanseniaspora*, *Candida*, *Metschnikowia*, *Debaryomyces*, *Pichia*, and *Lachancea* as well as fermentative species from the genera *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, *Dekkera*/*Brettanomyces*, *Schizosaccharomyces*, and *Saccharomycodes* (Setati et al., 2015). The grapes mycobiota also include fungal obligate parasites such as *Plasmopara viticola* and *Erysiphe necator*, responsible for downy and powdery mildew, respectively, as well as saprophytic moulds including *Botrytis cinerea*, causing grey rot, and other ubiquitous genera such as *Aspergillus*, *Cladosporium* and *Penicillium*, responsible for various grape rots or ochratoxin production (Barata et al., 2012). However, the surface of grape berries is an unstable habitat for microorganisms whose composition and the abundance are mainly driven by grape variety, the vineyard geographical position, local and regional climate (temperature, precipitation, relative humidity), soil, growth stage of the berries, health status of the grapevine, and the viticultural management practices (organic or conventional vineyard) (Bokulich et al., 2014; Chalvantzi et al. 2021; Cureau et al., 2021; Milanović et al., 2013; Rantsiou et al., 2020; Zhu et al., 2021). The vineyard mycobiota have been extensively studied using traditional culture-dependent methods that might miss up to 95% of the community due to low frequency or the presence of viable but non-culturable cells (Taylor et al., 2014). By contrast, metataxonomic methods can reveal larger microbial diversity than other fingerprinting methods, thus playing a fundamental role in the assessment of the grape microbiome (Rantsiou et al., 2020; Stefanini & Cavalieri, 2018). Despite the advantages, metataxonomic approaches are not free of pitfalls which are mostly related to low taxonomic

resolution level (family or genus). Indeed, only a higher taxonomic resolution at species or even strain level could determine an association between indigenous microbiota and specific wine characteristics thus giving a better view of wine microbial biogeography (Alexandre 2020; Chalvantzi et al. 2021). Many limiting steps such as nucleic acid extraction protocols, DNA library preparation, sequencing methods, and incomplete databases should be improved to achieve accurate taxonomic assignment (Belda et al., 2017; Morgan et al., 2017).

Vineyards in Croatia cover about 25,000 ha and include 197 cultivars, among which 103 are considered indigenous (Maletic et al, 2015). Croatian wine-growing zones are divided into continental (eastern and western) and coastal region. The latter, including Istria/Kvarner and Dalmatia (Northern Dalmatia, Dalmatian hinterland, Central and Southern Dalmatia) is located along the coast of Adriatic Sea and is characterized by Mediterranean climate (Regulation EU No 1308/2013). In contrast to the continental region, where native cultivars represent only a small fraction, in the coastal region, especially in Central and Southern Dalmatia, native cultivars are grown in more than 90% of the vineyards. Although the most cultivated white variety in Dalmatia is Trbljan (9.5%, 495 ha), followed by Kujunđuša (6.3%, 328 ha), Maraština (4.6%, 242 ha) and Pošip (4.3%, 227 ha) (Voncina et al., 2011), Maraština is the second (after Pošip) most important variety for wine sector due to its capacity for producing high quality wines. Maraština (synonyms Rukatac, Malvasia del Chianti, Malvasia binca lunga) is characterized by small- to medium-sized grapes of a golden yellow colour with small, brown spots, thick skin and the grapes tightly packed in bunches. Maraština is considered an autochthonous Croatian white variety, although Šimon et al. (2007) reported its high similarity with the Italian variety Malvasia del Chianti and the Greek variety Pavlos. By contrast, Crespan et al. (2009) reported just seven of the 11 simple sequence repeat loci of Maraština overlapping with Malvasia del Chianti.

Wines made from the same grape variety but from different geographical regions are appreciated for their differences in aroma, flavour, taste, and quality, thus leading to their higher price and market demand (van Leeuwen & Seguin, 2006). The fungal communities have been proposed as contributing to the concept of wine terroir; therefore, understanding fungal composition and dynamics among different vineyards or winegrowing regions is of great importance in the wine-making process (Alexandre, 2020). To the best of our knowledge, no report on indigenous mycobiota of Croatian grapevine cultivars is available. Hence, the aim of the present study was to employ a culture-independent metataxonomic approach to give the first insight into the fungal

communities associated with Croatian white grapevine cultivar Maraština as influenced by geographical position of the vineyards located within the Croatian coastal winegrowing region of Dalmatia, including sub-regions of Northern Dalmatia, Dalmatian hinterland, and Central and Southern Dalmatia. Correlations between the mycobiota composition and climate data, vineyard soil type and physicochemical characteristics of fresh musts were also calculated.

2. Materials and methods

2.1 Grape sampling

Healthy and undamaged vines were used for the collection of the grape berry samples from 10 commercial vineyards and the germplasm repository of native varieties cultivated at the Institute for Adriatic Crops and Karst Reclamation in Split as part of the Croatian National Collection. The vineyards were located along the Croatian coast in the winegrowing subregions of Northern Dalmatia [Smilčić (S), Nadin (Polača) (N), Stankovci (Z), Vukšić (V)], Dalmatian hinterland [Oklaj (O)], and Central and Southern Dalmatia [Institute for Adriatic Crops and Karst Reclamation in Split (IJK-RB), Kaštela (VP), Dračevica (DR), Prapatna 1 (P), Prapatna 2 (B), Kruševo (K)] as shown in Figure 1. The vineyards DR, P, B and K are situated in the island of Korčula. The air distance between the northernmost (S) and the southernmost vineyard (located on island Korčula) is 177 km. The detailed information, including the global positioning coordinates, altitude, the plantation year, soil type, and row distance per vine ~~and the trellis system~~ for each vineyard, is reported in Table 1.

On 11th, 12th and 16th September 2021, a total of 11 technologically mature samples of Maraština grapes were collected in biological triplicate. In detail, the experimental plan consisted of three randomized blocks in the middle of each vineyard. A block was formed by one row of vines. The sample for each block was composed of nine well-exposed bunches collected from three different vines from the beginning, middle and end of the row. Only healthy and undamaged grapes (around 3 kg per vineyard) were harvested using sterile scissors, placed in sterile bags, and transported to the laboratory in a cool bag. Once in the laboratory, 200 berries from different parts of the grape bunches (top, centre, and bottom) were aseptically cut off by scissors and

141 immediately transferred in a refrigerator to the Polytechnic University of Marche (Ancona, Italy) for
142 microbiological analyses. The remaining berries were pressed by hand and homogenized manually to obtain
143 fresh must for physicochemical analyses.
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145 *2.2 Climate data*
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147 Climate data collected from the nearest meteorological station for each vineyard (Table 1) were obtained from
148 the Croatian Meteorological and Hydrological Service. The average (Tav), maximum (Tmax) and minimum
149 (Tmin) temperature (°C) as well as the average daily precipitation (Dp) (mm) for each winegrowing sub-region
150 are reported in Supplementary Table 1.
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152 *2.3 Physicochemical analyses of fresh must*
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154 Standard physicochemical parameters were determined according to the International Organisation of Vine
155 and Wine reference methods for wine analysis (OIV, 2021) in a laboratory accredited according to HRN EN
156 ISO/IEC 17025 at Institute for Adriatic Crops and Karst Reclamation (Split, Croatia). The content of total
157 soluble solids, TSS (˚Brix), was measured using a refractometer (Hi 96814, Hanna Instruments, USA). The
158 pH was measured using a pH meter Titrino 718 (Metrohm, Switzerland) and total acidity (TA) was determined
159 by titrating the samples with 0.1 M sodium hydroxide solution to reach a pH end-point of 7. A FTIR Lyza
160 5000 Wine analyser (Anton Paar GmbH, Austria) was used to determine the following oenological parameters
161 of the fresh musts: glucose (g/L), fructose (g/L), malic acid (g/L), tartaric acid (g/L) and yeast assimilable
162 nitrogen, YAN [mg/L (N)]. Concentrations of D–glucose and D-fructose were confirmed by using an
163 enzymatic test K-FRUGL (Megazyme, Ireland). Also, concentrations of malic acid and tartaric were confirmed
164 by using the enzymatic tests for L-malic acid and tartaric acid (Megazyme, Ireland).
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166 *2.4 DNA extraction and sequencing*
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A total number of 33 fresh grape berry samples (three biological replicates for each of 11 vineyards) were crushed at 260 rpm by a Stomacher 400 Circulator machine (VWR International PBI, Milan, Italy) for 5 min. The 1.5 mL aliquots of the obtained homogenates were centrifuged at 16 000 g for 10 minutes to pellet the microbial cells that were then used for the extraction of the total microbial DNA using an E.Z.N.A. soil DNA kit (Omega Bio-tek, GA, USA). The quantity and the purity of the extracted DNA were checked by a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

A metataxonomic approach was applied to study the mycobiota composition of Maraština grapes collected from 11 geographical locations within three Dalmatian winegrowing subregions. The 26S rRNA gene of the extracted DNA was amplified by using the primers NL4R (5′-GGTCCGTGTTTCAAGACGG-3′) and LS2-MF (5′-GAGTCGAGTTGTTTGGGAAT-3′) following the procedure previously described by Mota-Gutierrez et al. (2019)~~.~~ and further successfully applied to study the mycobiota of grapes (Rantsiou et al., 2020) and other food (Biolcati et al., 2022; Franciosa et al., 2021) and non-food matrices (Ferrocino et al., 2022). The PCR products were purified, tagged, and pooled following the Illumina Sequencing Library Preparation guidelines. An Illumina MiSeq platform with V2 chemistry was used to generate 250-bp paired-end reads. After sequencing, the obtained raw files (*fastq*) were processed by QIIME2 software as described by Bolyen et al. (2019). *Cutadapter* was used to trim the sequence adapters and primers, and DADA2 algorithm (Callahan et al., 2016) was used to eliminate low quality reads. The DADA2 denoise paired plug-in of QIIME2 was implemented to remove chimeric sequences and join sequences shorter than 300 bp. The manually build database for the mycobiota was used for the taxonomy classification using the QIIME feature-classifier plugin against SILVA database implemented in Mota-Gutierrez et al. (2019). BLAST suite tools were used to confirm the taxonomic assignment. Data generated by sequencing were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and are available under the BioProject Accession Number PRJNA851272.

2.5 Statistical analyses

The diversity script of QIIME2 was used for alpha and beta diversity indices calculation. In R environment, the differences between alpha diversity parameters and Amplicon Sequence Variants (ASVs) relative

abundance were evaluated by non-parametric Kruskall Wallis test. Bray–Curtis distance matrix was used to perform PERMANOVA by the "vegan" package in R environment.

Principal Component Analysis (PCA), using the function *dudi.pca* of R, was used to analyse the differences of ASVs. Spearman correlation analysis between fungal ASVs and physicochemical parameters of fresh Maraština must was performed through the package *psyc* of R, and only the significant associations ($P < 0.05$) are shown in the plots drawn by the corr.plot function of R.

One-way analysis of variance (ANOVA) was used to evaluate differences in physicochemical characteristics of the samples collected from different vineyards by Tukey-Kramer's Honest Significant Difference (HSD) test and the one-tailed t-test (level of significance 0.05) using the JMP software version 11.0.0 (SAS Institute Inc., Cary, NC). Furthermore, prior to PCA, the entire data set related to physicochemical characteristics of fresh musts was subjected to factor analysis to examine whether there was a need to include all the data. The decision on the data inclusion was based on factor loading of ≥0.7 (Topić Popović et al., 2021), and only the ratio glucose/fructose was considered a variable that would not greatly affect the qualitative distribution of harvest locations. This data set was used to perform the PCA using statistical software for Excel, XLStat 2014, using the Varimax rotation and presented in a form of a distance biplot.

3. Results and discussion

3.1 Characterization of indigenous mycobiota

The indigenous grapevine microbial communities, especially yeasts and bacteria, –together with other biological and physical factors play a crucial role in shaping the organoleptic characteristics of wine. Consequently, wines produced from the same grapevine cultivar but in different geographical regions can be recognized for their different sensory characteristics, which in cases of specific regions may lead to increased consumer's acceptance and significant economic returns (Stefanini & Cavalieri, 2018). ~~Because fungi are reported to have greater impact on wine sensory attributes than bacteria (Liu et al., 2020),~~ The current study focused on indigenous mycobiota associated with the Croatian grapevine cultivar Maraština, thus laying a foundation for research on the composition of fungal communities of Croatian grapevine cultivars. Croatia,

like several other Mediterranean countries including Italy, France, Spain, and Greece, is a traditional wine-producing country characterized by a great number of small producers, thus making the Croatian market recognised as a market of numerous monovarietal wines often made from indigenous and rare grape varieties (Žurga et al., 2019). Indigenous varieties, especially those cultivated in Dalmatian winegrowing region, are playing an important role in Croatian viticulture, mainly due to their high genetic variability which could be of interest to other countries for breeding or production (Maletić et al., 2015). Additional interesting characteristic of Croatian indigenous wines is a high concentration of phenolic compounds which could be presumably associated with traditional prolonged maceration times and intrinsic natural richness in polyphenols of some Croatian varieties (Radeka et al., 2022).

The high-throughput sequencing methods revealed the local distribution patterns of microbial communities throughout different world winegrowing regions ~~, showing a strong correlation between local microbial terroir and wine organoleptic characteristics~~ (Li et al., 2022). To verify whether this pattern could be applied to Maraština, 33 grape berry samples collected from 11 vineyards located along the Croatian coastal area were subjected to metataxonomic analysis. A total of 14.007.462 paired reads were obtained by sequencing. After quality filtering, a total of 146,485,613 reads were used, with an average value of 44,389 reads/sample, and a mean sequence length of 375 bp. *Alternaria*, *Aureobasidium*, *Cladosporium*, *Filobasidium*, *Hanseniaspora* and *Metschnikowia* were ubiquitous and characterized by high relative abundance (Figure 2, Supplementary Table 2). *Aureobasidium* was the dominant ASV, with the relative abundance ranging between 19.7% (vineyard O, Dalmatian hinterland) and 94.6% (vineyard VP, Central and Southern Dalmatia), followed by *Cladosporium* varying from 3.6% (vineyard VP) to 47.6% (vineyard O), and *Metschnikowia* with relative abundance between 0.03% (vineyards IJK-RB and B, Central and Southern Dalmatia) and 33.3% (vineyard Z, Northern Dalmatia). *Aureobasidium* is commonly found on the surface of grape berries at all stages of maturation, probably due to its high tolerance to different environmental conditions and high antagonistic activity against plant pathogens due to production of volatile organic compounds and antimicrobials (Galli et al., 2021). Moreover, it has been reported to have a positive role on mycotoxin biocontrol and to produce valuable industrial enzymes such as amylases, proteases, pectinases, β-glucosidase, lipases, cellulases, xylanases and mannanases, with some of them very useful for the improvement of wine quality and aroma (Bozoudi & Tsaltas, 2018). In the present study, for most of the samples, the relative abundance of

Aureobasidium was inversely proportional with the relative abundance of the *Cladosporium*. The latter genus is considered ubiquitous but particularly frequent in geographical zones with mild Mediterranean climates such as Dalmatia, exerting negative influence on wine quality by diminishing aroma, flavour, and colour (Briceno & Latorre, 2008). The highest relative abundance of ASVs ascribed to genus *Metschnikowia* were detected in vineyards Z, DR, and O, showing distribution of this genus within different winegrowing regions. *Metschnikowia* is one of the most explored genera in oenology, frequently used in mixed fermentations with the aim to improve the organoleptic profile of wines by modulating the synthesis of secondary metabolites. It has also been reported that *Metschnikowia pulcherrima* has the strong antimicrobial activity against spoilage yeasts and fungi as well the ability to decrease the concentration of ochratoxin, thus making this species useful in the winemaking (Vicente et al., 2020). Moreover, *M. pulcherrima* showed the ability to decrease the ethanol concentration, which is particularly important for wines produced in regions characterized by warm climate (Vaquero et al., 2021). The last genus commonly present in Maraština samples with the relative abundances >10% (vineyards N and O) was *Hanseniaspora*, comprising the most abundant yeasts found in vineyards able to increase the concentration of acetate esters contributing to positive fruity aroma, as well as sulfur-containing compounds and higher concentration of alcohols (Capozzi et al., 2015). Finally, samples collected from the vineyard IJK-RB were characterized by the highest relative abundance (23.7%) of *Quambalaria*, known as plant pathogenic fungal genus (Narmani & Arzanlou, 2019).

Botrytis, *Buckleyzyma*, *Cryptococcus*, *Cystobasidium*, *Didymella*, *Eremothecium*, *Hyphopichia*, *Penicillium*, *Pichia*, *Plenodomus* and *Sporobolomyces* were detected in less than 50% of the samples with the low relative abundance (<1%). *Eremothecium* and *Plenodomus* were identified only in O and IJK-RB vineyards, respectively, whereas *Botrytis*, causing grey rot, was present only in vineyards located in Central and Southern Dalmatia (IJK-RB, DR, B, and K).

The previous studies based on high-throughput sequencing methods suggested that grapevine microbiota exhibits regional distinction mainly due to the dominance of a limited number of genera or species per region (Bokulich et al., 2014, Pinto et al., 2015; Wang et al., 2015). Generally, different DNA extraction procedures, PCR, and high-throughput sequencing methods including different target marker genes, are making difficult the comparison of the results obtained in different studies. Moreover, grapes and wine are complex samples that may contain numerous agents interfering molecular analysis, thus requiring further purification steps

which can create bias by modifying the composition of original microbial communities. Also, different fungal forms (anamorph or teleomorph) as well as incomplete databases can cause difficulties in taxonomic classification, especially at species level (Belda et al., 2017; Morgan et al., 2017). Nonetheless, several genera constituting Maraština mycobiota seem to be widely distributed among white grape varieties from other European wine-producing countries, especially those characterized by Mediterranean climate. Indeed, some highly abundant genera such as *Aureobasidium*, *Hanseniaspora*, and *Cladosporium*, as well as low abundant genera such as *Erysiphe*, *Aspergillus*, *Penicillium*, and *Alternaria*, detected in Maraština samples were dominant in Xynisteri grapes collected from five terroirs in Cyprus (Kamilari et al., 2021). Furthermore, the members of *Aureobasidium*, *Hanseniaspora*, *Penicillium*, *Botryotinia*, and *Pichia* genera were found also in Nosiola grapes used for the production of traditional Italian Vino Santo Trentino (Stefanini et al., 2016), together with *Saccharomyces*, *Candida*, and *Starmerella* genera, not detected in Maraština samples. Similarly, Alvarinho and Loureiro samples collected from different wine appellations in Portugal (Pinto et al., 2015) were characterized by high abundance of *Aureobasidium* and *Hanseniaspora*. Not only the white grape varieties collected from Europe, but also those collected from other continents, share the similar mycobiota including the genera *Aureobasidium*, *Cladosporium*, *Hanseniaspora*, *Botrytis*, and *Penicillium*, highly abundant in Sauvignon blanc grapes from the Central Valley of Chile (Mandakovic et al., 2020) and Chardonnay samples collected from across California in two separate vintages (Bokulich et al., 2014).

Even if several studies using high-throughput sequencing methods revealed a connection between grape microbiota and the geography of the region, thus assuming the concept of microbial terroir, very few studies have demonstrated the real association between grape microbial communities and wine aroma profiles (Knight et al., 2015; Bokulich et al., 2016). Hence, further research efforts are still required to support the concept of microbial terroir (Alexandre, 2020).

Since winemaking industry is strictly correlated with environmental, territorial, economic, and social challenges, the concept of microbial terroir could undoubtedly contribute to Sustainable Development Goals (SDGs) of the 2030 Agenda of the United Nations. Indeed, the unique taste, flavour, aroma, and quality of wines from different geographical regions are very appreciated by consumers which leads to their higher price and market demand, and, at the same time, supports a rural development, increase the wine culture, and the awareness of indigenous vines. Therefore, it is of great importance to evaluate the qualitative impact of

indigenous microorganisms on wine organoleptic characteristics and their role in grapevine disease biocontrol, as well as to evaluate the effect of climate changes on microbial diversity which could have great consequences on the wine style.

Samples collected from the vineyards IJK-RB, DR and Z showed the highest Shannon diversity index ($P<0.05$, Figure 3). Bray–Curtis distance matrix showed a significant separation between samples according to vineyards (PERMANOVA, $p = 0.001$).

The PCA analysis confirmed a separation of the samples based on their mycobiota composition (Figure 4). In detail, the samples collected from IJK-RB and DR vineyards, both from Central and Southern Dalmatia subregion, were well separated from the other samples. The samples from VP and N vineyards, although from different winegrowing regions, clustered together. These findings suggest a local-scale effect of the distribution of fungal ASVs~~., confirming the concept of microbial terroir.~~ Indeed, several ASVs were associated with different locations; *Aspergillus*, *Cryptococcus*, *Cystobasidium*, *Erysiphe*, *Filobasidium* and *Plenodomus* showed higher relative abundance in samples collected from IJK-RB vineyard ($P<0.05$), whereas *Cladosporium*, *Fusarium* and *Rhodotorula* showed the highest relative abundance in samples collected from the O vineyard located in Dalmatian hinterland ($P<0.05$, Supplementary Figure 1). Even though the genus *Fusarium* comprises numerous harmless species of filamentous fungi, some of them can cause grapevine wilt disease or even produce the mycotoxins (Desjardins, 2006; Gonzalez & Tello 2011). The *Rhodotorula* genus is frequently detected and isolated from grape berries, probably due to its ability to produce biofilms on berry surfaces (Lederer et al., 2013). Although some species from this genus can enhance the wine aroma complexity due to β-glucosidase and α-L-arabinofuranosidase activity, they are rarely used in wine production (Hu et al., 2016; Martínez et al., 2006). Samples belonging to vineyard Z located in Northern Dalmatia winegrowing subregion were characterized by the highest relative abundance of *Metschnikowia* and *Pichia* ($P<0.05$, Supplementary Figure 1). Different wine related species of the latter genus are reported to produce enzymes that positively influence wine organoleptic characteristics. Moreover, they can produce antimicrobial compounds, thus showing high potential for reducing the growth of wine spoilage microorganisms. However, only *Pichia kluyveri* strains are commercially available as a starter culture (Vicente et al., 2021). Finally, the highest abundance of the *Lachancea* was detected in the vineyard V from the same winegrowing subregion ($P<0.05$, Supplementary Figure 1). Members of this genus have been found in various habitats, with *Lachancea*

thermotolerans as a key species in wine fermentation processes principally due to its ability to reduce pH through lactic acid production, thus giving pleasant acidity to wine (Porter et al., 2019).

Considering the winegrowing subregions of Northern Dalmatia, Central and Southern Dalmatia, and Dalmatian hinterland as the main factor influencing the distribution of the ASVs, the significant separation of the samples was observed ($P<0.05$, Figure 5). Indeed, the one tailed t-test (Supplementary Table 3) indicated that the highest relative abundance of ASVs ascribed to *Botrytis* and *Plenodomus* was detected in the vineyards located in Central and Southern Dalmatia, whereas the ASVs ascribed to *Pichia* were characteristic for the samples collected from Northern Dalmatia. Finally, Dalmatian hinterland subregion was characterized by the highest relative abundance of *Buckleyzyma, Cladosporium*, *Eremothecium, Fusarium*, *Papiliotrema* and *Rhodotorula,* whereas the abundance of *Hanseniaspora* and *Metschnikowia* was the lowest in Central and Southern Dalmatia.

It has been suggested that the structure of grapevine microbial communities partly depends on climate conditions both inside and between vineyards, but it remains unclear which climate factor has the greatest impact (Liu et al., 2019). Here, due to lack of meteorological data for each single vineyard, the average values of air temperatures (average, maximum and minimum) (°C) and daily precipitations (mm) (Supplementary Table 1) were estimated only for the winegrowing subregion and were correlated with metataxonomic analysis results. The highest average (Tav) and minimum (Tmin) air temperatures as well as daily precipitations (Dp) were correlated with *Aspergillus*, *Aureobasidium*, *Botrytis*, *Cryptococcus*, *Cystobasidium*, *Didymella*, *Eremothecium*, *Erysiphe*, *Penicillium*, *Plenodomus*, *Sporobolomyces* and *Quambalaria,* all associated with Central and Southern Dalmatia (Figure 6). Regarding *Aureobasidium,* Chalvantzi et al. (2021) reported its positive correlation with net precipitation amounts in different Greek vineyards. Furthermore, *Filobasidium* and *Alternaria* were well correlated with the maximum temperature values (Tmax).

Soil has been proposed to be a possible natural source of microbial communities associated with grapevines, thus making the wind-blown soil dust the principal vector for their distribution (Zarraonaindia et al., 2015). In the current study, the correlation between the vineyard soil type and fungal ASVs present on grape berries was calculated. As reported in Table 1, the vineyards were planted on different soil types including brown soil on limestone, red soil, loam, sand, reclaimed karst and brown soil. Samples collected from grapevines planted on brown soil on limestone were well separated from the other samples (Figure 7) principally due to the highest

relative abundance of *Alternaria, Aspergillus, Botrytis, Didymella, Erysiphe, Plenodomus* and *Quambalaria* ($P<0.05$, Supplementary Figure 2), all known for their negative influence on the grapevine sanitary status. Moreover, samples collected from grapevines grown on brown soil were characterized by the presence of *Metschnikowia* and *Cladosporium* ($P<0.05$, Supplementary Figure 2).

3.2 Physicochemical characteristics of fresh musts

The physicochemical analyses were performed to characterize the fresh grape musts obtained from the collected samples. The results, expressed as the average values coupled with standard deviations are reported in Table 2. The pH values ranged between 3.3 (vineyards Z and O) and 3.6 (vineyards IJK-RB, P, K, S), which is comparable with the results commonly reported in the literature for fresh grape must samples (Unluturk & Atilgan, 2015). Grape musts are mainly composed of water (70-80%), carbohydrates (15-25%) with glucose and fructose commonly present in equal amounts (1:1 ratio), plus several organic acids (Granato et al., 2016). Glucose/fructose ratio in Maraština samples was about 1, with glucose concentration ranging from 92.3 g/L (vineyard DR) to 118.1 g/L (vineyard K) and fructose concentration between 77.4 g/L (vineyard DR) and 115.7 g/L (vineyard O). The TSS (˚Brix) content in Maraština samples was between 18.1 (vineyard DR) and 23.4 (vineyard V), which, according to OIV (1990) indicated the stage of technical maturity (14–25 ˚Brix). Even if the TSS level usually determines the grape price, it does not always correspond to the best overall maturity. Indeed, it has been reported that in cultivars such as Merlot and Chardonnay a concentration of 24 to 25 °Brix possibly establishes the upper limit beyond which an additional increase of TSS is associated mainly with deterioration and dehydration of the berries (Bondada et al., 2017; Tillbrook & Tyerman, 2008). After sugars, organic acids such as malic, tartaric, acetic, citric, succinic, and lactic acid are the most abundant solids in grape musts directly impacting the flavour, colour and wine stability (Eyduran et al., 2015). The concentration of organic acids is directly linked to TA which commonly ranges between ~~0.40~~4 and ~~7.08~~ g/L in fresh grape musts (Granato et al., 2016). The Maraština samples were characterized by TA values ranging from 3.7 g/L (vineyard K) to 6.4 g/L (vineyard IJK-RB), which is in line with the results previously reported for Maraština sampled and analysed during three consecutive years (2009-2011) (Preiner et al., 2013). Malic and tartaric acids account for 70-90% of the total acids. The concentration of tartaric acid, responsible for taste

392 and the wine biological stability, is relatively constant during ripening and independent of climate conditions,
393 thus making it characteristic of a grape cultivar (Ribereau-Gayon et al., 2006). Conversely, the concentration
394 of malic acid is variable depending on several factors, including climate conditions, soil type, sunlight exposure
395 and grape variety (Granato et al., 2016). Here, the concentration of tartaric acid in fresh Maraština musts ranged
396 from 2.3 g/L (vineyard K) to 4.0 g/L (vineyard O), and that of malic acid from 0.2 g/L (vineyards B and VP)
397 to 0.9 g/L (vineyard DR). These values are lower than those previously reported for the same cultivar during
398 the 2009-2011 triennial (average concentration of 4.92 g/L for tartaric and 1.21 g/L for malic acid, Preiner et
399 al., 2013). A negative correlation between malic acid and high air temperatures has been reported previously
400 (Conde et al., 2007).
401 One of the most important parameters for wine fermentation is nitrogen (N) availability because it is essential
402 for the metabolism of yeast cells. Different N sources such as amino acids, ammonium, and small peptides are
403 present in must, but not all of them can be used by yeasts. The content of YAN in grape musts is commonly
404 between 50 and 450 mg/L, although a minimum of 140 mg/L has been established as crucial to prevent stuck
405 or sluggish fermentations (Verdenal et al., 2021). Only samples collected from VP vineyard (142 mg/L)
406 satisfied the minimum acceptable YAN concentration, whereas the lowest YAN value was registered for the
407 samples collected from DR vineyard (90.7 mg/L) (Table 2).
408 The PCA was used to assess the distribution of samples collected from different vineyards based on the results
409 of physicochemical analysis, including primary parameters such as acidity (TA, malic and tartaric acids), sugar
410 concentration (glucose, fructose), and analytical data such as TSS (°Brix) and pH, as well as secondary
411 parameters such as YAN (Figure 8). The samples collected from IJK-RB, VP and O vineyards grouped
412 together due to their high YAN and tartaric acid concentration, similar to the samples collected from vineyard
413 Z. Even though the concentration of tartaric acid in fresh musts obtained from the samples collected in vineyard
414 DR was not significantly lower than in the samples from the IJK-RB, VP and O vineyards, the high
415 concentration of malic acid in the DR samples caused a separation into the second quadrant, opposite to sugar-
416 related parameters (glucose and fructose) that were low at 92.3 g/L and 77.4 g/L, respectively. The P samples
417 were distinguished from all the other samples mainly due to their relatively high concentration of malic acid
418 and low YAN concentration. The last group containing samples collected from vineyards V, B, S, N and K
419 was positively correlated with pH and fructose concentration. Interestingly, samples from the same

winegrowing region were scattered among different PCA quadrants, thus indicating that local conditions such as climate, soil and vineyard practices may impact the physicochemical characteristics of fresh grape musts and consequently organoleptic characteristics of resulting wines. This was further confirmed by the fact that the samples DR, K, B and P, geographically close to each other (all on Korčula island) were distributed in all four quadrants.

During ripening, grapes undertake various physiological and biochemical modifications that may influence the mycobiota of grape berries (Conde et al., 2007). The availability of nutrients such as sugars, organic and amino acids is undoubtedly an important factor shaping the fungal ecology on grapes. Prakitchaiwattana et al. (2020) have recently demonstrated the presence of nutrients on grape surfaces, with their concentration increasing during ripening, which was associated with more abundant fungal population. Given these premises, the correlation analysis between mycobiota and principal physicochemical parameters of fresh Maraština musts was performed. As shown in Figure 9, concentration of malic acid was associated positively with the relative abundance of *Metschnikowia* and negatively with that of *Fusarium*. Indeed, the relative abundance of *Fusarium* in the analysed samples followed the opposite trend compared to *Metschnikowia*. Some non-*Saccharomyces* wine yeasts are assumed to metabolize malic acid, especially *M. pulcherrima*, decomposing around 10% of malic acid during fermentation (Vicente et al., 2020). Regarding negative correlation between *Fusarium* and malic acid, a similar result was recently reported by Lv et al. (2021), whereby malic acid had a significant inhibitory effect on the occurrence of *Fusarium* wilt in faba bean. Finally, the relative abundance of *Erysiphe* showed a positive correlation ($P<0.05$) with pH, whereas the relative abundance of *Sporobolomyces* and *Cystobasidium* was negatively ($P<0.05$) associated with glucose concentration (g/L) (Figure 9). The species from the latter two genera may represent a source of biocontrol agents effective in regulation of different grapevine diseases (Patanita et al., 2022).

4. Conclusions

The current study aimed to fill a knowledge gap on indigenous mycobiota associated with the Croatian grapevine cultivar Maraština, hence laying a foundation for further research on the composition of microbial communities related to Croatian grapevines. The high-throughput metataxonomic analysis revealed a

significant regional as well as local scale differences in fungi distribution., ~~thus further supporting the concept of microbial terroir.~~ The climate conditions and the vineyard soil type as well as the physicochemical characteristics of fresh musts (such as pH and the concentrations of malic acid and glucose) partly contributed to local distribution patterns of fungal communities. *Aureobasidium* dominated the surface of Maraština grapes followed by *Cladosporium*, *Metschnikowia*, *Hanseniaspora*, *Alternaria* and *Filobasidium*. The knowledge of Maraština indigenous mycobiota provided a basis for examining the role of *Aureobasidium*, *Cryptococcus*, *Cystobasidium*, *Metschnikowia*, *Pichia*, *Sporobolomyces* and *Vishniacozyma* in grapevine disease biocontrol and wine quality. Of special interest are the yeasts from the genera *Hanseniaspora*, *Metschnikowia*, *Lachancea*, *Pichia* and *Hyphopichia* because they are known for their potential positive contributions to organoleptic characteristics of wine. To preserve the role of the microbial terroir, future research will be oriented toward isolation and oenological characterization of indigenous Maraština non-*Saccharomyces* yeasts for their potential as wine starter cultures. Additionally, to support the hypothesis of microbial terroir, the metabolic profile and sensory analyses of the resulting wines are crucial to establish whether the use of indigenous strains could reach to human-perceptible variances in wine.

CRediT authorship contribution statement

Vesna Milanović: Conceptualization, Investigation, Formal analysis, Writing - Original Draft; **Federica Cardinali:** Investigation, Formal analysis; **Ilario Ferocino:** Investigation, Formal analysis, Writing - Review & Editing; **Ana Boban:** Investigation, Formal analysis; **Irene Franciosa:** Investigation, Formal analysis; **Jasenka Gajdoš Kljusurić:** Formal analysis; Writing - Review & Editing; **Ana Mucalo:** Investigation; **Andrea Osimani:** Validation, Visualization, Resources; **Lucia Aquilanti:** Visualisation, Resources; **Cristiana Garofalo:** Resources; Writing - Review & Editing; **Irena Budić-Leto**: Conceptualization, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure captions

Figure 1. Position of the vineyards located along the Croatian coastal area in the winegrowing subregions of Central and Southern Dalmatia [Institute for Adriatic Crops and Karst Reclamation at Split (IJK-RB), Kaštela (VP), Dračevica (DR), Prapatna 1 (P), Prapatna 2 (B), Kruševo (K)], Dalmatian hinterland [Oklaj (O)], and Northern Dalmatia [Smilčić (S), Nadin (Polača) (N), Stankovci (Z), Vukšić (V)].

Figure 2. Relative abundance (%) of fungal genera detected in Maraština grape samples collected from 11 different vineyards located along the Croatian coastal area in the winegrowing subregions of Central and Southern Dalmatia [Institute for Adriatic Crops and Karst Reclamation at Split (IJK-RB), Kaštela (VP), Dračevica (DR), Prapatna 1 (P), Prapatna 2 (B), Kruševo (K)], Dalmatian hinterland [Oklaj (O)], and Northern Dalmatia [Smilčić (S), Nadin (Polača) (N), Stankovci (Z), Vukšić (V)].

Figure 3. Boxplot showing the alpha diversity index (Shannon index and observed ASVs) for Maraština grape samples.
The samples are labelled as indicated in Figure 1.

Figure 4. Principal component analysis (PCA) showing a separation of the samples collected from 11 vineyards located along Croatian winegrowing region of Dalmatia based on their mycobiota composition.
PC1 = 15.52%; PC2 = 13.47%; Significance = 0.001.
The samples are labelled as indicated in Figure 1.

Figure 5. Principal component analysis (PCA) showing grouping of the samples based on their mycobiota composition according to winegrowing subregions of Northern Dalmatia, Dalmatian hinterland, and Central and Southern Dalmatia.
PC1 = 15.52%; PC2 = 13.47%; Significance = 0.002.

Figure 6. Principal component analysis (PCA) showing distribution of the samples based on their mycobiota composition according to winegrowing subregion and climate data.

Figure 7. Principal component analysis (PCA) showing grouping of the samples based on their mycobiota composition according to vineyard soil type.
PC1 = 15.52%; PC2 = 13.47%; Significance = 0.001.

Figure 8. Principal component analysis (PCA) showing grouping of the samples based on Yeast Assimilable Nitrogen (YAN) vs measured parameters indicated as significant after Factor analysis.

Figure 9. Correlation analysis between fungal ASVs and physicochemical parameters of fresh Maraština must (only significant associations are shown, $P < 0.05$). The colour intensity and the circle dimension represent the degree of correlation where red dots represent a negative degree of correlation and blue dots a positive degree of correlation.

Fig. 1.

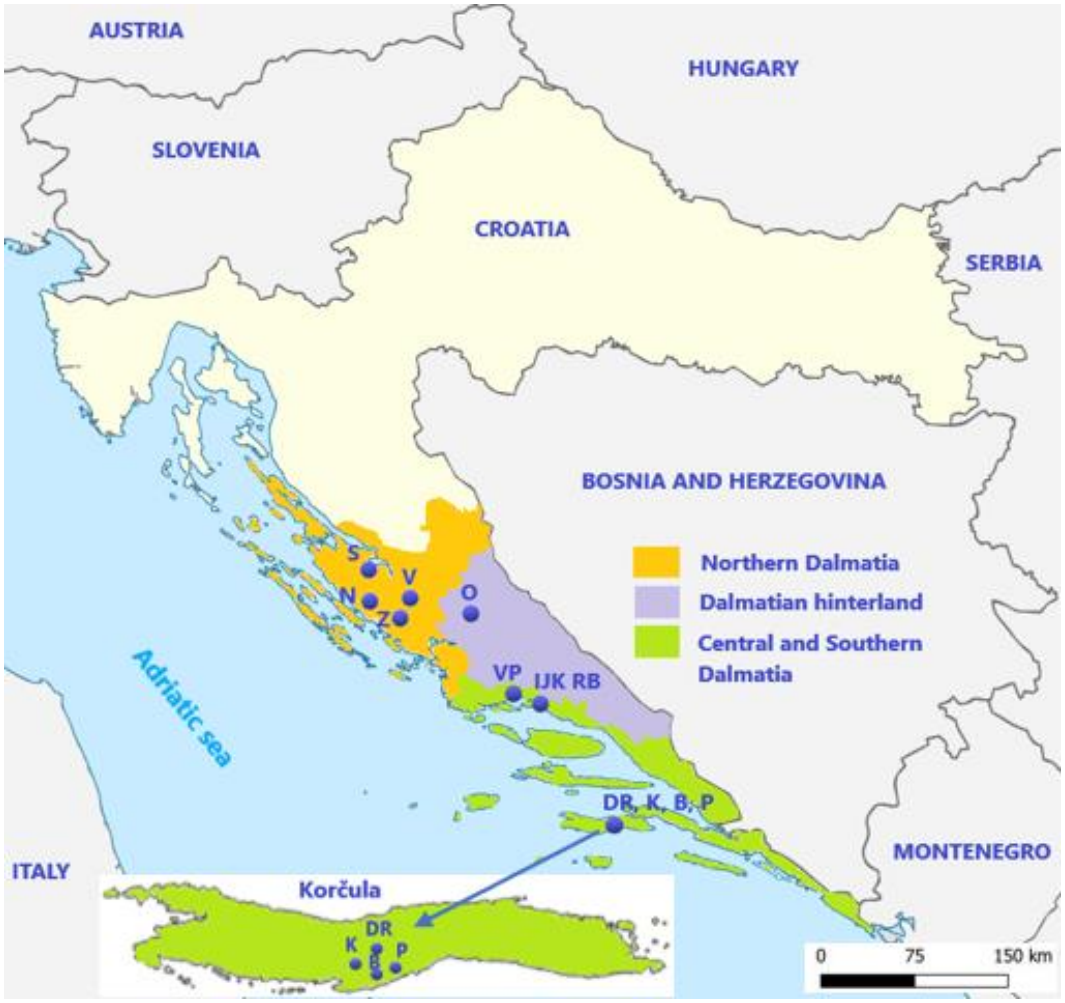

AUSTRIA
HUNGARY
SLOVENIA
CROATIA
SERBIA
BOSNIA AND HERZEGOVINA
Northern Dalmatia
Dalmatian hinterland
Central and Southern Dalmatia
S
V
N
Z
O
VP
IJK
RB
DR, K, B, P
Adriatic sea
ITALY
MONTENEGRO
Korčula
DR
K
P
0
75
150 km

Fig. 2.

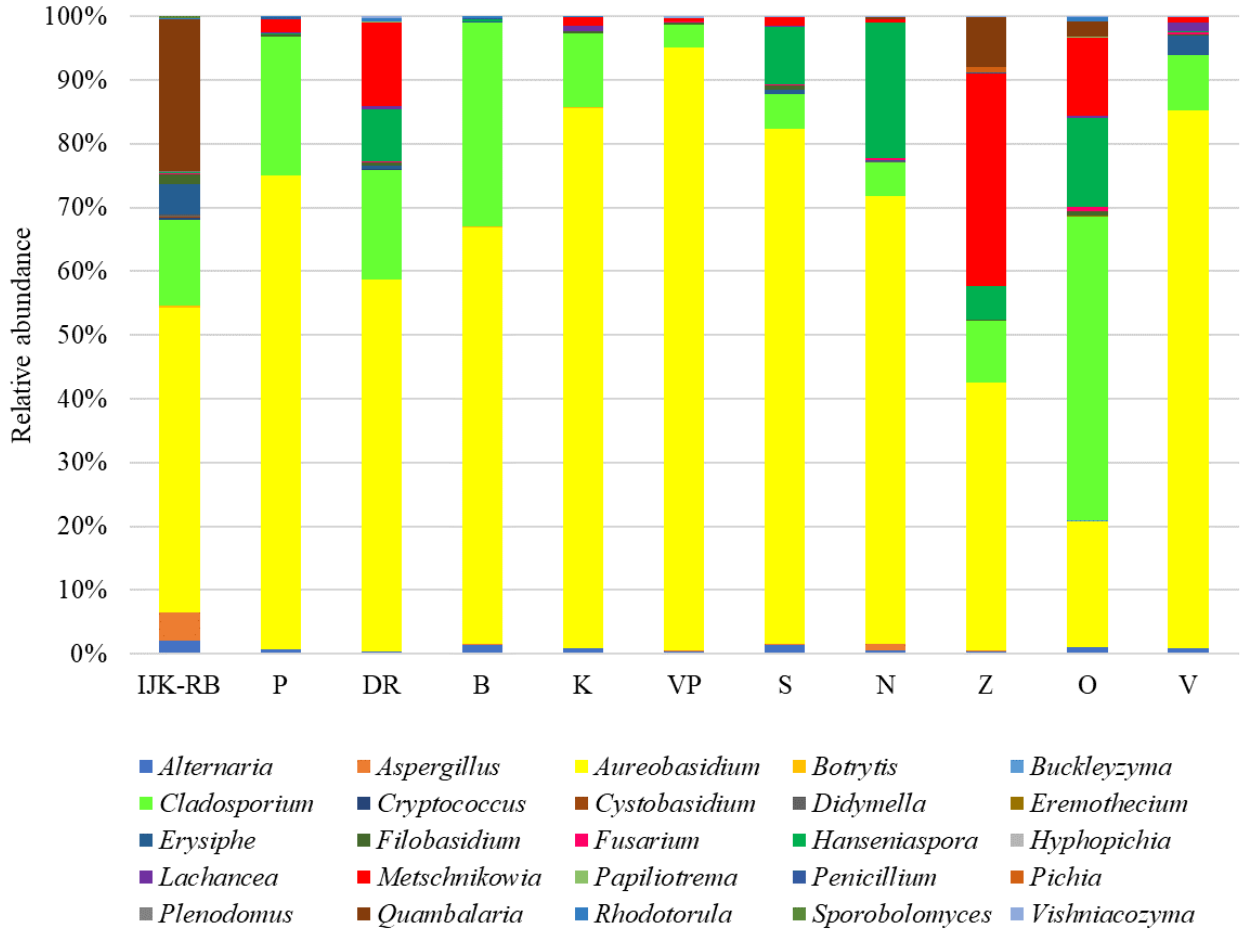
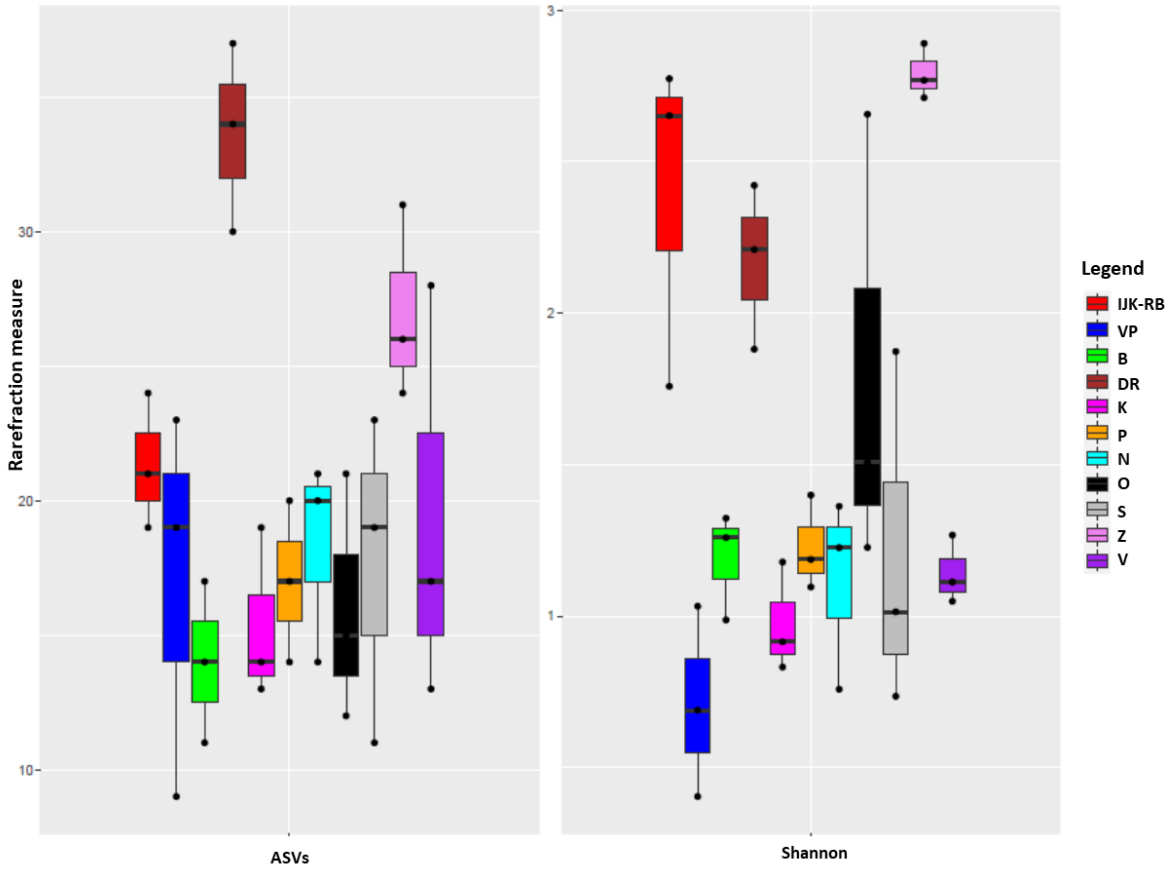


Fig. 3.



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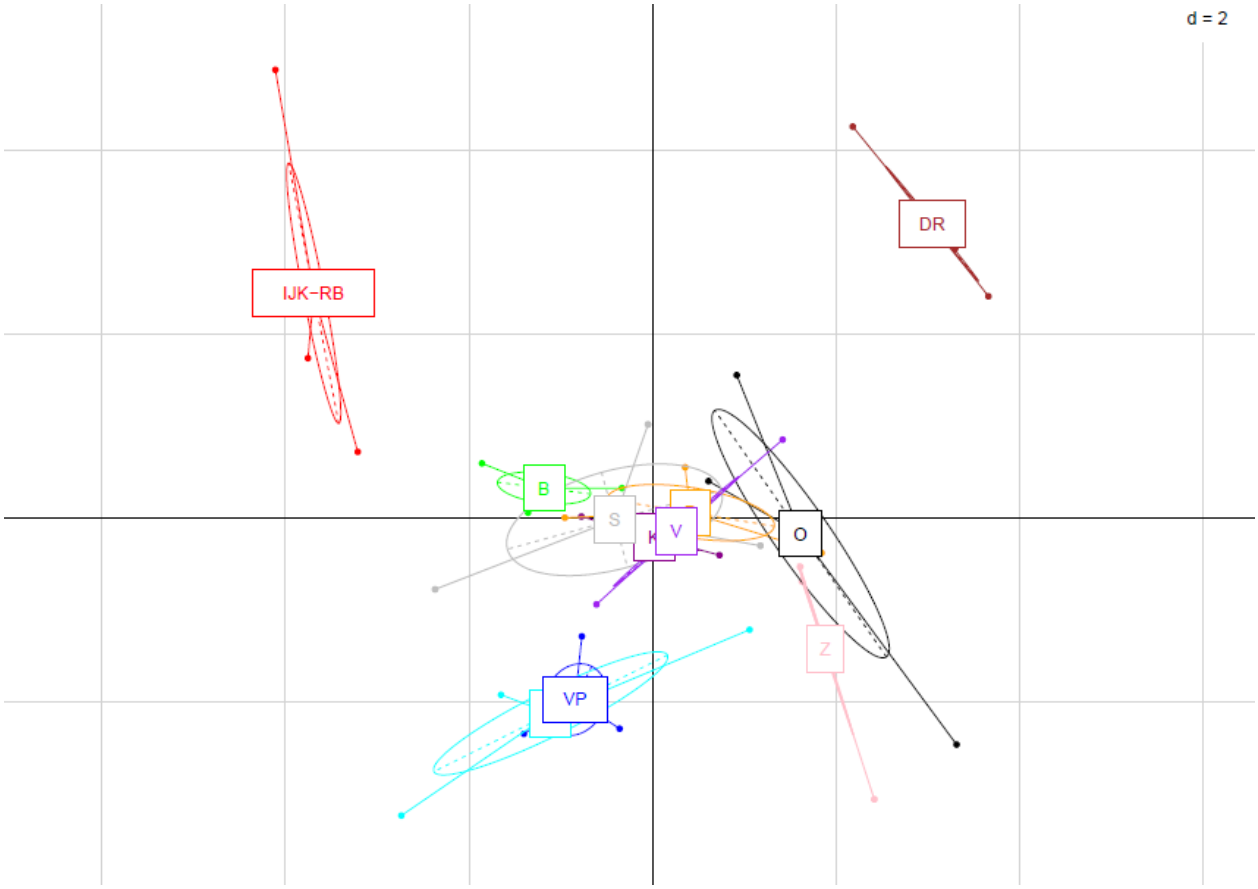

Fig. 5.

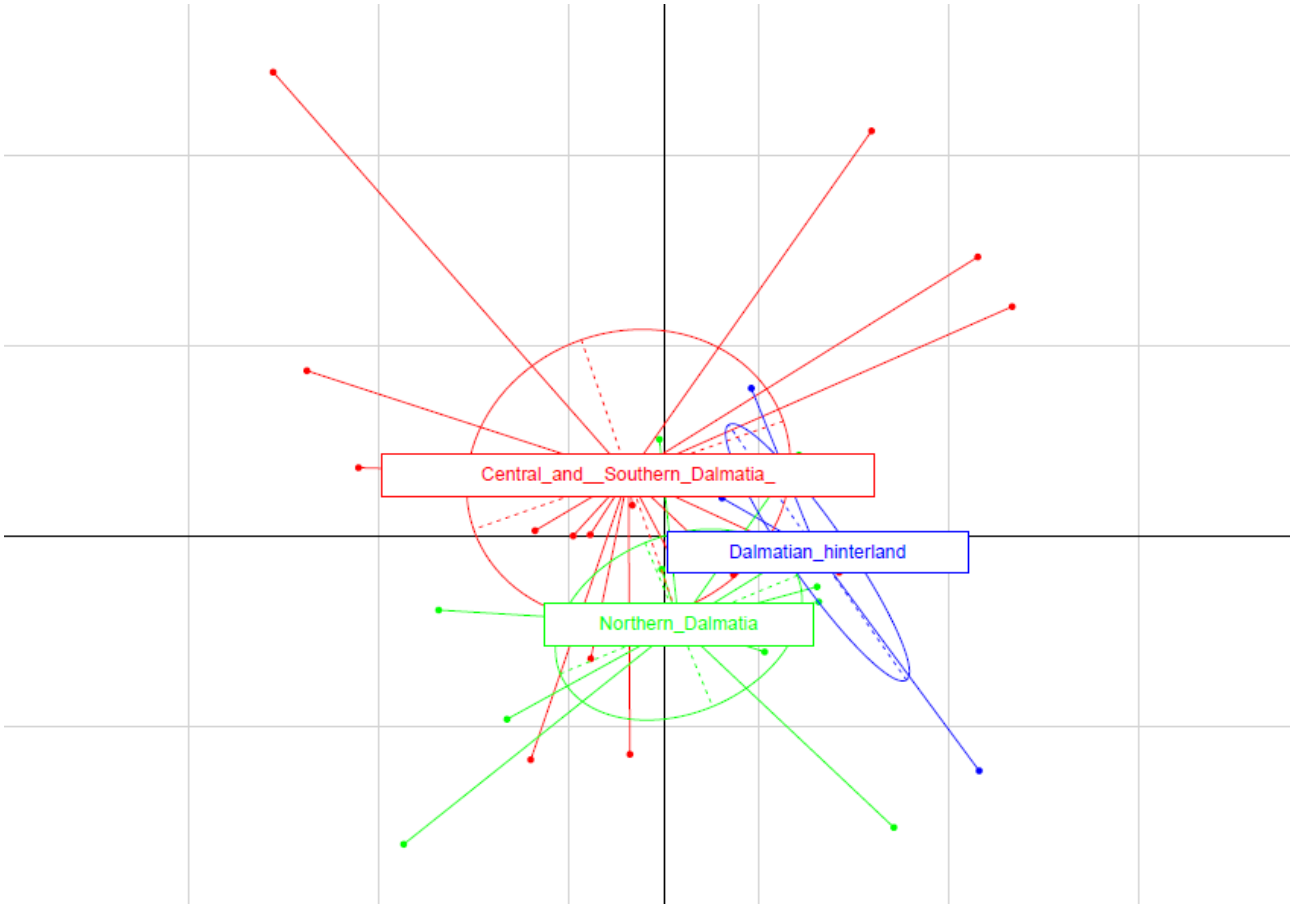
Central_and__Southern_Dalmatia_
Dalmatian_hinterland
Northern_Dalmatia

Fig. 6.

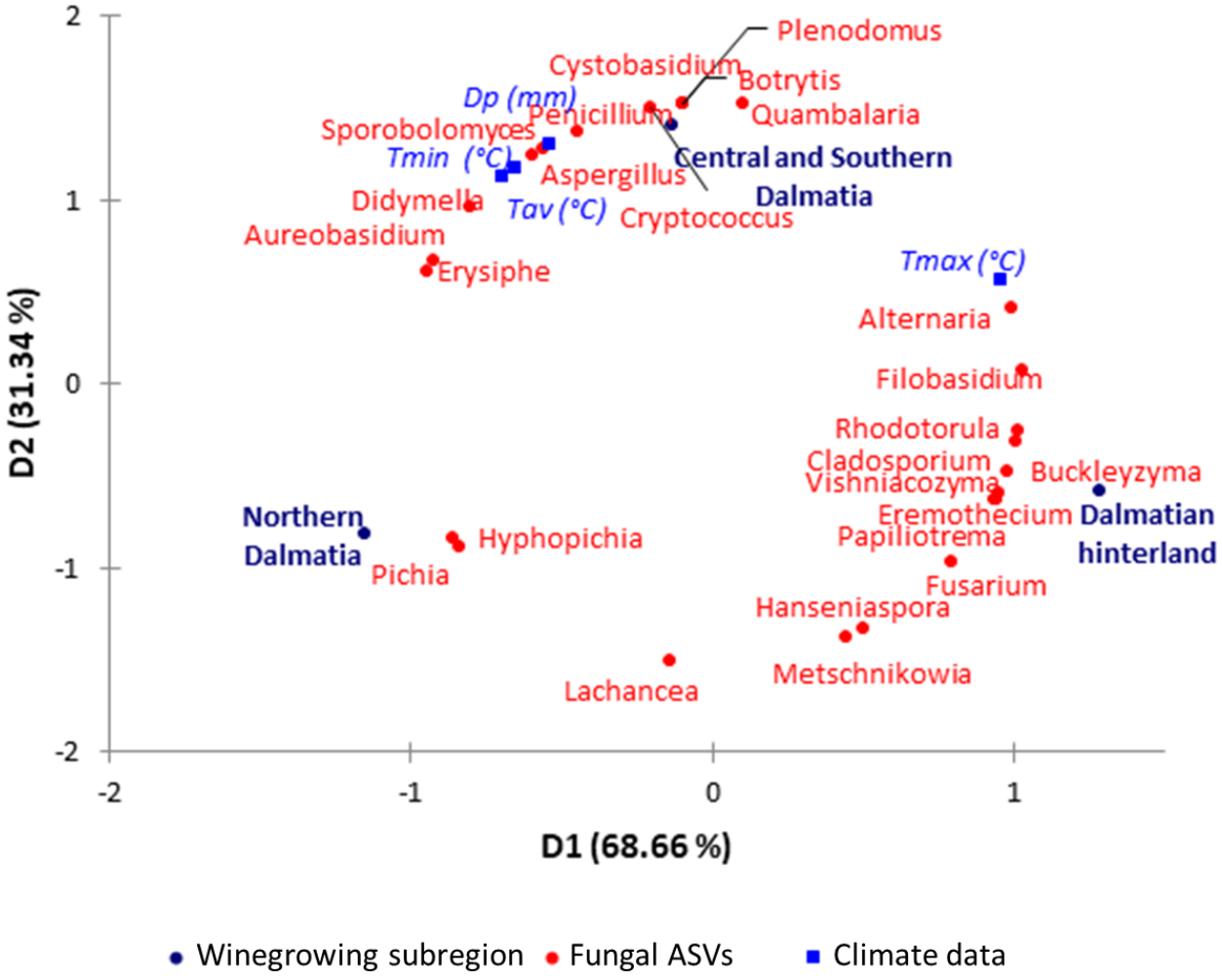
Plenodomus
Cystobasidium
Botrytis
Dp (mm)
Penicillium
Quambalaria
Sporobolomyces
Tmin (°C)
Central and Southern
Aspergillus
Dalmatia
Didymella
Tav (°C)
Cryptococcus
Aureobasidium
Erysiphe
Tmax (°C)
Alternaria
Filobasidium
Rhodotorula
Cladosporium
Buckleyzyma
Vishniacozyma
Eremothecium
Dalmatian
Northern
Dalmatia
Hyphopichia
Papiliotrema
hinterland
Pichia
Fusarium
Hanseniaspora
Metschnikowia
Lachancea
D2 (31.34 %)
D1 (68.66 %)
2
1
0
-1
-2
-2
-1
0
1
Winegrowing subregion
Fungal ASVs
Climate data

Fig. 7.

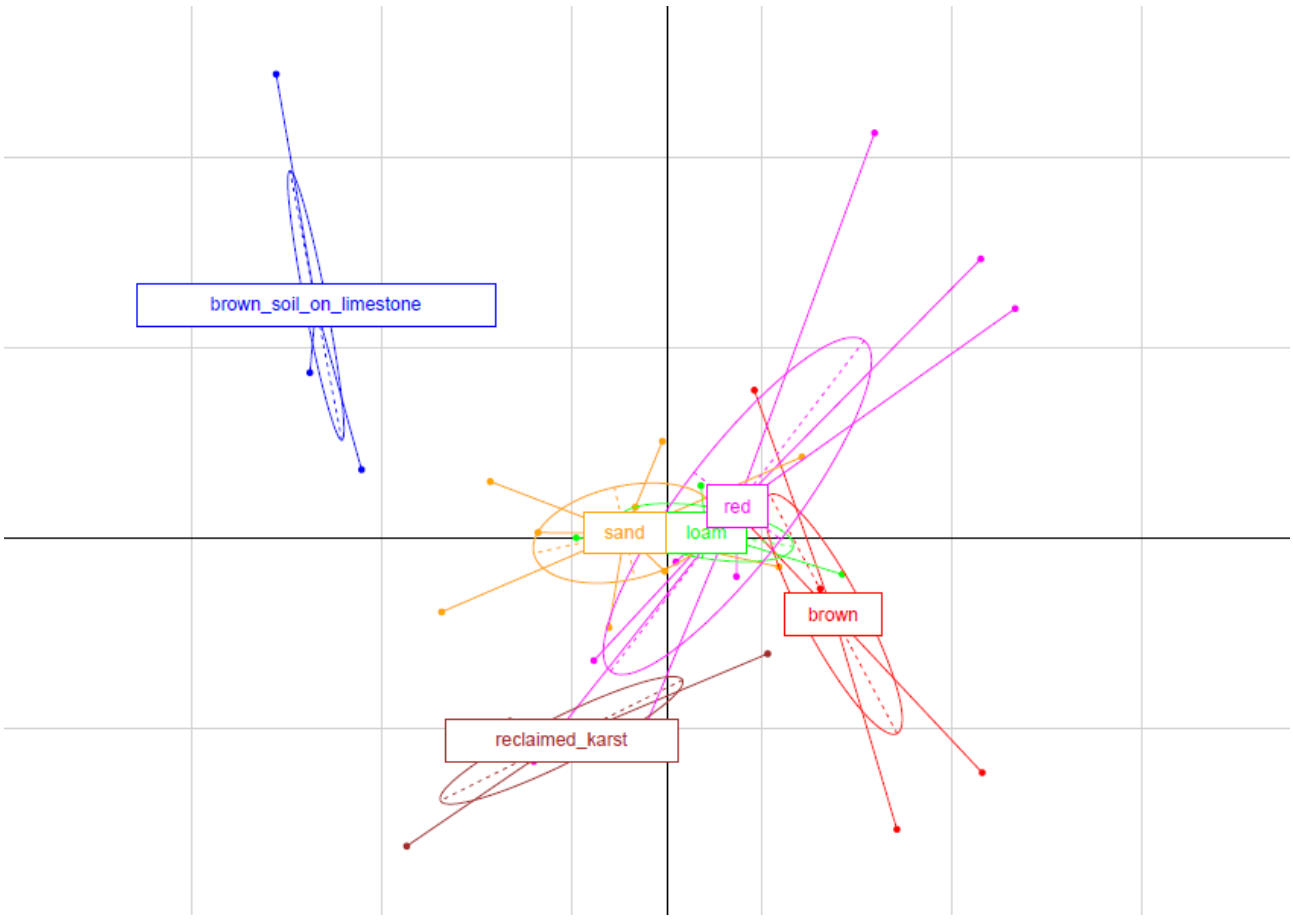
brown_soil_on_limestone
red
sand
loam
brown
reclaimed_karst

Fig. 8.

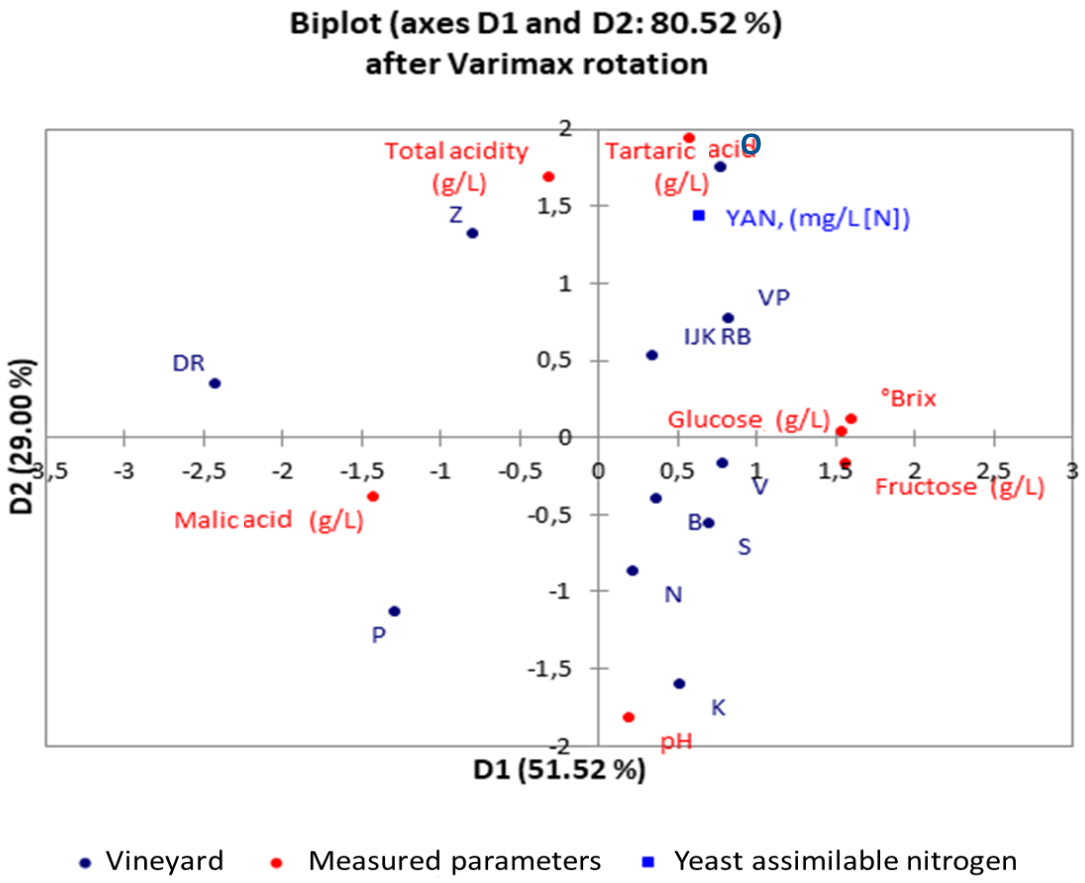

Fig. 9.

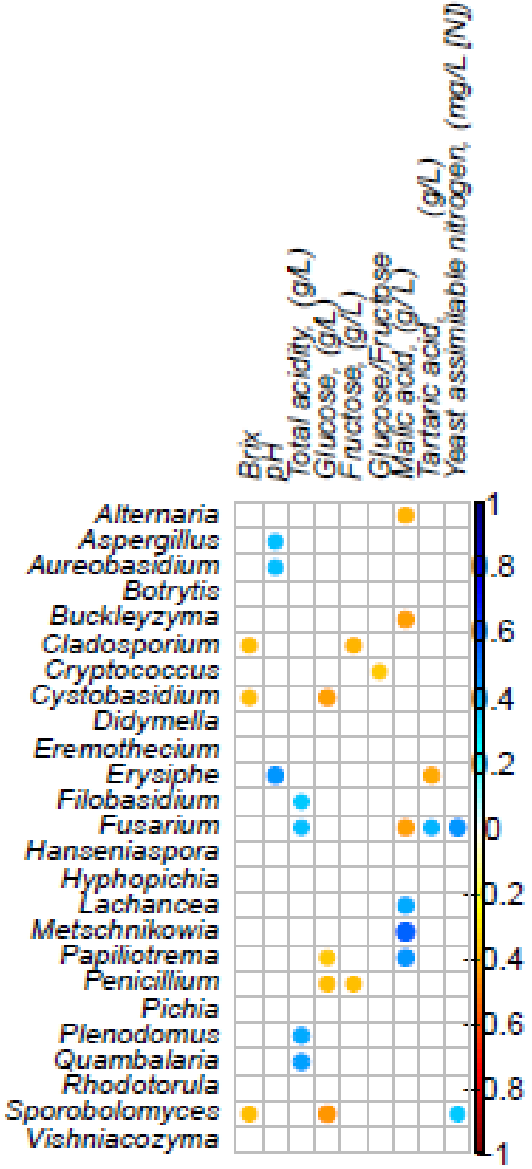

Table 1. General vineyard parameters

Vineyard	Abbreviation	Geographical position	Altitude (m)	Wine-growing subregion	Meteorological station	Plantation year	Soil type	Row distance x vine (m)
Institute for Adriatic Crops and Karst Reclamation, Split	IJK RB	43˚30' 35" N, 16˚29' 85" E	14	Central and Southern Dalmatia	Split	2005	brown soil on limestone	2.0 x 1.0
Dračevica (Korčula)	DR	42˚55' 36" N, 16˚ 54' 0" E	62	Central and Southern Dalmatia	Vela Luka (Korčula)	1941	red	1.8 x 1.0
Prapatna 1 (Korčula)	P	42˚54' 51" N, 16˚54' 58" E	40	Central and Southern Dalmatia	Vela Luka (Korčula)	2000	loam	1.5 x 1.2
Prapatna 2 (Korčula)	B	42˚ 54' 50" N, 16˚ 54' 52" E	40	Central and Southern Dalmatia	Vela Luka (Korčula)	2008	sand	1.2 x 1.0
Kruševo (Korčula)	K	42˚ 55' 20" N, 16˚ 53' 54" E	68	Central and Southern Dalmatia	Vela Luka (Korčula)	1999	red	1.8 x 1.0
Kaštela (Kaštel Kambelovac)	VP	43˚ 33' 34" N, 16˚ 22' 32" E	94	Central and Southern Dalmatia	Split	2011	red	1.6 x 0.9
Smilčić	S	44˚ 7' 23" N, 15˚ 28' 52" E	60	Northern Dalmatia	Zadar	2009	sand	2.6 x 1.0
Nadin (Polača)	N	44˚ 0' 28" N, 15˚ 29' 41" E	103	Northern Dalmatia	Benkovac	2010	reclaimed karst	2.2 x 1.1
Stankovci	Z	43˚ 57' 20" N, 15˚ 43' 6" E	130	Northern Dalmatia	Benkovac	2008	brown	1.9 x 1.0
Oklaj	O	43˚ 56' 55" N, 16˚ 4' 56" E	260	Dalmatian hinterland	Knin	2006	brown	2.0 x 1.0
Vukšić	V	43˚ 56' 37" N, 15˚ 43' 46" E	130	Northern Dalmatia	Benkovac	2003	sand	2.0 x 1.0

Table 2. Physicochemical characteristics of fresh musts obtained from Maraština samples collected from 11 different vineyards.

Vineyard	TSS (°Brix)	pH	Acidity	Glucose	Fructose	Glucose /Fructose	YAN	Malic acid	Tartaric acid
			g/L	g/L	g/L		mg/L [N]	g/L	g/L
IJK RB	22.4±0.5^{a}	3.6±0.0^{a}	6.4±0.2^{a}	109.9±4.9^{abc}	105.4±10.9^{ab}	1.0±0.1^{a}	116.5±23.3^{abc}	0.3±0.3^{bc}	3.3±0.1^{ab}
DR	18.1±0.6^{b}	3.5±0.1^{ab}	5.7±0.7^{ab}	92.3±5.1^{d}	77.4±3.9^{b}	1.2±0^{a}	90.7±4.9^{c}	0.9±0.2^{a}	2.8±0.9^{ab}
P	18.8±0.6^{b}	3.6±0.1^{a}	4.1±0.3^{cd}	94.0±2.9^{cd}	98.1±6.1^{ab}	1.0±0.1^{a}	97.3±2.9^{c}	0.7±0.3^{ab}	2.4±0.3^{b}
B	22.4±1.0^{a}	3.5±0.1^{ab}	3.9±0.2^{d}	109.1±6.1^{abc}	101.8±8.9^{ab}	1.1±0.1^{a}	113.0±2.6^{abc}	0.2±0.1^{c}	2.8±0.7^{ab}
K	21.9±1.4^{a}	3.6±0.0^{a}	3.7±0.3^{d}	118.1±8.2^{a}	114.9±6.3^{a}	1.0±0.1^{a}	98.0±8.5 ^{bc}	0.6±0.0^{abc}	2.3±0.2^{b}
VP	22.1±1.5^{a}	3.5±0.1^{a}	4.9±0.1^{bc}	115.6±8.0^{ab}	111.3±17.0^{a}	1.1±0.2^{a}	142.0±2.8^{a}	0.2±0.1^{bc}	3.9±0.4^{a}
S	23.0±0.9^{a}	3.6±0.0^{a}	4.1±0.1^{d}	112.3±4.1^{ab}	113.1±12.2^{a}	1.0±0.1^{a}	99.0±9.0^{bc}	0.5±0.2^{bc}	3.2±0.3^{ab}
N	21.9±1.2^{a}	3.6±0.1^{a}	3.7±0.1^{d}	104.0±4.9^{abcd}	107.4±5.1^{a}	1.0±0.0^{a}	106.3±4.0^{abc}	0.4±0.0^{bc}	3.0±0.4^{ab}
Z	20.5±1.5^{ab}	3.3±0.0^{b}	5.0±0.3^{b}	102.1±4.6^{bcd}	98.3±16.0^{ab}	1.1±0.1^{a}	134.0±4.0^{ab}	0.7±0.1^{abc}	3.7±0.1^{a}
O	22.6±0.3^{a}	3.3±0.0^{b}	5.7±0.1^{ab}	112.9±3.8^{ab}	115.7±4.1^{a}	1.0±0.0^{a}	117.0±8.0^{abc}	0.3±0.1^{bc}	4.0±0.2^{a}
V	23.4±1.0^{a}	3.5±0.1^{a}	5.0±0.2^{b}	115.6±5.4^{ab}	114.5±6.0^{a}	1.0±0.1^{a}	109.3±34.1^{abc}	0.4±0.2^{bc}	2.9±0.5^{ab}

YAN, Yeast Assimilable Nitrogen.

Values are expressed as means ±standard deviations. Within each column, means followed by different superscript letters (^{a}, ^{b}, ^{c}) are significantly different ($P < 0.05$).

Supplementary Figure 1

Supplementary Figure 2

Graphical Abstract