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Brief Report

Above-ground parts of white grapevine *Vitis vinifera* cv. Furmint share core members of the fungal microbiome

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Summary

Grapevine (*Vitis vinifera*) is a reservoir of fungal endophytes that may affect its growth, health status and grape production. Although there is growing interest in comparing fungal communities of mainly red grape varieties across various factors using only high-throughput sequencing, the small-scale mycobiome variations in geographically close vineyards need further examination. We aimed to characterize the fungal microbiome of the above-ground tissues of *V. vinifera* cv. Furmint in different plant parts, seasons and sites using culture-dependent and culture-independent methods, and *in planta* fluorescent microscopic visualization techniques. Samples were collected from four sites of the Tokaj wine region in Mád and two reference sites in Eger, Hungary, across different seasons for 2 years. Fungal endophytes of young and mature leaves, flowers and grape bunches were collected at different phenological stages. Based on each technique, *Aureobasidium pullulans*, *Cladosporium* spp. and the complex species *Alternaria alternata* dominated the community at every site, season and plant organ. We found no significant difference among communities in distinct neighbouring vineyards, nor when compared with the distant reference sites. We can conclude that the different shoot parts of the Furmint grapevines harbour a common core group of fungal community in these regions.

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Introduction

Most terrestrial plants form symbioses with diverse fungal endophytes, which are important members of the plant microbiome (Vandenkoornhuyse *et al*., 2015). Communities of fungal endophytes, which colonize plant tissues without causing any visible symptoms in the hosts, can be found in both natural and managed ecosystems (Porras-Alfaro and Bayman, 2011). Grapevine varieties (*Vitis vinifera*) also represent reservoirs of these endophytes, which may affect their growth, health status and grape production (Morgan *et al*., 2017). To date, information on endophyte presence and role have been mostly unknown compared with that on fungal pathogens; however, there is an intensifying focus on different microbial communities in distinct wine-growing regions. This could lead to a better understanding of their potential influences on grape variety and production year (Bokulich *et al*., 2014). Relatively less attention has been paid to the fungal microbiota of grapevines compared to the bacterial community and the above-ground compared with the below-ground community (Martinez-Diz *et al*., 2019; Deyett and Rolshausen, 2020; Liu and Howell, 2021).

Numerous studies have addressed questions on the seasonal, temporal and regional variations of fungal communities inhabiting different above-ground organs of several varieties of grapevines using high-throughput sequencing (HTS) and other methods (Pinto *et al*., 2014; Setati *et al*., 2015; Varanda *et al*., 2016; Jayawardena *et al*., 2018; Martinez-Diz *et al*., 2019; Deyett and Rolshausen, 2020; Swift *et al*., 2020). However, most of these studies compared communities of different vineyards, mainly red grape varieties, with large geographic

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distances among them. Although fungal endophyte isolation can support and supplement the results from culture-independent methods (Dissanayake *et al*., 2018; Eichmeier *et al*., 2018; Jayawardena *et al*., 2018), there are few grapevine studies where these techniques have been applied. Moreover, the *in planta* visualization of the fungal microbiome of grapevines has largely been neglected. Nevertheless, studies focusing on bacterial communities have localized these endophytic microorganisms (Compant *et al*., 2010, 2011).

Fungal diversity differences among vineyards could play a role in forming the terroir effect (Gilbert *et al*., 2014); therefore, understanding the fungal community dynamics among vineyards could be useful for the wine-making process. The Tokaj wine region, a historically and economically important wine-producing territory in Hungary, is ideal for such studies (Szepesi *et al*., 2017). This region produces the world's oldest botrytised 'aszú' wines, growing almost exclusively indigenous white varieties such as Furmint (Kovács *et al*., 2017). However, almost no information is available on the fungal microbiome of grapevines in the region, except for the characterization of a few potential fungal pathogens collected from here (Kovács *et al*., 2017; Váczy, 2017; Váczy *et al*., 2018). A small-scale geographical study focusing on above-ground fungal communities of white grape varieties from neighbouring vineyards could provide new information on *V. vinifera*, which has been investigated from several other aspects.

We aimed to test whether organs, sites and seasons affected the fungal community structure in the above-ground tissues of *V. vinifera* cv. Furmint. To determine this, we (i) studied the fungal microbiome using culture-dependent and -independent methods and identified the common and dominant members of the community, (ii) compared the endophytic fungal communities of different plant organs and evaluated their seasonality on a fine-scale regional variation and (iii) visualized the fungal endophytes within the plant tissues.

Results

Here, we present the first data on fungal microbiomes of different above-ground tissues of the white grapevine variety Furmint, one of the most cultivated white grape varieties in Hungary. The sampling was carried out in four different vineyards in Mád (Tokaj wine region); Betsek (BET), Király (KIR), Szent Tamás (STT) and Úrágya (URA) (Fig. S1), and two reference sites in Eger wine region; Nagy-Eged (NEG) and Hangács (HAN) vineyards (see Supplementary Information Experimental Procedures). Young and mature leaves and grape bunches were examined in spring, summer and autumn for 2 years (Fig. 1), and the fungal microbiome was studied by an HTS-based method, culture-dependent methods and fluorescence-based microscopic visualization (see Supplementary Information Experimental Procedures).

High-throughput sequencing

After paired-end alignments, quality filtering, and deletion of singletons, chimeric and non-fungal sequences, a total of 4 053 313 fungal ITS sequences were generated from 140 plant samples (Supporting Information Table S1). The average read number of the samples was 28 952 and the median was 4875. The samples collected in autumn had generally higher read numbers compared to those from other seasons (Supporting Information Table S1). After exclusion of samples with low read numbers and random subsampling to 3000 reads, 853 OTUs represented the fungal community of the grapevine (Supporting Information Table S2). A vast majority of the sequences belonged to Ascomycota (92%), and the most abundant orders were Dothideales (51%), Capnodiales (16%), Pleosporales (11%), Helotiales (9%) and Filobasidiales (6%). Within these orders, generally 1–1 dominant genus comprised the majority of the reads and shaped the community: *Aureobasidium* (51%), *Cladosporium* (16%), *Alternaria* (9%), *Erysiphe* (4%) and *Filobasidium* (4%) respectively (Fig. 2). Ascomycota (70%) and Basidiomycota (30%) represented almost all of the total OTUs detected (Supporting Information Table S3). The order Pleosporales represented the most OTUs (221) followed by Agaricales (105), Tremellales (51), Helotiales (47) and Capnodiales (43). The 25 most abundant OTUs comprised more than 95% and the top 10 OTUs almost 91% of the sequences (Supporting Information Table S2). The most dominant OTU represented *Aureobasidium pullulans*, which was dominant in all seasons, sites and plant parts (Fig. 1). The dominant presence of *Cladosporium* spp. and *Alternaria* aff. *alternata* was in itself remarkable. These three OTUs represented over 70% of all reads in general, and at least 60% of the reads grouped by seasons, distinct sites and different shoot parts. To have an overview of the similarity of the samples, Principal Coordinate Analysis (PCoA) with Bray–Curtis distances was used, and the most conspicuous finding was the arrangement of the communities by sampling season (Fig. 3). To reveal the groupings of the samples according to sampling sites, plant parts, sampling years and seasons general linear model (GLM) using generalized least squares were applied for the first two extracted PCoA coordinates. Changes in samples' Chao1 diversities were also tested the same way. Generally, sampling sites were not separated from each other based on community composition nor based on diversity (Fig. 4A and B; Supplementary Information Fig. S2A).

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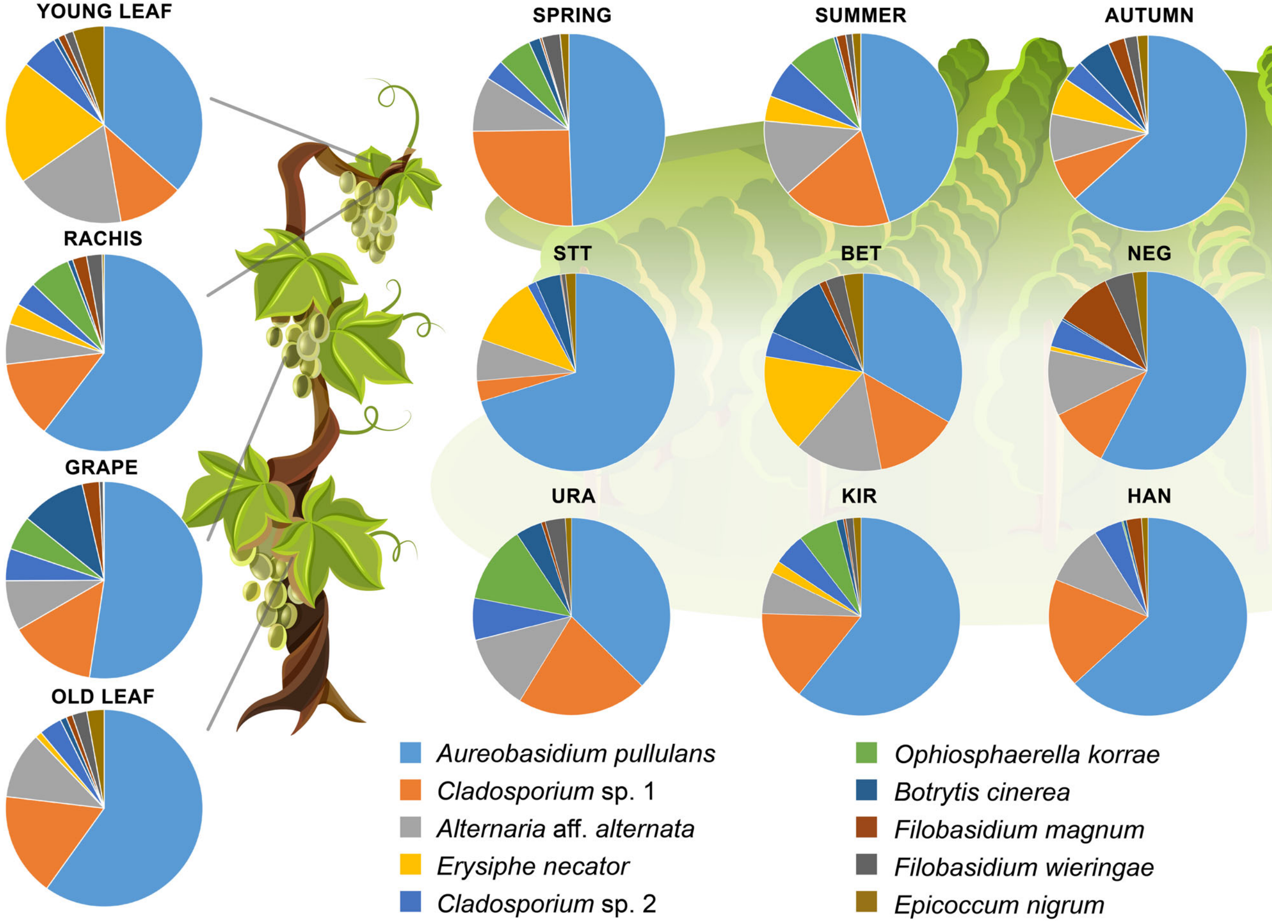

Fig. 1. Ratio of the 10 most abundant OTUs of all samples from different seasons, sites [Betsek (BET), Király (KIR), Szent Tamás (STT), Úrágya (URA) vineyards in Mád, and Hangács (HAN), Nagy-Eged (NEG) vineyards in Eger], plant parts [grape berry (GRAPE), small juvenile leaves (YOUNG LEAF), mature leaves (OLD LEAF) and woody part of the grape cluster (RACHIS)].

However, there were significant differences in fungal diversity between seasons and also between different plant parts with a decreasing fungal diversity from spring to autumn and young leaves to grapes. (Fig. 4C, D, G and H). Furthermore, the two sampling years differed significantly (Fig. 4E and F).

Culture-dependent technique

During the isolation process to acquire fungi from the internal tissues, more than 3400 samples/parts of different plant materials were surface-sterilized and laid onto PDA media. Endophytes growing out from the tissues of the same plant sample with identical colony morphology were considered to represent the same taxa and were sub-cultured and used for further analyses. Altogether, 353 isolates were obtained from different organs of the Furmint grapevine from Mád and Eger (Supporting Information Fig. S3; Table S5). Most of the collected isolates belonged to the phylum Ascomycota, representing diverse orders. Only a few basidiomycetes were found, including the yeast *Curvibasidium* and *Peniophora* species (Supporting Information Fig. S3; Table S5). Based on molecular phylogenetic identification, the majority of the clades could be identified at the species or genus level, whereas others could only be identified at higher taxonomic ranks (Supporting Information Figs S3 and S4; Table S5). Based on the analysis of the ITS sequences, the isolates represented 44 lineages introduced here as clade 1 to clade 44. The most numerous clade, representing almost one-third of the collected fungi, consisting of 122 isolates, was the complex species *Alternaria* aff. *alternata* (clade 1), followed by clade 7 (*Epicoccum nigrum*), clade 11 (*Botrytis cinerea*) and clade 13 (*Aureobasidium pullulans*), with 46, 34 and 32 isolates respectively (Supporting Information Fig. S3; Table S5). Most of the isolates (331) belonged to non-singleton clades, while 22 clades contained only one isolate. Pleosporales was the most represented order with 209 isolates and 10 clades (209/10), followed by Helotiales (35/2), Dothideales

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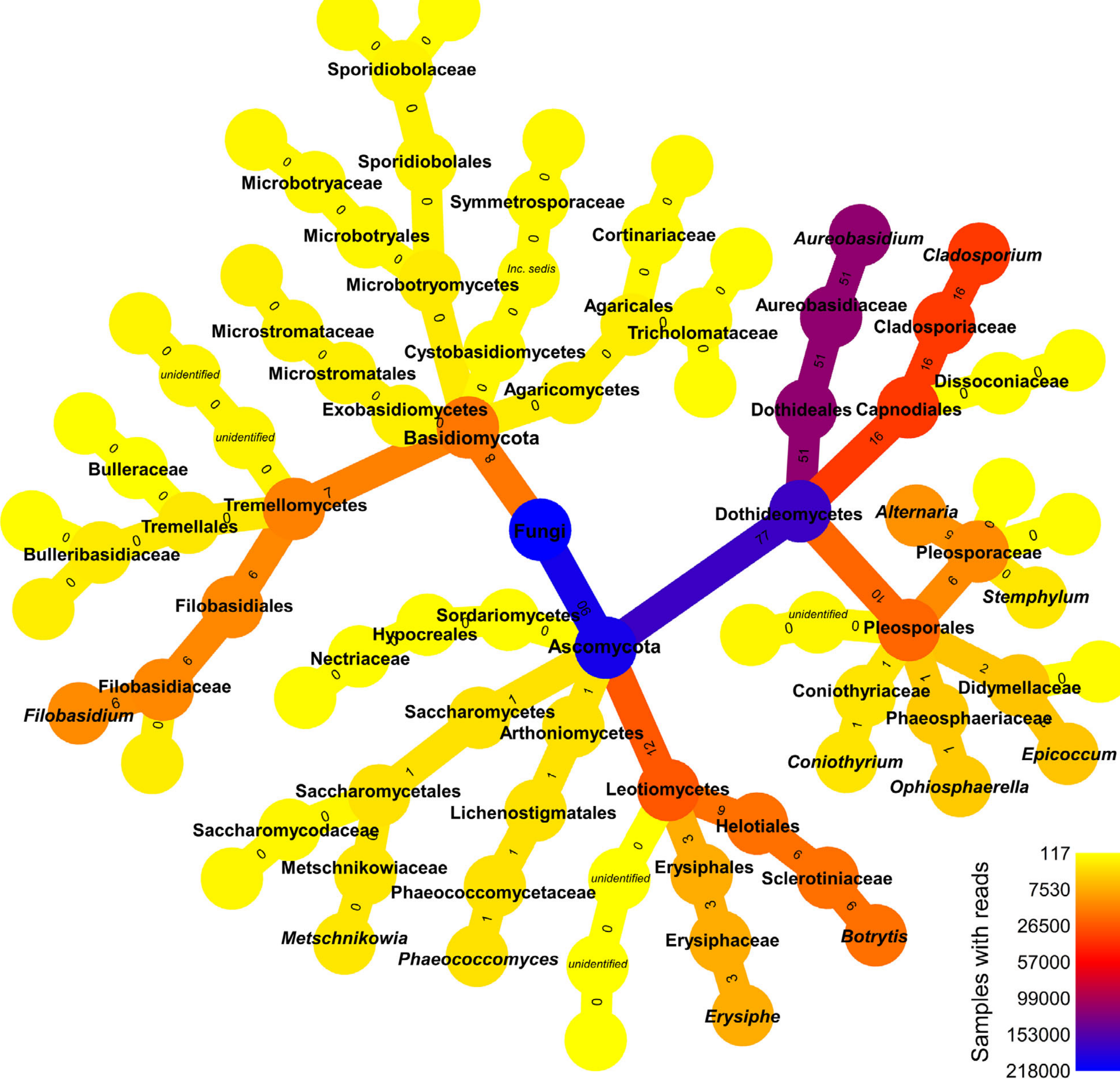

Fig. 2. Taxonomic composition of fungal communities of *V. vinifera* cf. Furmint based on nrDNA ITS2 region amplicon sequencing. Each node represents a taxon from the kingdom to the genera. Taxa representing more than 0.05% of the 200 most abundant OTUs were visualized in the heat tree. Taxon names are shown at the nodes and only genera with more than 1000 reads are indicated. The colour of the nodes ranges from blue (100% relative abundance) through red to yellow (0% relative abundance) according to the abundance of the taxa in each sample. Small numbers on the edges give the relative abundance of the taxa that follow the given edge.

(32/1) and Capnodiales (31/2); however, all these orders, especially the last three, were dominated by only one or two clades. Among the cultivable fungal taxa *A.* aff. *alternata* dominated the above-ground plant tissues, and *E. nigrum*, *B. cinerea* and *A. pullulans* also had a considerable presence. Compared to the OTUs revealed by HTS, the dominant culturable filamentous fungi were also identified by the culture-dependent method (Supporting Information Table S5). However, ascomycetes were overrepresented by isolation-based techniques, and e.g. basidiomycete groups (Agaricales and Tremellales) frequent in the HTS analyses were not isolated.

Because of *A. alternata* and related species that belong to *Alternaria* sect. *Alternaria*, where the ITS region is not adequate for species delimitation (Woudenberg *et al*., 2015), 24 isolates originating from different sites, plant parts and seasons were selected for further identification, and the partial RPB2 region was sequenced

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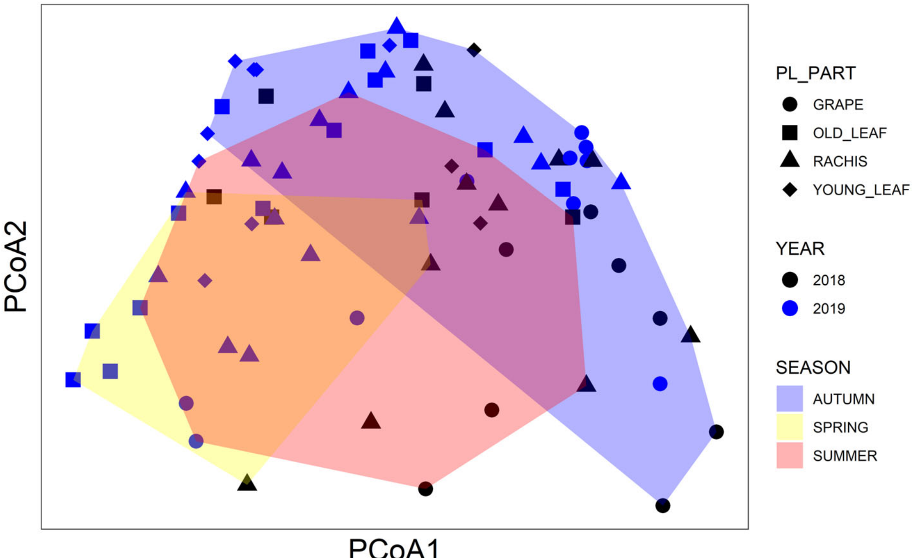

Fig. 3. Similarity of fungal community composition based on nrDNA ITS2 region amplicon sequencing with Principal Coordinate Analysis (PCoA) based on Bray–Curtis distance matrix using the relative abundances of the logarithmic (base 10) transformed OTU table. Different small symbols denote plant part, colour of the symbols denote sampling year and background convex polygons denote sampling season.

(Supporting Information Table S5). The phylogenetic analysis resulted in at least six well-supported different lineages within *Alternaria* sect. *Alternaria* (Supporting Information Fig. S5). The isolates represented different clades and morphospecies within *A. alternata* and two lineages in the *A. arborescens* species complex (Supporting Information Fig. S5).

Visualization

The *in planta* fungal colonization of each collected sample could be detected by fluorescent microscopy (Fig. 5). Using different excitation wavelengths and barrier filters for epifluorescence microscopy, the specificity and applicability of WGA-AlexaFluor488 dye were tested, and we found only weak autofluorescence of the plant tissues in the background at an excitation of 488 nm (Fig. 5A–C). Fungal colonization seemed to be restricted to the intercellulars of the leaves. The hyphal structures did not show typical intracellular growth; in most cases, hyphae formed relatively long branches in the intrafoliar space (Fig. 5E). Although we did not aim for any quantitative measurement, the results of the visualization showed that the colonization of the leaves and young grape clusters was weakest at the beginning of the growing season and strongest in autumn. In particular, in the six young leaves collected in early spring of 2019, almost no colonization or presence of fungal structures could be detected.

We detected only septate hyphae within the plant tissues (Fig. 5H). In case of some fungal lineages that were found to be dominant by both culture- and non-culture-dependent techniques, morphological identification of a particular taxonomic level may be feasible based on specific structures of the fungi. The most common fungal structures were longitudinal hyphae, which generally formed arthroconidia (Fig. 5D–E). Although we cannot strongly conclude much about these species, these structures might be formed by *Aureobasidium pullulans* based on its known morphological features and significant abundance in the grapevine (Fig. 5D–E). In numerous samples, obclavate conidia with both transverse and longitudinal septations were present (Fig. 5G), which are characteristic of *Alternaria* species.

Discussion

This study reports the first observations of the fungal community within different above-ground tissues of one grapevine variety in different seasons and vineyards located in a limited geographic proximity. We also present the first information on the fungal microbiome of white grapevine *V. vinifera* cv., Furmint, historically cultivated in the Tokaj wine region of Hungary. To date, only the occurrence of grapevine trunk diseases (GTDs) and pathogens involved in botryosphaeria dieback (Kovács *et al.*, 2017; Váczy, 2017; Váczy *et al.*, 2018), as well as the yeast population of the berries (Naumov *et al.*, 2002; Sipiczki, 2016) have been investigated in this area. These data provide important information as most diversity studies applying HTS focusing on fungal communities of grapevines generally present mycobiomes of different red varieties (Pinto *et al.*, 2014; Setati *et al.*, 2015; Varanda

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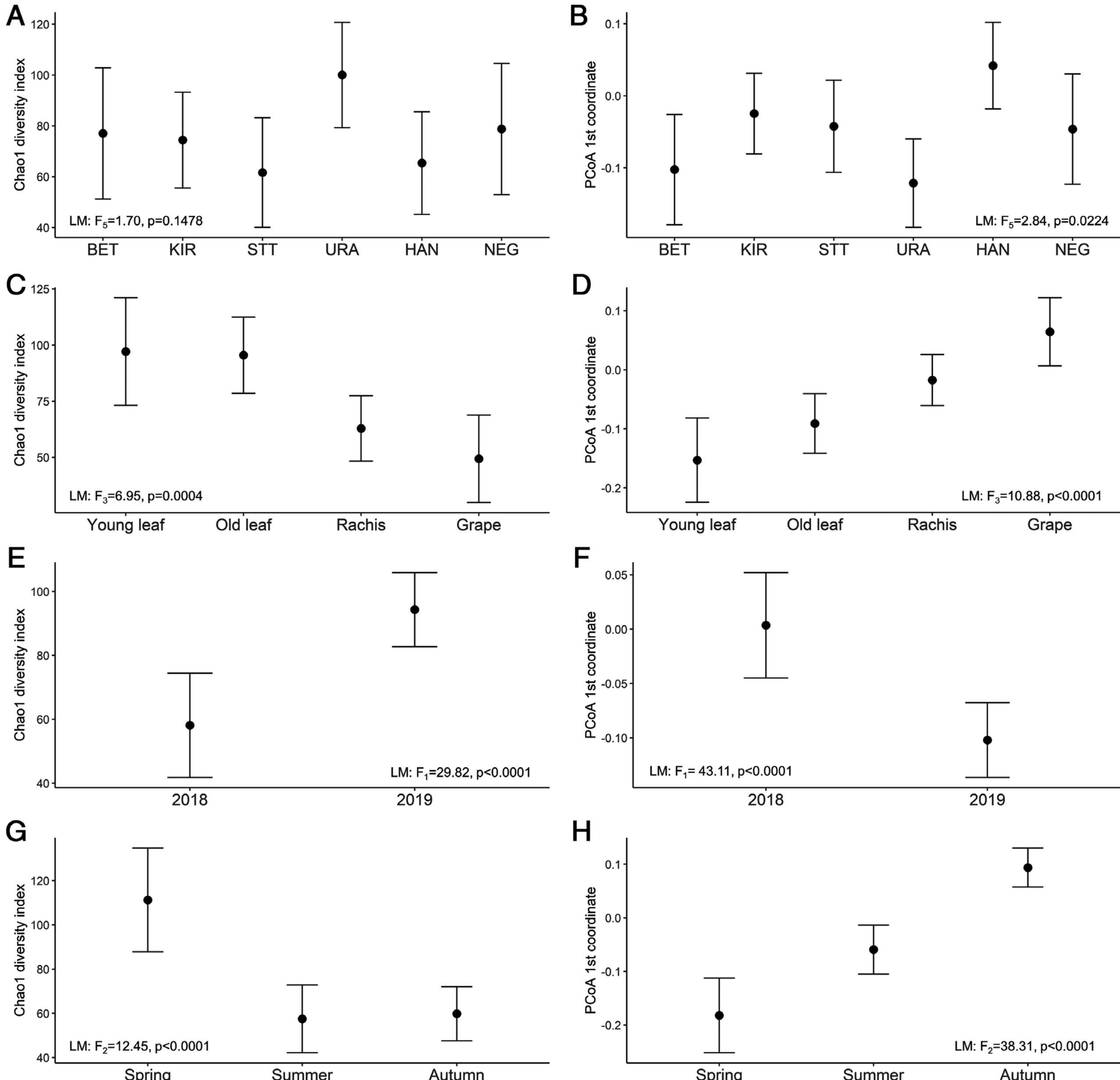

Fig. 4. Effects of (A–B) sampling site, (C–D) plant part, (E–F) year and (G–H) season on (A, C, E, G) fungal Chao1 diversity and on (B, D, F, H) community composition (extracted first PCoA coordinates of the samples) Sites: Betsek (BET), Király (KIR), Szent Tamás (STT), Úrágya (URA) vineyards in Mád, and Hangács (HAN), Nagy-Eged (NEG) vineyards in Eger; plant parts: grape berry (Grape), small juvenile leaves (Young leaf), mature leaves (Old leaf) and woody parts of the grape cluster (Rachis). Values indicate the estimated marginal means ±95% confidence intervals calculated from the general linear models.

et al., 2016; Jayawardena *et al*., 2018; Martinez-Diz *et al*., 2019; Deyett and Rolshausen, 2020), and only a few studies present data from white varieties (e.g. Bokulich *et al*., 2014, 2016).

Diversity

Although various techniques are commonly used to assess OTU or amplicon sequence variant (Deyett and Rolshausen, 2019; Deyett and Rolshausen, 2020) numbers in fungal communities of grapevines using different primer sets (Pinto *et al*., 2014) and sequencing techniques (Kernaghan *et al*., 2017), our 853 OTUs of the filtered samples correspond to those of similar studies, in which the OTU number ranges between 200 and 2200 (e.*g*. Pinto *et al*., 2014; Jayawardena *et al*., 2018; Martinez-Diz *et al*., 2019, 2020; Fan *et al*., 2020; Liu and Howell, 2021). Random subsampling of the samples to a

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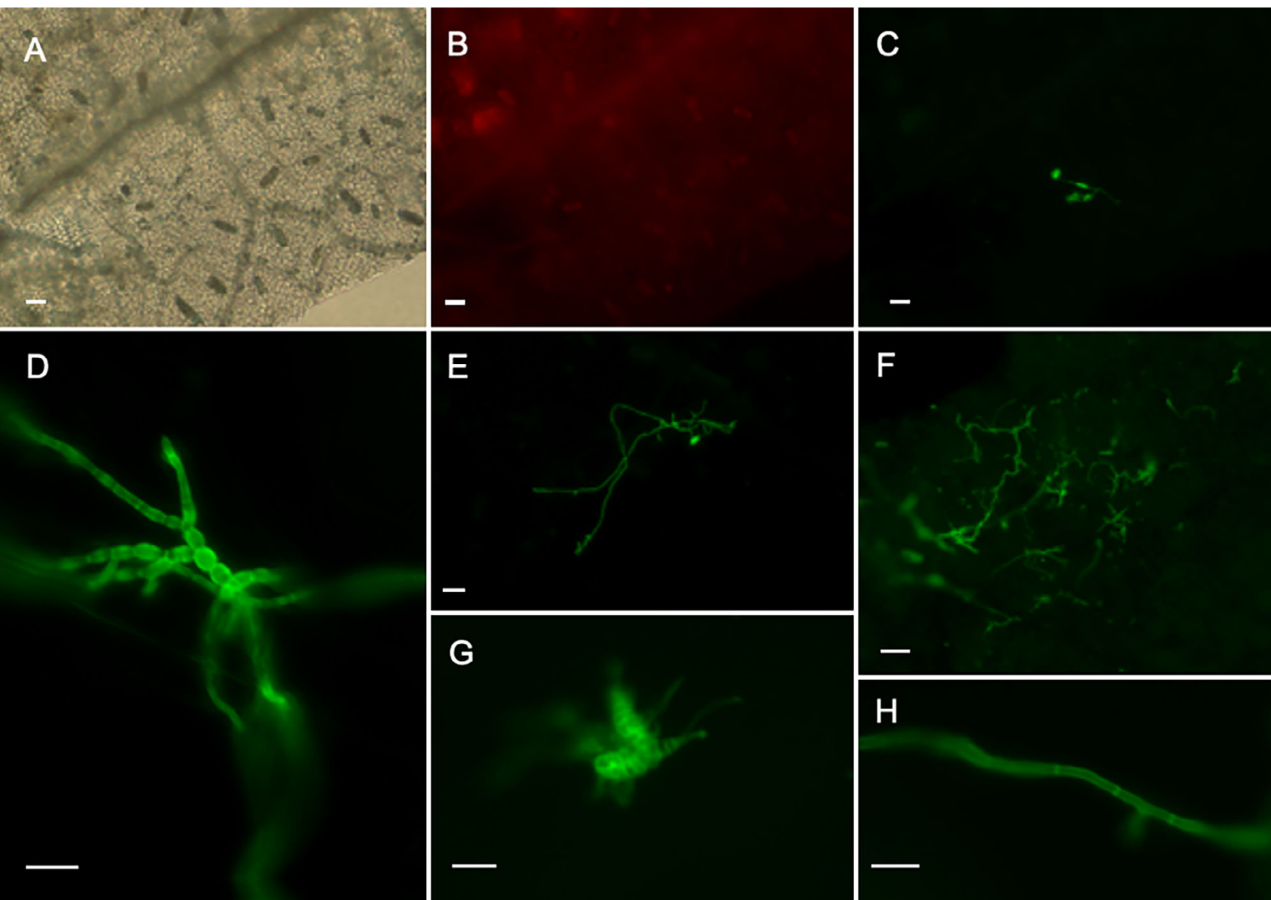

Fig. 5. Fungal colonization of different tissues of *V. vinifera* cf. Furmint and stained fungal structures using WGA-AlexaFluor488 dye. Micrographs of cleared matured grapevine leaf in brightfield (A) and in fluorescence mode using filter wheel with excitation and emission filters for a non-specific excitation (546 nm) (B). Fungal structures are invisible in brightfield mode, and weak red background is present in case of non-specific excitation caused chiefly by the veins and idioblasts comprising of calcium oxalate raphides crystals. Using a filter wheel with excitation and emission filters for a specific excitation for visualization of WGA labelling (488 nm) shows fungal structures stained by WGA-AlexaFluor488 (C). Fungal colonization of different tissues of *V. vinifera* cf. Furmint and stained fungal structures by WGA-AlexaFluor488 dye. Arthroconidia and longitudinal hyphal branches resembling the growing characteristics of *Aureobasidium pullulans* within old leaves (D, E). Characteristic conidia of likely *Alternaria* species (F). Extensive hyphal colonization at the inner side of the peal of a matured grape berry (G). Septate hyphae within the intercellular space of an old leaf (H). Scale bars = 5 μm.

few thousand reads is not unusual as seen in various analyses of fungal microbiomes of different above-ground grapevine plant materials (Bokulich *et al*., 2014, 2016; Pinto *et al*., 2014; Fan *et al*., 2020). The relatively low number of fungal sequences in the internal plant tissues can be caused by the sampling of only the above-ground tissues, where the fungal abundance and diversity of the organs, especially the leaves, is generally below that of other plant parts. Based on several studies focusing on the mycobiome of roots, rhizosphere and different shoot tissues, the fungal dominance and diversity reported to be higher in below-ground than that in above-ground parts of several *Vitis* varieties (Zarraonaindia *et al*., 2015; Deyett and Rolshausen, 2020; Liu and Howell, 2021). Although there might be clear correlations and differences in ITS-based diversities, we should bear in mind the substantial biases in fungal diversity due to highly variant rDNA copy numbers, different cell and growing characteristics, and the number of nuclei in a specific region (Lofgren *et al*., 2018).

General microbiome, dominant members of the community

Grapevine hosts a diverse spectrum of fungal endophytes, and the vast majority of these fungi belong to various orders of the phylum Ascomycota, as detected by different techniques (Dissanayake *et al*., 2018; Jayawardena *et al*., 2018; Liu and Howell, 2021). Using both culture-dependent and independent methods, studies showed that healthy-looking grapevine plants live together with a core community consisting of *Alternaria*

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alternata (and related *Alternaria* species), *Aureobasidium pullulans*, *Epiccocum nigrum*, and sometimes *Botrytis cinerea* along with *Cladosporium*, *Fusarium*, *Penicillium*, *Phoma*/*Didymella* and other species (González and Tello, 2011; Brum *et al*., 2012; Pancher *et al*., 2012; Setati *et al*., 2012; Bokulich *et al*., 2014; Pinto *et al*., 2014; Setati *et al*., 2015; Kernaghan *et al*., 2017; Dissanayake *et al*., 2018; Jayawardena *et al*., 2018; Mezzasalma *et al*., 2018; Wei *et al*., 2018; Deyett and Rolshausen, 2020; Liu and Howell, 2021). In the current study, the most abundant OTUs were represented mainly by the taxa mentioned above; however, some lineages considered as dominant by other studies were not present, such as *Mycosphaerella*, which is usually a member of the core community (Deyett and Rolshausen, 2020; Liu and Howell, 2021). The isolation technique confirmed the dominant presence of the most common lineages found by molecular investigations. Species representing six of the 10 most common OTUs, such as *A*. *alternata* and close relatives, *A*. *pullulans*, *B*. *cinerea*, *Cladosporium* sp1, *Cladosporium* sp2 and *E*. *nigrum* were present in high numbers, and *O*. *korrae* was also isolated. One of the primary importance of the isolation technique was finding numerous isolates belonging to the *Alternaria alternata* within *Alternaria* sect. *Alternaria*, which could not be separated into different species by only the ITS2 or the whole ITS region (Woudenberg *et al*., 2013, 2015). The obligate biotrophic pathogen of grape powdery mildew *Erysiphe* (*Uncinula*) *necator* and two yeasts, *Filobasidium magnum* and *F*. *wieringae* belonging to Filobasidiales (Basidiomycota) could not be cultured.

The top three OTUs were *A*. *pullulans*, *Cladosporium* sp. and *A*. aff. *alternata* representing over 70% of the fungal sequences obtained from grapevine, and they were also found to be common in different studies in grapevine fungal microbiomes. *Aureobasidium pullulans* is a cosmopolitan dimorphic fungus known as black yeast due to its melanin production, representing one of the most abundant members of the grapevine fungal microbiome worldwide (González and Tello, 2011; Kernaghan *et al*., 2017; Deyett and Rolshausen, 2020). Although our knowledge has been limited to the accurate identity, taxonomy and function of *Cladosporium* species related to grapevines, their dominance within plant tissues has been found in several studies (Zhang *et al*., 2017; Dissanayake *et al*., 2018; Deyett and Rolshausen, 2019, 2020).

In this study, most isolates, and a significant number of the reads in the HTS analysis belonged to the genus *Alternaria*, which has been found among the dominant members of the grapevine community in different regions and varieties (González and Tello, 2011; Pancher *et al*., 2012; Varanda *et al*., 2016; Kernaghan *et al*., 2017). Polizzotto *et al*. (2012), using a combination of morphological, molecular and chemical analyses, also reported that grapevines are associated with a dominant and unique *Alternaria* endophyte community. As a genus, *Alternaria* is a biologically, morphologically and ecologically diverse group of fungi producing multi-celled conidia (Simmons, 2007; Lawrence *et al*., 2016). Many of its species are considered to be cosmopolitan saprobes, endophytes, pathogens, or the causal agents of postharvest rots of numerous agronomic plants (Ragazzi *et al*., 2001; Polizzotto *et al*., 2012; Armitage *et al*., 2015). The complex *A*. *alternata* species comprises 35 morphospecies (Woudenberg *et al*., 2015) often called small-spored *Alternaria*, with species such as *A*. *alternata*, *A*. *arborescens* and *A*. *gaisen*. Our findings showed that at least six lineages of *A*. *alternata* and *A*. *arborescens* species complex are present in grapevines masked by identical ITS sequences. Isolation of these fungi is important to assess the structure of the community of even the closely related *Alternaria* species within the plant because major differences in their strategy and effects on the host might be present. Since endophytic fungi belonging to this complex species may play an essential role in the biocontrol of plant pathogens such as *Plasmopara viticola* (Musetti *et al*., 2006, 2007), the accurate identification of members of this dominant group is of vital importance.

Season, plant parts and site

Seasonal changes in the endophytic community of grapevines were found in the bacterial microbiome (Bulgari *et al*., 2009; Campisano *et al*., 2017); however, with fungal endophytes, these seasonal changes are not entirely understood. Pinto *et al*. (2014) reported significant alterations in the fungal community structure of leaves from July to August in Portugal. Martinez-Diz *et al*. (2020) investigated wood-inhabiting fungi of grapevines at three sampling times, November, February and May, in Spanish vineyards, and they found a correlation between fungal diversity and sampling time. Fungal richness and diversity were lower from February to May. Deyett and Rolshausen (2019) also found temporal variation in the sap microbiome of grapevines in California. Significant variation in fungal richness and diversity were also recorded at each grapevine habitat over time in Australian vineyards (Liu and Howell, 2021), where differences were observed in fungal community composition over time regardless of grapevine habitat. Our results using endophyte isolation, microscopy and HTS pointed to fewer isolate numbers, structures and reads in spring when the colonization and probably the whole fungal biomass are likely less dominant. According to these

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findings, communities in spring differed from those in other seasons. The community structure showed significant variations across seasons and years based on alpha diversity and PCoA coordinates (Figs 3 and 4E–H). We also found substantial variation in the community among sampling years, similar to that by Deyett and Rolshausen (2019).

Investigation of potential variations among the different shoot parts of the grapevine has been addressed in several studies (Wei *et al*., 2018; Deyett and Rolshausen, 2020; Fan *et al*., 2020); however, more information is needed on the different phenological stages of generative parts and leaves. Here, we found significant differences in community diversity. As discussed above, the young leaves, mainly in spring, were inhabited by fewer fungi. The community structure of the mycobiome of both the leaves and grape clusters were more similar (Fig. 4C and D).

Our geographical sampling setup that included four nearby vineyards in Mád and two reference sites indicated the presence of a core fungal microbiome and similar community structures (Fig. 4A and B, Supporting Information Fig. S2A). Therefore, the terroir effect is not likely driven by the mycobiome of the above-ground plant tissues. Similarly, Martinez-Diz *et al*. (2020) found no significant differences between the diversity of fungal communities of grapevine wood samples in different sampling plots. Liu and Howell (2021) also reported that geographic location only slightly affected the microbial diversity and composition, except for fungal diversity associated with roots; however, regional and small-scale variation in the below-ground fungal microbiome is relatively common (Zarraonaindia *et al*., 2015; Knight *et al*., 2019; Deyett and Rolshausen, 2020). Based on our study and that by others, we may assume that small-scale geographical differences are usually not evident in the mycobiome of the shoot; nevertheless, the fungal microbiome of the must from the same cultivars (including the white variety, Chardonnay) can be significantly different among vineyards (Bokulich *et al*., 2014, 2016).

Visualization

In the present study, we carried out fungal visualization within plant tissues using fluorescence microscopy. Although endophytic bacterial communities of the grapevine are often visualized (e.g. Compant *et al*., 2010, 2011; Pacifico *et al*., 2019), we are not aware of studies focusing on the fungal microbiome of grapevine using microscopy to prove the presence and structure of endophytic fungi. Here, we used a relatively simple technique of fluorescent staining of the cell wall of fungi for *in planta* visualization, which is generally used for visualization of fungal endophytes (Andrade-Linares *et al*., 2011; Knapp *et al*., 2019). Our microscopic observations suggest that the colonization of plant tissues is weaker in spring and young organs becoming relatively more severe towards the end of the growing season. These findings were in putative correlation with the results of the isolations, especially with the low read-numbered samples from spring and young leaves in the HTS dataset. The sole presence of septate hyphae within the plant is also consistent with Ascomycota and Basidiomycota dominated communities of the plants found using further approaches.

We were able to visualize the most common members of the mycobiome community with characteristic structural features. *Aureobasidium pullulans* has three distinctive forms: long-branched-septate filaments, large chlamydospores and smaller yeast-like cells (Chi *et al*., 2009). *Alternaria* species commonly produce typical septate conidia (Simmons, 2007). The confirmed presence and localisation of specific endophytic taxa within different plant organs using *in planta* fluorescence *in situ* hybridisation (FISH) may enable us to answer important functional questions (Compant *et al*., 2011), which could also be used for fungi. Using WGA to assess general colonization and implementation of *in planta* RNA FISH for visualization of living fungi, which may be used simultaneously for more taxa (Vági *et al*., 2014), could assist us in the provision of important information on the fungal microbiome of the grapevine.

Conclusion

Here, we investigated the above-ground fungal microbiome of the white grapevine variety Furmint in different seasons, phenological stages, plant tissues and vineyards within a fine geographical scale. We also present the first report on the mycobiome of *V. vinifera* in the Tokaj wine region, Hungary. We found no major differences among communities in distinct neighbouring vineyards, even compared to that of the reference sites in Eger. Our findings indicate that the shoot of the Furmint grapevines has a similar suit of fungal community, and the terroir effect is not likely driven by the fungal microbiome of the plant tissues. For potential biocontrol agents and further applied utilisations, our future focus should be on *Aureobasidium pullulans*, *Cladosporium* spp. and the complex *Alternaria* aff. *alternata* dominating the community at every site, season and plant organ. The isolation technique used was found to be important in uncovering different *Alternaria* species, which may have different functions and roles in the plants, and we have revealed that visualization through microscopy can also be a useful tool in studying the grapevine mycobiome.

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Supporting Information

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Appendix 1. Supporting Information Experimental Procedures

Fig. S1. The four sampling sites at the vineyards: Betsek (BET), Király (KIR), Szent Tamás (STT) and Úrágya (URA) in Mád, Hungary.

Fig. S2. Effects of (A) sampling site, (B) plant part, (C) year and (D) season on community composition (extracted 2nd PCoA coordinates of the samples) Sites: Betsek (BET), Király (KIR), Szent Tamás (STT), Úrágya (URA) vineyards in Mád, and Hangács (HAN), Nagy-Eged (NEG) vineyards in Eger; plant parts: grape berry (Grape), small juvenile leaves (Young leaf), mature leaves (Old leaf), and woody parts of the grape cluster (Rachis). Values indicate the estimated marginal means ±95% confidence intervals calculated from the general linear models.

Fig. S3. Phylogenetic tree of isolates collected from *Vitis vinifera cf*. *Furmint*. The 50% majority rule consensus phylogram was inferred from the Bayesian analysis of the ITS sequences. Bayesian posterior probabilities (≥ 90) are shown as percentages of branches. VVFIS_340 served as the outgroup. The scale bar indicates 0.5 expected changes per site per branch.

Fig. S4. Maximum Likelihood (RAxML) phylogenetic tree of ITS sequences of representative isolates in each clades and reference sequences from GenBank. ML bootstrap support values ≥70% are shown above branches. VVFIS_340 served as the outgroup. The scale bar indicates 0.1 expected changes per site per branch.

Fig. S5. Maximum likelihood (RAxML) phylogenetic tree of representative strains of *Alternaria* Sect. *Alternaria* and RPB2 sequences of 24 *Alternaria* aff. *Alternata* isolates from the clade 1 collected from *Vitis vinifera cf*. *Furmint*. Isolates collected from grapevine are shown in bold, GenBank accession number are indicated before the isolate names. The lineages are named and labelled sensu Woudenberg *et al*. (2015). ML bootstrap support values (≥ 70) are shown at the branches. *Alternaria alternantherae* CBS 124392 served as the outgroup. The scale bar indicates 0.01 expected changes per site per branch.

Table S1. Steps of the bioinformatics pipeline with indication of number of sequences and OTUs and after each step

Table S2. The OTU table containing subsampled reads per sample used for analyses

Table S3. The BLAST table showing identity of the most abundant sequences of each OTU

Table S4. Diversity indices and rarefaction curves of the samples

Table S5. Details of isolates collected from different tissues of Furmint grapevine

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