Endophytic fungal community in grape is correlated to foliar age and domestication

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Abstract

Purpose: The composition of endophytic communities has been shown to depend on grape genotypes and viticultural managements in leaves, stems, and berries of grape, but there have been relatively few reports exploring fungal endophytes associated with wild grape and foliar age.

Methods: The regions of internally transcribed spacer (ITS) were sequenced using the Illumina HiSeq to determine the diversity of fungal endophytes associated with European grape (Vitis vinifera cv. Red Globe) and Chinese wild grape (Vitis amurensis cv. Shuangyou) in young and mature leaves.

Results: A total of 3 phyla, 23 classes, 51 orders, 97 families, and 150 fungal genera were identified. Young leaves have significantly higher diversity and richness than that in mature leaves in both cultivars. Endophytic fungal diversity was greater in wild grapevines (119 genera) than in cultivated grapevines (81 genera) in both young and mature leaves. Endophytic fungal community structure was also significantly different between young leaves and mature leaves as well as in both cultivars based on statistical tests of ANOSIM and MRPP.

Conclusions: Our results suggest that endophytic fungal communities were strongly affected by foliar age and domestication, which are crucial factors in establishing symbiotic associations with a selective enrichment for specific endophytes.

Keywords: Wild grapevine, Endophytic fungi, Community, Foliar age

Introduction

Endophytes are organisms living within plant tissues and often provide beneficial effects to their hosts, without causing any disease symptoms. Endophytic colonization may protect plants from pathogen attack (Kuldau and Bacon 2008) and environmental stresses (Baltruschat et al. 2008; Sherameti et al. 2008), fix nitrogen (Dixon and Hartmann 2017), and increase host growth (Bae et al. 2009; Varma et al. 1999). However, the establishment of an endophytic fungal species largely depends on host genotype (Horton et al. 2014), sampling site (González and Tello 2011), the developmental stage of host (Oono et al. 2015), the leaf age (Arnold et al. 2003), and the environmental contexts (Pancher et al. 2012) such as seasons (Sadeghi et al. 2019), average temperature, annual rainfall, and latitude (Arnold and Lutzoni 2007).

The endophytic diversity is associated with host genotype. However, most the studies that investigated the diversity of endophytic fungal communities in grapevines mainly focused on Vitis vinifera cultivars (Casieri et al. 2009; González and Tello 2011; Martini et al. 2009; Pancher et al. 2012), whereas comparisons of endophytic communities between cultivars and Chinese wild grape are scant. Chinese wild grape germplasms have been identified as highly resistant to multiple pathogenic fungi (Wan et al. 2015; Wang et al. 1995; Yu et al. 2013). The elucidation of endophytic fungal species diversity between cultivars and wild grape genotypes of grapevine will help us understand the domestication and host specificity, as well as distribution, frequency, and beneficial effects of endophytes.
Endophytic fungal communities are also likely to be correlated with plant age including seedlings (Oono et al. 2015) and leaves (Sanchez-Azofeifa et al. 2012) at different stages of development. Comparisons of endophyte communities across leaf age are helpful for an in depth understanding of the interaction between endophytic fungi and leaf characteristics, including leaf physiology and leaf development. It was reported in previous studies that endophytic fungal species richness and diversity could vary according to the age of the leaves from an increase to no difference or an initial increase and then decrease in species richness (Arnold et al. 2003; Espinosa García and Langenheim 1990; Fröhlich et al. 2000; López-González et al. 2017; Sanchez-Azofeifa et al. 2012). Therefore, studying the effects of different foliar ages on fungal endophyte communities will provide clues about the recruitment and accumulation of species along foliar development under natural conditions in grapes.

Little information is known about how endophytic fungal community is affected by cultivated grape and wild grape. This is why we have decided to study foliar endophytes. A comprehensive study of foliar fungal endophytes associated with Chinese wild grape (Vitis amurensis) and European grape (V. vinifera) in young and mature leaves under their natural environment is lacking. Therefore, we determined the relative abundance and diversity of foliar endophytes of Chinese and European grapes and evaluated the effect of leaf age on the colonization frequency, species richness, and diversity of the endophytic community. Our study provides an important baseline to further expand the ecological understanding of foliar fungal endophytes in grape.

Materials and methods

Plant species and study site

This study utilized 3-year-old grape plants grown in the germplasm nursery of Northwest A&F University, Shaanxi, China (latitude = 34.16, longitude = 108.07, annual precipitation = 635.1–663.9 mm, annual average temperature = 12.9 °C). The soil in the nursery contains 1.02 g/kg total nitrogen, 1.03 g/kg total phosphorus, and 40.22 g/kg total potassium, with an average pH of 8.5. Grape plants were asexually propagated and spaced 2–4 m. All grape plants were periodically sprayed with fungicide and insecticide products such as lime sulfur, Bordeaux mixture, deltamethrin, and dimethoate. Two genotypes, V. vinifera cv. Red Globe and V. amurensis cv. Shuangyou, were used in this study.

Sampling

Asymptomatic leaves from two cultivars were collected respectively from the second or third node and the seventh or eighth node from the shoot apex respectively for young leaves and mature leaves (Fig. 1). A total of 20 complete leaves were randomly chosen and pooled from five grape plants for each cultivar at different foliar ages; each assay was repeated five times in the summer of 2017 to provide biological replicates. All leaves were washed thrice with sterile water to eliminate ephiphytes, immersed in 3% sodium hypochlorite for surface sterilization, and then washed again thrice with sterile water. To check the efficacy of this method of surface sterilization, random surface sterilized leaves were incubated on potato dextrose agar (PDA) plates to check for the absence of colony growth from the outside (Dissanayake et al. 2018).

DNA extraction and sequencing

Each sample was frozen with liquid nitrogen and extracted with DNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. These DNA samples were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The primer used for fungal ITS amplification were ITS1-F (CTTGGTCATTGAAGGATTA) and ITS2 (GCTCGGTTCTCTAGCAGTGC) (White et al. 1990). PCR protocols were performed according to a previously described method (Pancher et al. 2012). PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and then quantified with Quant-iT PicoGreen Kit (Invitrogen, Grand Island, NY, USA). The amplicons were sequenced on an Illumina HiSeq (Illumina Inc., San Diego, CA, USA).

Data processing

Raw sequence reads were processed using FLASH v1.2.7 (Magoč and Salzberg 2011) and filtered with QIIME 1.8.0 (Caporaso et al. 2010). Sequencing adapters were removed from raw reads of each sample, and the chimeric sequences were deleted from the remaining sequences with USEARCH 6.1 algorithm (Edgar et al. 2011). Reads were checked by average quality score (20 and 30) to identify regions of low sequence quality and then clustered into operational taxonomic units (OTUs) using Uparse v7.0.1001 with a threshold of 97% identity (Edgar 2013). Each representative sequence of each OTU sequence was blasted with National Center for Biotechnology Information (NCBI) and UNITE database (Abarenkov et al. 2010). Alpha and beta diversity values were calculated using relative abundance as the number of sequences representing each OTU. The Shannon diversity index and the non-parametric Chao1 index were performed with the QIME (Caporaso et al. 2010) to quantify endophytic fungal diversity and richness, respectively. Comparison between cultivars and foliar ages was performed through a principal coordinate analysis (PCoA) calculated using weighted Unifrac distance.
(Chen et al. 2012). Two different statistical approaches, ANOSIM (analysis of similarity) and MRPP (multiple-response permutation procedure), were used to test the community structure of endophytic fungus among *V. vinifera* and *V. amurensis* of young and mature leaves. Significant differences were assessed by testing for the least significant difference (*P* < 0.05) and noted in the text or figure captions.

**Results**

**General characterization of endophytic fungal community**

After quality filtering, a total of 727,074 reads of the original 775,440 reads were obtained with an average length of 238.7 bp (Table S1). As shown in the rarefaction curves, all of the samples reached saturation with at least 4000 sequences, suggesting that the majority of the operational taxonomic units (OTUs) were recovered in this study (Fig. S1). In total, 198 OTUs were assessed for taxonomic affiliation. Ascomycota was the dominant phylum and accounted for 62.36% of all OTUs, followed by Basidiomycota (21.57%) and Zygomycota (2.10%) (Table 1). Ascomycota has several taxonomic orders, such as Eurotiales (12.89% OTUs), Capnodiales (8.15% OTUs), Hypocreales (7.89% OTUs), and Pleosporales (7.89% OTUs), which represented the majority of OTUs; Agaricales (3.15% OTUs), Malasseziales (3.15% OTUs), Sporidiobolales (3.15% OTUs), and Polyporales (2.63% OTUs) were the most abundant order in the Basidiomycota. A total of 150 endophytic fungal genera were identified as the closest hits of individual OTUs. The ten most abundant endophytic fungal genera were *Aspergillus, Penicillium, Rhodotorula, Candida, Fusarium*,

![Fig. 1 Foliar morphology of young (a, b) and mature leaves (c, d) from V. vinifera cv. Red Globe and V. amurensis cv. Shuangyou. Bars: a, b = 1 cm, c, d = 2 cm](image)

| Phylum          | Order        | % OTU |
|-----------------|--------------|-------|
| Ascomycota      | Eurotiales   | 12.89 |
|                 | Capnodiales  | 8.15  |
|                 | Hypocreales  | 7.89  |
|                 | Pleosporales | 7.89  |
|                 | Sordariales  | 2.63  |
|                 | Saccharomycetales | 2.10 |
|                 | Incertae sedis | 4.47 |
|                 | Unidentified | 4.47  |
|                 | Other Ascomycota | 11.84 |
| Basidiomycota   | Agaricales   | 3.15  |
|                 | Malasseziales| 3.15  |
|                 | Sporidiobolales | 2.63 |
|                 | Polyporales  | 2.63  |
|                 | Filobasidiales | 1.84 |
|                 | Russulales   | 1.84  |
|                 | Other Basidiomycota | 6.31 |
| Zygomycota      | Mortierellales | 1.31 |
|                 | Mucorales    | 0.78  |
|                 | Unidentified | 13.94 |

*Includes orders Xylariales, Microascales, Pezizales, Botryosphaeriales, Lecanorales, Chaetosphaeriales of the phylum Ascomycota*

*Includes orders Erythrobasidiales, Cantharellales, Auriculariales, Cystofilobasidiales, Agaricostilbales of the phylum Basidiomycota*
Pyrenochaetopsis, Gibellulopsis, Amorphotheca, Cladosporium, and Simplicillium (Fig. 2).

Differences in endophytic fungal communities between foliar ages
Endophytic fungal diversity differed significantly between foliar ages ($P < 0.05$), and endophytic fungal diversity in young leaves was higher than mature leaves in V. vinifera cv. Red Globe and V. amurensis cv. Shuangyou based on Shannon index and Chao 1 index (Fig. 3). PCoA revealed that the compositions of all samples were clustered into three groups, suggesting partial overlap for young leaves and obvious difference for mature leaves between two grape hosts (Fig. S2). Two non-parametric multivariate statistical tests, ANOSIM and MRPP, showed that the endophytic fungal community structures of young and mature leaves were significantly different in both cultivars (Table 2). In all 150 endophytic fungal genera, 65 fungal genera were associated with young leaves and 30 fungal genera were associated with mature leaves in ‘Red Globe’ (RG), whereas 90 were associated with young leaves and 67 with mature leaves in ‘Shuangyou’ (SY) (Fig. S3). Aspergillus and Fusarium were the most dominant genera in young leaves of RG and SY, whereas Gibellulopsis and Mycosphaerellaceae reached the highest relative abundance values in mature leaves (Table S2).

Differences in endophytic fungal communities between cultivars
Community structure of endophytic fungi in SY was significantly different from RG. Similar results were also obtained in the mature leaves of both cultivars, whereas no significant difference was observed in young leaves (Table 2). In total, 150 foliar fungal endophytes at genus level were identified in both cultivars, in which 20% and 46% non-overlapped endophytic fungi of the total endophytic were detected in RG and SY, respectively (Fig. 4a; Table S3). The genera of Gibellulopsis, Aspergillus, Cladosporium, and Malassezia were significantly less abundant in SY than in the RG in 50 overlapped endophytic fungi (Fig. 4b). Hyphoderma, Oedocephalum, Lambertella, and Phialosimplex genus were the most abundant in RG, whereas Pyrenochaetopsis, Montagnulaceae, and Phoma genus were the most abundant in SY (Fig. 4c).

Discussion
The endophytic fungal community associated with grape leaves was mainly composed of fungi belonging to the phylum Ascomycota with a small proportion of the phyla Basidiomycota and Zygomycota. The dominance of endophytic Ascomycota in the leaves has been documented in many plant species, such as Arabidopsis thaliana (García et al. 2013), loblolly pine (Oono et al. 2015), lima bean (López-González et al. 2017), and grape (González and Tello 2011). Within Ascomycota, the three most detected classes were Dothideomycetes, followed by Eurotiomycetes and Sordariomycetes, which have shown to be the main components in other woody plants (Materatski et al. 2019). At genus level in grape, most fungi in this study are already known as grape endophytes and found in similar abundances in previous reports, such as high abundant genus of Aspergillus, Penicillium, Cladosporium, and Fusarium and low

![Fig. 2](image-url) Relative abundance of fungi identified as endophytes in grape samples at genus level. Stacked bar plots represent mean relative abundances of fungal endophytes within genera.
abundance genus of *Gibberella* and *Phoma* (González and Tello 2011; Pancher et al. 2012).

The number of species of fungal endophytes in wild grape *V. amurensis* cv. Shuangyou was higher than in *V. vinifera* cv. Red Globe both in young and mature leaves (Fig. S3). Kernaghan et al. (2017)'s study found similar patterns as ours, suggesting that foliar fungal endophytes from wild populations of *Vitis riparia* have higher richness and diversity than hybrid Vitis (Kernaghan et al. 2017). In addition, the diversity of endophytic bacteria was also greater in wild than in domesticated grapevines (Campisano et al. 2015). Compared to cultivated grapes, this higher endophyte diversity in wild grapes may be due to different living environment, fungicide treatments, defense traits, and higher general biodiversity in a wild environment (Campisano et al. 2015). Fungicide treatments can possibly result in changes in fungal species composition and decrease endophyte colonization (Nettles et al. 2016).

Our results clearly show that the richness and diversity of the endophytic fungal community in young leaves were significantly higher than that in mature leaves for both cultivars. Similar patterns were found in other woody plants (Oono et al. 2015); this finding suggests that the diversity of the endophytic fungal community decreased with plant age (Espinosa Garcia and Langenheim 1990). A similar behavior has also been found in mycorrhizal communities (Husband et al. 2002). Conversely, a recent study showed that endophytic fungal community tends to expand with foliar age in lima bean (López-González et al. 2017). This discrepancy can be due to the differential specificity of woody plants and herbaceous plants to endophytic species. The changes of endophytic fungal diversity may be concerned with foliar physiology. This phenomenon has been documented; for example, carbohydrates, phosphorus, and phenolic compound utilization are related to microbial community (Lin et al. 2014; Kolton et al. 2017). Broeckling (2008)'s study found that root exudates of *Arabidopsis thaliana* and *Medicago truncatula* influenced the fungal community, suggesting fungal diversity is associated with physiological metabolism of plants. In addition, young leaves may have poor structural barriers in

Table 2  Community structure of endophytic fungus among *V. vinifera* (Red Globe) and *V. amurensis* (Shuangyou) at young and mature stages tested by ANOSIM and MRPP

| ANOSIM* | Samples characteristics | R value | P value |
|---------|-------------------------|---------|---------|
| RG YL - RG ML | 0.73 | 0.013 |
| SY YL - SY ML | 0.44 | 0.022 |
| RG YL - SY YL | 0.11 | 0.168 (ns) |
| RG ML - SY ML | 0.83 | 0.007 |
| RG - SY | 0.28 | 0.007 |

| MRPPb | Samples characteristics | A value | Observed delta | Expected delta | P value |
|-------|-------------------------|---------|---------------|---------------|---------|
| RG YL - RG ML | 0.09 | 0.70 | 0.78 | 0.007 |
| SY YL - SY ML | 0.04 | 0.73 | 0.76 | 0.037 |
| RG YL - SY YL | 0.008 | 0.78 | 0.79 | 0.186 (ns) |
| RG ML - SY ML | 0.11 | 0.66 | 0.75 | 0.009 |
| RG - SY | 0.03 | 0.77 | 0.80 | 0.005 |

Significance with P < 0.05 is indicated in bold (ns, not significant)

*Analysis of similarity (ANOSIM) indicates the degree of difference between groups, where R value closer to 1 indicates that no members are shared, and R value closer to 0 indicates that communities are identical.

Multiple response permutation procedure (MRPP) returns a delta value representative of the observed versus permuted within-group distances.
structural characteristics for most fungal endophytic species and become vulnerable to a diverse group of fungal species as compared with mature leaves.

Endophytic fungi from Chinese wild grape are considered as potential candidate biocontrol agents. For instance, we found some strains of *Alternaria* that are known to be effective against *Plasmopara viticola* in grape (Musetti et al. 2006) and strains of *Phoma* that act as biocontrol agents of weeds (Zhou et al. 2004). Several studies showed that *M. verrucaria* functions as a bioherbicide against bacteria (Zou et al. 2011) and a nematocide against root-knot nematode (Fernández et al. 2001). Interestingly, four out of seven fungal endophytes isolated from young leaves inhibited the growth of the pathogen *Colletotrichum lindemuthianum* in lima bean (López-González et al. 2017). Additionally, Pujade-Renaud et al. (2019)’s study reported that endophytes from wild rubber trees can act as antagonists of the pathogen *Corynespora cassicola*, suggesting that some endophytes from wild plants are promising candidates as biocontrol agents.

Most fungi isolated as endophytes may be latent pathogens within the host tissue. It seemed to reflect the life history strategies of fungal species from mutualistic to parasitic (Delaye et al. 2013). Although endophytes have shown that they can protect their host against abiotic and biotic stress, stress condition, excessive humidity, and poor nutrition can clearly shift the interaction relationship from endophytes to pathogenic fungi (Photita et al. 2004; Romero et al. 2001). Additionally, although the grape leaves look healthy, they have been colonized by pathogens that may cause disease until suitable conditions. In this study, we identified two important genera (*Botryosphaeria* and *Erysiphe*) from young leaves of *V. amurensis* cv. Shuangyou. Among these, *Botryosphaeria dothidea* is the causal agent of grapevine canker (Phillips 1998) and *Erysiphe necator* cause grape powdery mildew (Gadoury et al. 2012). Further research is needed to clarify and minimize negative impact of endophytes on plants as biocontrol agents.

Conclusion
Our results showed that endophytic fungal community is quite different in domesticated and wild grape as well as in young and old leaves of the same shoot. Some of these endophytes are already known as biocontrol agents, possibly explaining the enhanced resistance to fungal pathogens of *V. amurensis*. Future investigation of the richness and diversity of endophytes from different sites and more wild grapes will likely provide a deeper understanding of the endophytic colonization, distribution, and their effective role in plant fitness.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13213-020-01574-9.

Additional file 1: Figure S1. Rarefaction curve of observed OTUs from young and mature leaves of *V. vinifera* cv. Red Globe and *V. amurensis* cv. Shuangyou. Figure S2. Principal coordinate analysis (PCoA) plot based
on the weighted UniFrac distance at different cultivars and foliar ages. Points that are closer together on the ordination have communities that are more similar. Red: RG young leaves; green: RG mature leaves; blue: SY young leaves; purple: SY mature leaves. RG: V. vinifera cv. Red globe; SY: V. amurensis cv. Shuangyou. **Figure S3.** Numbers of endophytic fungal genera in young leaves (LY) and mature leaves (ML) of RG and SY. **Table S1.** Sequencing data of all samples in this study. **Table S2.** Relative abundances of endophytic fungus at genus level. Values represent relative abundances for each family and sample type. RG: V. vinifera cv. Red globe; SY: V. amurensis cv. Shuangyou; YL: young leaves; ML: mature leaves. **Table S3.** Host-specificity of endophytes of endophytic fungal genera in young leaves (LY) and mature leaves (ML) of V. vinifera cv. Red globe and V. amurensis cv. Shuangyou.

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**Authors’ contribution**

YF and LG carried out all experiments and wrote this manuscript. PC performed the experiment. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

This article does not contain any studies with human participants or animals performed by any of the authors.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no conflict of interest.

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