Fungal Diversity (2018) 90:85–107
https://doi.org/10.1007/s13225-018-0399-3

Direct comparison of culture-dependent and culture-independent molecular approaches reveal the diversity of fungal endophytic communities in stems of grapevine (*Vitis vinifera*)

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Received: 24 October 2017 / Accepted: 9 March 2018 / Published online: 13 April 2018

Abstract

Grapevines (*Vitis vinifera*) are colonized by ubiquitous microorganisms known as endophytes, which may have advantageous or neutral effects without causing disease symptoms. Certain endophytes are uncultivable, so culture-independent approaches such as next generation sequencing (NGS) can help for a better understanding of their ecology and distribution. To date, there are no studies which directly link NGS results with taxa derived from a culturing approach, integrating morphological and multi-gene phylogenetic analysis of endophytes. In this study, a culture-dependent and high-resolution culture-independent approach (next generation sequencing) were used to identify endophytes in grapevine stems. In the culture-dependent approach, a total of 94 isolates were recovered from 84 of 144 healthy grapevine stem fragments (colonization rate = 58.3%). The study is unique as we used subsets of combined multi-gene regions to identify the endophytes to species level. Based on each multi-gene phylogenetic analysis, 28 species belong to 19 genera (*Acremonium*, *Alternaria*, *Arthrinium*, *Ascorhizoctonia*, *Aspergillus*, *Aureobasidium*, *Bipolaris*, *Botryosphaeria*, *Botrytis*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Hypoxylon*, *Lasiodiplodia*, *Mycosphaerella*, *Nigrospora*, *Penicillium*, *Phoma*, *Scopulariopsis*) were identified. A higher number of culturable fungi were obtained from 13 year-old vines, followed by eight and three year-old vines. In the culture-independent approach, a fungal richness of 59 operational taxonomic units (OTU) was detected, being highest in 13 year-old grapevines, followed by eight and three years. Even though the cultivation approach detected lower fungal richness, the results related to stem are consistent for fungal community composition and richness. Comparison of the fungal taxa identified by the two approaches resulted in an overlap of 53% of the fungal genera. Due to interspecific variability of the sequences from NGS, in many cases the OTUs (even with the highly abundant ones) were only assignable to order, family or genus level. Incorporating multi-gene phylogenies we successfully identified many of the NGS derived OTUs with poor taxonomic information in reference databases to the genus or species levels. Hence, this study signifies the importance of applying both culture-dependent and culture-independent approaches to study the fungal endophytic community composition in *Vitis vinifera*. This principle could also be applied to other host species and ecosystem level studies.

Keywords Molecular data · Morphological characteristics · Multi-gene phylogeny · Mycobiome · Next generation sequencing

Introduction

Endophytes are microorganisms that reside asymptomatically within interior tissues of living plants for all or part of their life cycle without damaging the host plant (Navarro-Meléndez and Heil 2014; Zhou et al. 2015). Fungal endophytes have been found in all plant species studied in the plant kingdom (Hyde and Soytong 2008; Sánchez et al. 2010; Tejesvi et al. 2010; García et al. 2013; Bonfim et al. 2016; Busby et al. 2016), and have been associated with

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13225-018-0399-3) contains supplementary material, which is available to authorized users.

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lichens (Li et al. 2007; Chagnon et al. 2016; Muggia et al. 2017) and sea grasses (Supaphon et al. 2017). The biodiversity of endophytes in a plant can be significant; in certain species, more than 100 endophytic taxa have been discovered (Tan and Zou 2001; Stone et al. 2004). Over the last three decades, endophytic fungi have fascinated taxonomists, mycologists, ecologists (Promputtha et al. 2007, 2010; Purahong and Hyde 2011), chemists and evolutionary biologists (Garoé et al. 2012).

Vitis vinifera L. is an extensively grown, highly important crop and naturally hosts a reservoir of microorganisms. Therefore, a complete survey of the grapevine endophytes under natural conditions is of upmost importance, as grape production and quality can be affected by the vineyard's active microbial community (Pinto et al. 2014; Busby et al. 2016). Recent studies have shed some light upon the bacterial endophytic communities in grapevines (Bulgari et al. 2011; Compant et al. 2011; Andreolli et al. 2016; Lòpez-Fernàndez et al. 2017), while investigations on fungal endophytic communities have been rare and often limited to culture-dependent approach (Compant et al. 2011; González and Tello 2011; Campisano 2012; Morgan et al. 2017). However, knowledge of the diversity, distribution or influence of endophytic fungi in the development or prevention of certain fungal diseases is still incomplete and the majority of studies have involved European grapevines (Bruez et al. 2016; Rondot and Reineke 2016; Varanda et al. 2016).

Although fungal endophyte research has received considerable attention, their ecology and community composition are poorly characterized, due to methodological limitations. Endophytic fungi have traditionally been studied and described based on culture-dependent approach and characterization of morphological characters in culture (Hyde and Soytong 2008; González and Tello 2011; Busby et al. 2016). This approach is still used worldwide (Ghimire et al. 2011; González and Tello 2011; Ko et al. 2011; Rocha et al. 2011; Heinonsalo et al. 2016; Bhattacharyya et al. 2017; Mahmoud et al. 2017; Morgan et al. 2017), but the results must be considered with some caution, since it is a selective method and subject to factors including surface sterilization techniques, culturing media, incubation conditions, and ability of some fungi to sporulate in culture (Clay et al. 2016; Steinrucken et al. 2016). Many factors such as sampling site, tissue specificity, plant age, physiology or associated vegetation can influence the composition of endophytic communities (Martín-García et al. 2011; Vivas et al. 2015; Donayre et al. 2014; Wicaksono et al. 2015; Christian et al. 2016; Yadav et al. 2016; Dastogeer et al. 2017). The fungal endophyte colonization frequency vary with the age of the host (Arnold and Lutzoni 2007; Goveas et al. 2011; Park et al. 2012; Gupta and Chaturvedi 2017). Nascimento et al. (2015) reported that the rates of endophyte colonization varies with the plant age/development. Several other studies also found that endophyte colonization varied with the plant age (Osono and Mori 2005; Olejniczak and Lembicz 2007; Gupta and Chaturvedi 2017; Fuchs et al. 2017; Liu et al. 2017b).

Endophyte research could benefit from advances in molecular techniques that infer the genetic structure of cultures and the taxonomic composition of endophyte communities. In this sense, multi-locus genetic analyses of isolates are much-needed to obtain consistent information from evolutionary and ecologically determinant loci (Cai et al. 2009; Hyde et al. 2014, 2016; Ariyawansa et al. 2015; Liu et al. 2015). Even though a culture-dependent approach may contain biases, it provides reliable morphological and molecular taxonomic information of fungal endophytes (Ko et al. 2011).

Recently, meta-barcoding approaches have become important tools for assessment of the mycobiomes (Setati et al. 2015; Deagle et al. 2017; Lobo et al. 2017). The use of whole plant tissues for DNA extraction and molecular analysis of the fungal barcode is an alternative tool for the study of endophytic fungi (Duong et al. 2006; McKinnon 2016; Tejesvi et al. 2016; Liu et al. 2017a; Ruiz-Pérez and Zambrano 2017). These culture-independent approaches have been used to investigate the genetic diversity and population structure of endophytes, especially for those taxa that do not grow on standard media (Bullington and Larkin 2015; Ting et al. 2015; David et al. 2017; Purahong et al. 2018). Lücking and Moncada (2017) suggested that bulk of new fungal taxa is revealed through environmental high throughput sequencing with an astounding extent of information. However, these techniques have serious limitations in identifying the majority of unknown taxa into species level and obtaining correct names, since many sequences deposited in GenBank are associated with erroneous taxon names and many species groups cannot be discriminated by using ITS or other portions of the rDNA, in particular in the Ascomycota. Another fact is that many fungi have not been sequenced (Cai et al. 2009; Crouch et al. 2009; Nilsson et al. 2012, 2015).

NGS are mainly based on ITS regions, the fungal DNA barcode (Schoch et al. 2012), but using short fragments such as the ITS2 fragment. Such short sequences or even the whole sequences of ITS do not give the reliable sequence alignments to derive a phylogenetic tree at the species level. Furthermore due to their high inter- and intra-specific variability the taxonomic assignments at the generally agreed threshold of 97% similarity are not consistent for species level identification (Nilsson et al. 2008). Thus, the fungal taxonomic results derived from NGS are probably reasonable only to the genus level. Thus, they are usually reported at the genus level or even higher taxonomic levels such as family or order (Ovaskainen et al.

2010; Purahong et al. 2017a). To date there are no studies that directly link the NGS based fungal OTUs to the culture-dependent morphologically and molecularly (incorporating phylogenetic analysis using multiple genes) identified fungal species that are derived from the same sample.

The present study focused on (i) the comparison of a culture-dependent approach (culturing applying identification using morphological and phylogenetic analysis of multiple genes based identification), versus a culture-independent approach (meta-barcoding of the fungal ITS rDNA barcode) for characterization of diversity and community composition of fungal endophytes associated with stems of grapevines (*Vitis vinifera* cv. Summerblack) with different ages (3, 8 and 13 years old), (ii) assessing the shared community between two approaches detected from the same grapevine stem and (iii) revealing the potential functions of the endophytic fungal communities inhabiting the grapevine stem. For a better comparison, the same plant organ (stems of grapevines) located in the same vineyard was investigated. We hypothesized that (i) diverse fungal endophytes inhabit stems of grapevines and that the different approaches will reveal different fungal communities implying a higher diversity from the culture-independent approach as compared to culture-dependent approach, (ii) fungal endophytic communities are influenced by the age of grapevine plants and (iii) frequent taxa should be detected with both culture-dependent and culture-independent approaches.

Material and methods

Site description and sampling strategy

Samples were collected during summer of 2015 from a vineyard in Beijing, which comprised three age levels of grapevines (3, 8 and 13 years old *Vitis vinifera* cv. Midnight beauty). Growers spray a fungicide with pyraclostrobin and lime sulphur for four-to-five times a year. The cultivation style is 'rain-shelter cultivation'. This region has a temperate and continental monsoon climate, with a mean annual temperature of 26 °C. Mean annual precipitation ranges from 550 to 960 mm of which more than 45% usually falls in August (China Agriculture Yearbook 2014). Asymptomatic grape samples from four stems (or trunks; two inner and two outer parts) were collected from one grapevine. Three healthy grapevines (without any disease symptom) for each age level (3, 8 and 13 years) were selected as replicates and processed within 24 h for fungal endophyte isolation. The remaining samples from these three grapevines per age were subsampled, pooled and homogenized for culture-independent mycobiome analysis through paired-end Illumina sequencing.

Culture-dependent approach

Isolation and identification of endophytic fungi

Following pilot tests, the optimum conditions for surface sterilization were established (Kaewkla and Franco 2016). Samples were cut into 0.5×0.5 cm^2 sections. Under sterile conditions, tissue segments were surface disinfected in 70% ethanol for 1 min, 1.5% sodium hypochlorite solution for 1 min and three times in sterile-distilled water. To test the efficacy of this method, random surface-disinfected samples were repeatedly imprinted on PDA petri dishes, followed by incubation for two weeks at 20 °C to confirm the absence of epiphytes. After disinfection, samples were placed on PDA with the vascular vessels facing the medium. The plates were incubated for 7–15 days at 20 °C, and all morphologically different colonies were isolated. Fungal isolates were selected and grouped together as morphotypes (Lacap et al. 2003), according to the morphological characters such as the spore production, spore length and morphology, aerial mycelium colour, texture and form, exudates and growth rate.

DNA isolation and PCR

Fungal material for DNA extraction was harvested from 1 to 2 weeks-old cultures grown on potato dextrose agar (PDA) by scraping the mycelium. Specific gene regions were amplified with particular primers, i.e. ITS1 and ITS4 to amplify the internal transcribed spacers (ITS) (White et al. 1990), LROR and LR5 to amplify the large subunit rDNA (LSU) (Vilgalys and Hester 1990), NS1 and NS4 to amplify region of nuclear small subunit rDNA (SSU) (White et al. 1990), a fragment of translation elongation factor 1-α (TEF) was amplified using EF-728F and EF-986R (Carbone and Kohn 1999), GPD1 and GPD2 to amplify glyceraldehyde 3-phosphate dehydrogenase (GPDH) (Berbee et al. 1999), RPB2–5F and RPB2–7cR to amplify RNA polymerase second largest subunit (RPB2) (Sung et al. 2007), HSP60for+ and HSP60rev+ to amplify heat shock protein (HSP60) (Staats et al. 2005), BT2A and BT2B to amplify β- tubulin (TUB) (Glass and Donaldson 1995), ACT-512F and ACT783R to amplify partial actin gene (ACT) (Carbone and Kohn 1999). The amplification reactions were performed in 25 µl final volumes and consisted of TaKaRa Ex-Taq DNA polymerase 0.3 µl, 12.5 µl of 2× PCR buffer with 2.5 µl of dNTPs, 1 µl of each primer, 9.2 µl of double-distilled water and 100–500 ng of DNA template. PCR products were checked on 1% agarose electrophoresis gels stained with ethidium bromide. PCR

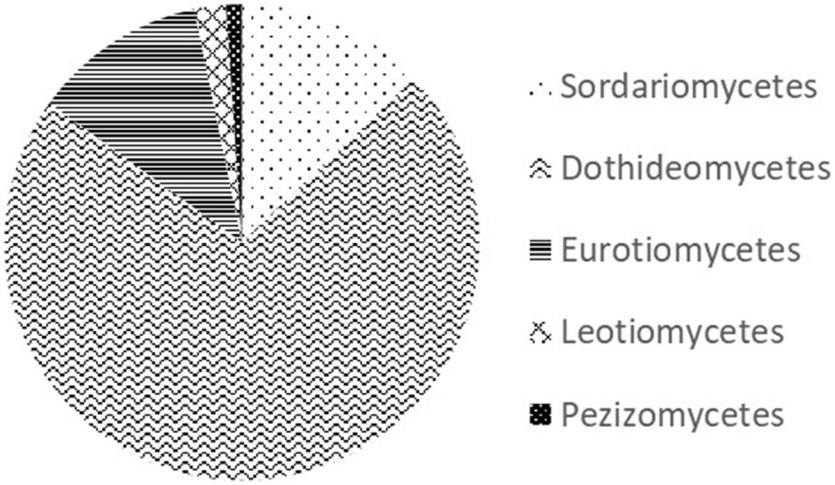

Fig. 1 Distribution of species of the culture-dependent method in class level

Table 1 The community composition of endophytic fungi in stems of *Vitis vinifera*, isolated from culture-dependent approach

Genus	Species	No. of isolates	Relative abundance (%)
Acremonium	*Acremonium alternatum*	2	2.1
Alternaria	*Alternaria alternata*	27	28.7
Arthrinium	*Arthrinium rasikravindrii*	2	2.1
Ascorhizoctonia	*Ascorhizoctonia* sp.	2	2.2
Aspergillus	*Aspergillus pseudoglaucus*	4	4.2
	Aspergillus pseudodeflectus	4	4.2
	Aspergillus japonicus	1	1.1
	Aspergillus niger	1	1.1
Aureobasidium	*Aureobasidium pullulans*	3	3.2
Bipolaris	*Bipolaris sorokiniana*	3	3.2
Botryosphaeria	*Botryosphaeria dothidea*	5	5.3
Botrytis	*Botrytis cinera*	2	2.1
Chaetomium	*Chaetomium globosum*	3	3.2
Cladosporium	*Cladosporium cladosporioides*	4	4.2
	Cladosporium ramotenellum	4	4.2
	Cladosporium silenes	1	1.1
	Cladosporium sphaerospermum	4	4.2
	Cladosporium tenellum	3	3.2
	Cladosporium tenuissimum	4	4.2
Curvularia	*Curvularia americana*	4	4.2
Hypoxylon	*Hypoxylon lateripigmentum*	1	1.1
Lasiodiplodia	*Lasiodiplodia theobrpmae*	1	1.1
Mycosphaerella	*Mycosphaerella graminicola*	1	1.1
Nigrospora	*Nigrospora oryzae*	1	1.1
	Nigrospora sphaerica	1	1.1
Penicillium	*Penicillium digitatum*	2	2.1
Phoma	*Phoma herbarum*	3	3.2
Scopulariopsis	*Scopulariopsis brevicaulis*	1	1.1

products were Sanger sequenced by Sunbiotech Company, Beijing, China.

Sequence alignment and phylogenetic analyses

A BLAST search with the ITS sequence data was used to reveal the closest matching taxa of endophytes. After they

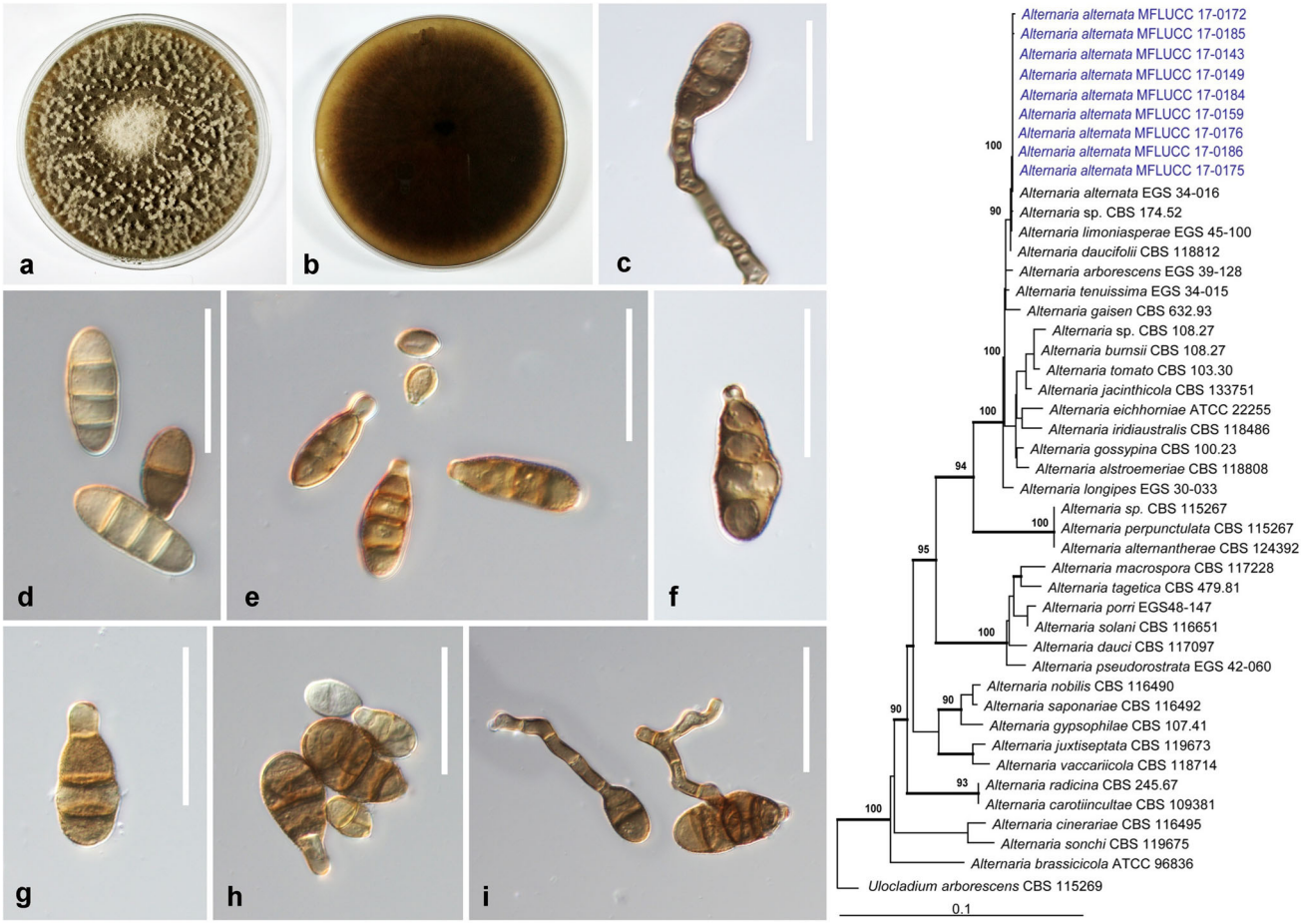

Fig. 2 *Alternaria alternata*. **a**, **b** Colonies on PDA (14 days old) from surface and reverse, **c** Conidia attached to conidiophore, **d**–**i** conidia. Scale bars: **c**–**i** = 20 µm. Phylogenetic tree inferred from maximum likelihood (ML) and Bayesian inference (BI) using combined ITS, LSU, GPDH, EF and RPB2 sequence data of the genus Alternaria. Only the topology generated from the ML analysis is shown. ML values greater than the 90% are indicated. Bayesian Posterior Probability greater than 0.90 are indicated with thick branch. Taxa isolated in this study are in blue

were identified in to genus level, other necessary gene regions were sequenced for particular genera. The sequences obtained in this study were aligned with sequences retrieved from GenBank using MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/) (Katoh and Toh 2010) and were manually optimized with BioEdit (Hall 2006). All available type sequences of each genus were included in a preliminary phylogenetic analysis and phylogenetically closely related species were selected for further analysis of the combined gene regions. Maximum parsimony analysis (MP) was performed using phylogenetic analysis using PAUP (v. 4.0b10) (Swofford 2003). Ambiguously aligned regions were excluded from all analyses and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Branches of zero length were collapsed, and all equally most parsimonious trees were saved. The trees were visualized with TreeView v. 1.6.6 (Page 1996).

For the Bayesian analyses, the models of evolution were estimated using MrModeltest v. 2.3 (Nylander 2004). Posterior probabilities (PP) were determined by Bayesian Markov Chain Monte Carlo (BMCMC) sampling in MrBayes 3.0b4 (Ronquist and Huelsenbeck 2003), using the estimated model of evolution. Six simultaneous Markov chains were run for 1,000,000 generations, and trees were sampled every 100th generation (resulting in 10,000 total trees). The first 2000 trees, which represented the burn-in phase of the analyses, were discarded and the remaining 8000 trees were used to calculate PP in the majority-rule consensus tree. The sequences used for phylogenetic analysis were deposited in GenBank and provided in the Supporting Information (Table S1).

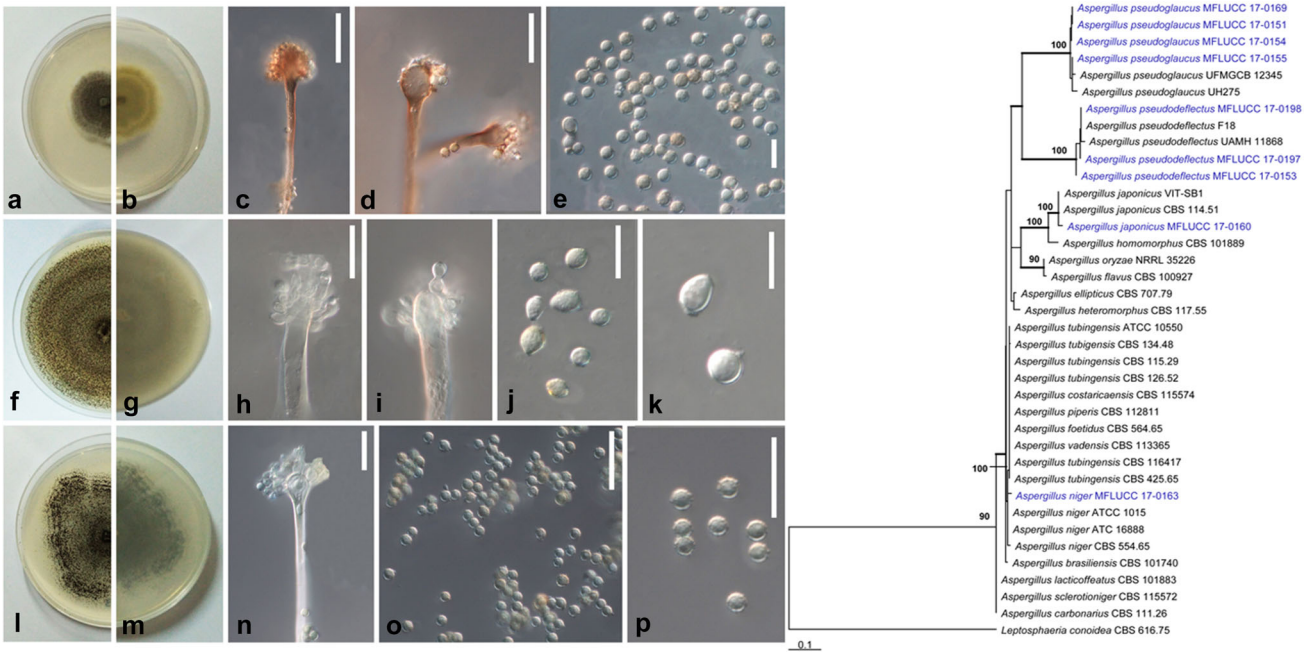

Fig. 3 *Aspergillus pseudoglaucus* (**a**–**e**), *Aspergillus japonicas* (**f**–**k**), *Aspergillus niger* (**l**–**p**). **a**, **b** Colonies on PDA (14 days old) from surface and reverse, **c**–**f** conidia. **g, h** Colonies on PDA (14 days old) from surface and reverse, **i**–**m** conidia. **n**, **o** Colonies on PDA (14 days old) from surface and reverse, **p**–**s** conidia. Scale bars: **c**–**f** = 20 μm, **i**–**m** = 20 μm, **p**–**s** = 20 μm. Phylogenetic tree inferred from maximum likelihood (ML) and Bayesian inference (BI) using ITS sequence data of the genus *Aspergillus*. Only the topology generated from the ML analysis is shown. ML values greater than the 90% are indicated. Bayesian Posterior Probability greater than 0.90 are indicated with thick branch. Taxa isolated in this study are in blue

Culture-independent approach

Endophytic mycobiome analysis: paired-end sequencing

Total genomic DNA from homogenized stem samples was extracted using the CTAB/SDS method. DNA concentration and purity was monitored on 1% agarose gels. Accordingly, the DNA was diluted to 1 ng/μL using sterile water and used as PCR template. Nuclear ribosomal internal transcribed spacer (ITS1) region was amplified using specific primers (ITS5-1737F and ITS2-2043R) (Huang et al. 2016) with sample specific barcodes. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Thirty-five cycles (95 °C for 45 s, 56 °C for 45 s, and 72 °C for 60 s) were performed with a final extension at 72 °C for 7 min. Samples with amplified products of 400–450 bp were chosen for further analysis. These PCR products were quantified using SYB green and all products were mixed in equimolar ratios. The PCR product mix was then purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated.

Endophytic mycobiome analysis: bioinformatic analysis

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. The unique barcode sequence for each sample is provided in the Supporting Information (Table S2). Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg 2011), and the splicing sequences were called raw tags as described in Bokulich et al. (2013). Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al. 2013) according to the QIIME (V1.7.0, http://qiime.org/index.html) (Caporaso et al. 2010) quality controlled process. The tags were compared with the reference database (Unite Database, https://unite.ut.ee/) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar et al. 2011) to detect chimera sequences, and then the chimera sequences were removed (Haas et al. 2011). Sequences analyses of the clean tags were performed using the Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) (Edgar

2013). Sequences with ≥ 97% similarity were assigned to the same OTUs. Representative sequences of each OTU were screened for further annotation. Taxonomic assignment of the representative sequences was done against the Unite Database (https://unite.ut.ee/) (Kõljalg et al. 2013) using the Blast algorithm. OTUs abundance was normalized to the sample with the least sequences (55, 822). Singletons were removed from the dataset. All subsequent analyses were performed based on this normalized dataset. Raw Illumina reads were submitted to the Sequence Read Archive (SRA) of National Center for Biotechnology Information (NCBI) under the BioProject number PRJNA433252.

Comparison of the NGS and culture based endophytes

Similarity of the endophyte community derived from the NGS analysis with that of the culture-based approach was done using the CD-HIT-EST-2D algorithm (http://weizhong-lab.ucsd.edu/cd-hit/) to compare ITS2 sequence similarity between two datasets using a 90% similarity to see the genus level similarity of the two databases followed by a manual BLAST based identification of the respective OTUs. Functional group assignment of each OTU was done using the FUNGuild data base to (Nguyen et al. 2016; https://github.com/UMNFuN/FUNGuild).

Statistical analysis

All statistical analyses were performed using PAST (Hammer et al. 2001). To visualize the endophytic community compositions among different age levels of grape plants derived from culture-dependent and culture-independent approaches, we used non-metric multidimensional scaling (NMDS) analysis based on Jaccard distance measure (presence-absence OTU matrix). The stress values from NMDS were zero in both cases. To test for the difference in endophytic community compositions among different age levels of grape plants we used cluster analysis based on Jaccard (presence-absence OTU matrix) distance measure. To assess the coverage of the sequencing depth in mycobiome analysis, individual rarefaction analysis (with 95% confidence) was performed for each sample using the "diversity" function.

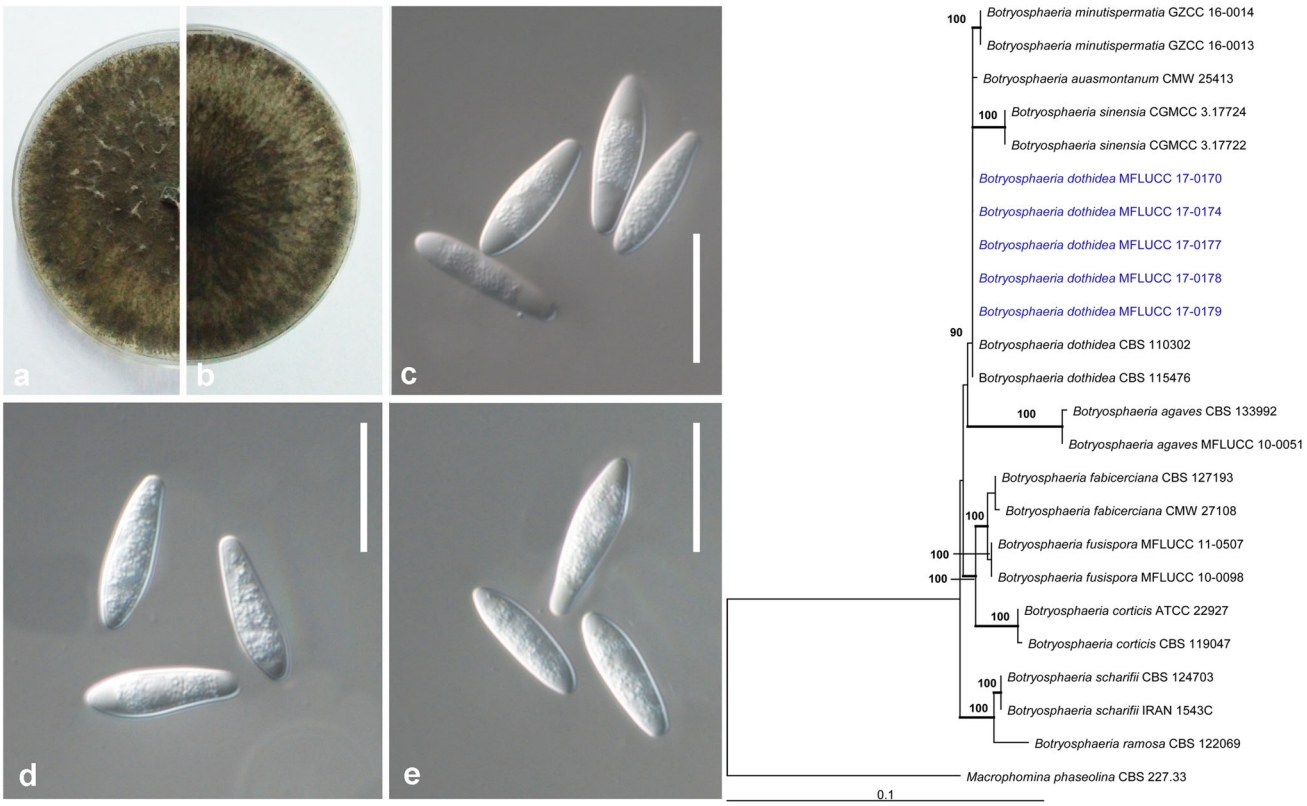

Fig. 4 *Botryosphaeria dothidea*. **a**, **b** Colonies on PDA (14 days old) from surface and reverse, **c**–**e** conidia. Scale bars: c–e = 20 µm. Phylogenetic tree inferred from maximum likelihood (ML) and Bayesian inference (BI) using combined ITS and TEF sequence data of the genus Botryosphaeria. Only the topology generated from the ML analysis is shown. ML values greater than the 90% are indicated. Bayesian Posterior Probability greater than 0.90 are indicated with thick branch. Taxa isolated in this study are in blue

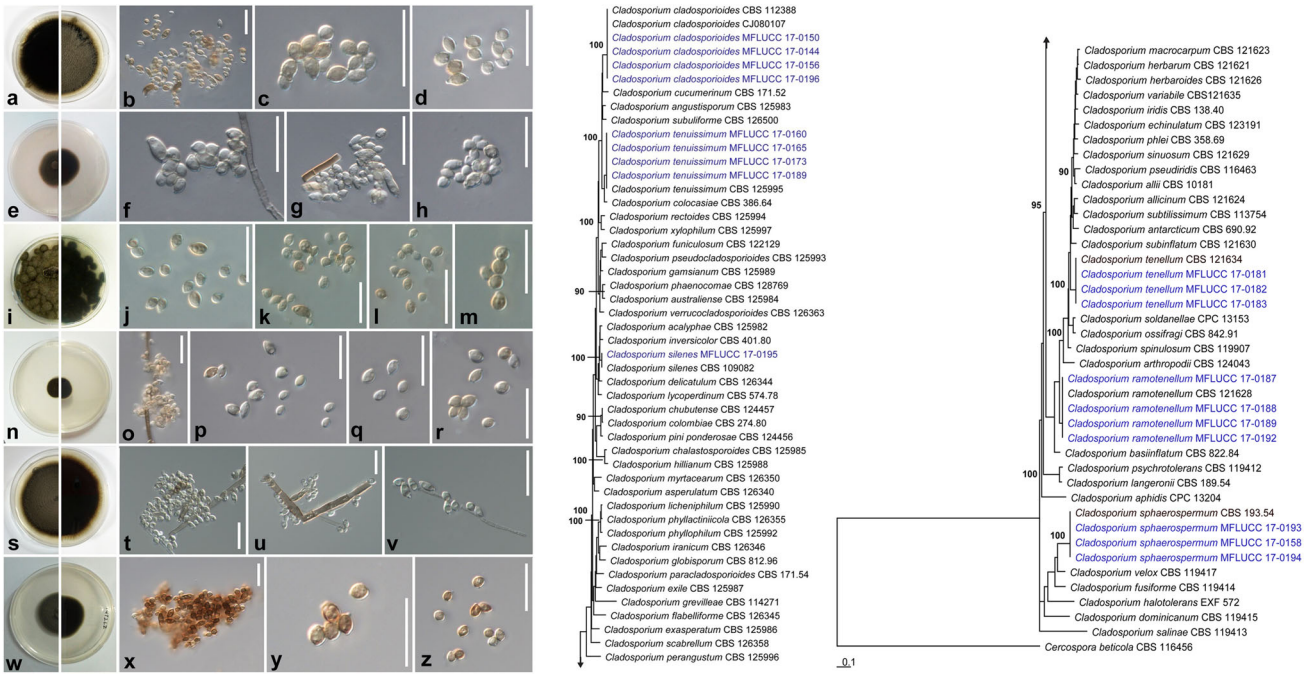

Fig. 5 *Cladosporium cladosporioides* (**a**–**d**), *Cladosporium ramotenellum* (**e**–**h**), *Cladosporium silenes* (**i**–**m**), *Cladosporium sphaerospermum* (**n**–**r**), *Cladosporium tenellum* (**s**–**v**), *Cladosporium tenuissimum* (**w**–**z**) **a**, **e**, **i**, **n**, **s**, **w** Colonies on PDA (14 days old) from surface and reverse, **b**–**d**, **f**–**h**, **j**–**m**, **o**–**r**, **t**–**v**, **x**–**z** Conidia. Scale bars: b–d, f–h, j–m, o–r, t–v, x–z = 20 µm. Phylogenetic tree inferred from maximum likelihood (ML) and Bayesian inference (BI) using combined ITS, TEF and ACT sequence data of the genus *Cladosporium*. Only the topology generated from the ML analysis is shown. ML values greater than the 90% are indicated. Bayesian Posterior Probability greater than 0.90 are indicated with thick branch. Taxa isolated in this study are in blue

Results

Culture-dependent approach: low fungal diversity and strong effect of grapevine age

In total, 94 isolates were recovered from 84 of the 144 stem samples analyzed (colonization rate = 58.3%); thus, 60 stem fragments did not yield any endophytic fungi. All of the culturable endophytic fungi recovered were ascomycetes, and were distributed in five classes (Dothideomycetes, Eurotiomycetes, Leotiomycetes, Pezizomycetes and Sordariomycetes). As shown in Fig. 1, 71.2% of the isolates were assigned to Dothideomycetes, of which Pleosporales and Capnodiales were dominant, accounting for 55.2 and 31.3% of this group. The remaining Dothideomycetes isolates belonged to Botryosphaeriales and Dothideales, accounting for 8.9 and 4.4%, respectively. There were 12.7% of isolates in Sordariomycetes. Of the Sordariomycetes group, Sordariales (27.2%) and Xylariales (27.2%) were prominent, while 18.1, 18.1, 9.0% respectively were in Hypocreales, Trichosphaeriales and Microascales. A considerable fraction of isolates (11.7%) were Eurotiomycetes with all belonging to Eurotiales. In addition, 2.1 and 1.1% of isolates belong to Leotiomycetes and Pezizomycetes correspondingly.Based on morphology and phylogenetic analysis of subsets of combined ITS, LSU, SSU, TEF, GPDH, RPB2, HSP60, TUB and ACT sequence data, the isolates obtained from the culture-dependent method were identified as 28 species (Table 1), 9 of which were observed only once. *Alternaria alternata* was the most abundant (relative abundance = 28.7%), followed by *Cladosporium* (21.1%), *Aspergillus* (10.6%), *Botryosphaeria dothidea* (5.3%), *Aureobasidium pullulans* (3.2%), *Bipolaris sorokiniana* (3.2%), *Chaetomium globosum* (3.2%) and *Phoma herbarum* (3.2%). The relative abundance of the other species were between 2.1 and 1.1% (Table 1). Fungal morphology and phylogenetic analysis of most frequent taxa are presented in Figs. 2, 3, 4 and 5 and the other prominent taxa are presented in Figs. 6, 7 and 8. Fungal richness of the culture-dependent approach ranged from 23 isolates (three year-old grapevines) to 32 isolates (13 year-old grapevines). Non-metric multidimensional scaling (NMDS) ordination and cluster analysis showed different fungal endophytic community composition among different ages of grapevine (Fig. 10). In three year-old stems, we detected 13 species, in eight year-old, 16 species and in 13 year-old, 17 species. The fungal endophytic community comprised several taxa, known as plant pathogens (Table 1). As an example, *Botryosphaeria dothidea* (associated with Botryosphaeria dieback in grapevine) could be considered

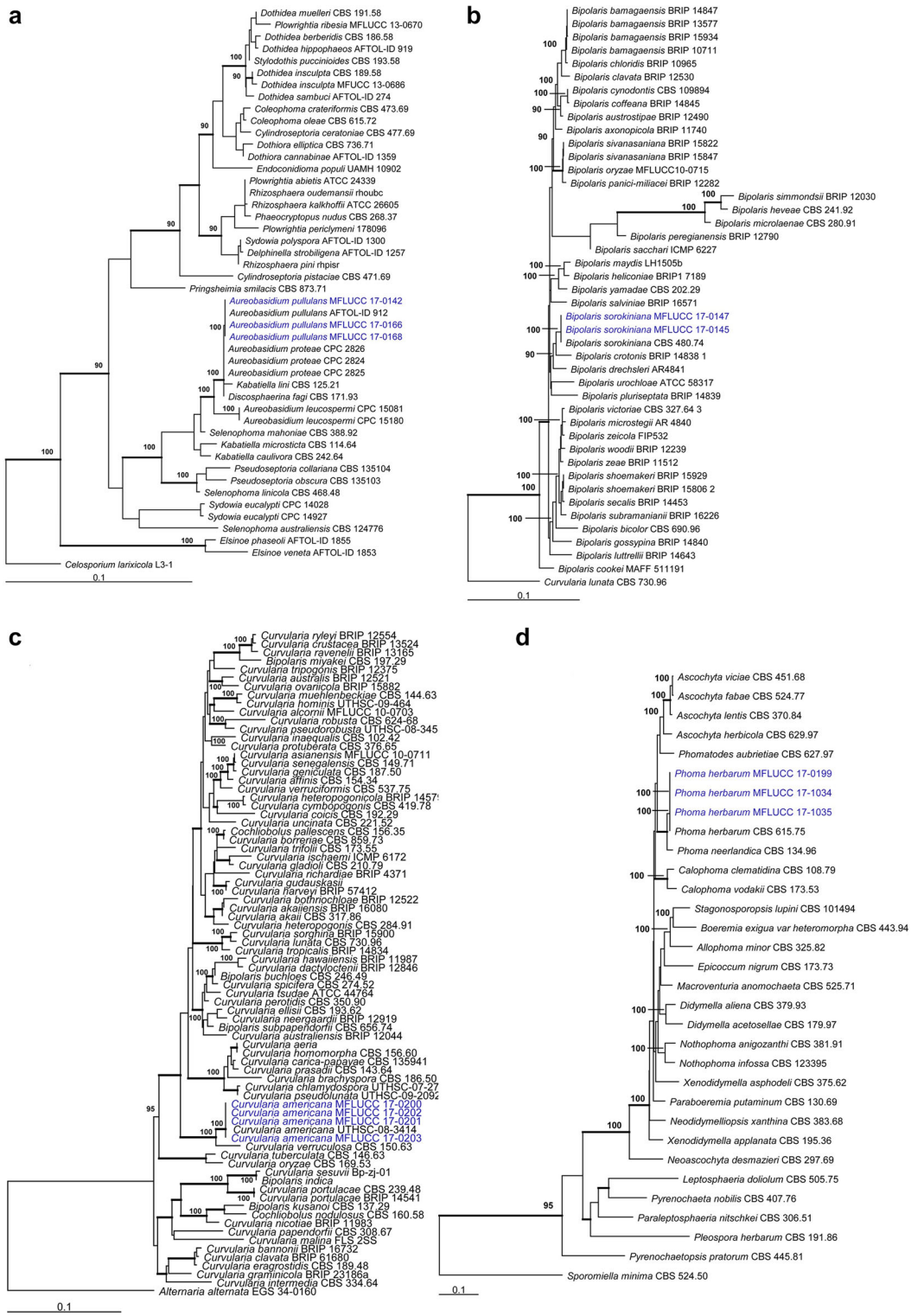

Fig. 6 Phylogenetic trees inferred from maximum likelihood (ML) and Bayesian inference (BI). **a** Combined LSU, SSU and ITS sequence data of the genus *Aureobasidium*, **b** combined ITS, GDPH, TEF and LSU sequence data of the genus *Bipolaris*, **c** combined ITS, GDPH, TEF and LSU sequence data of the genus *Curvularia*, **d** combined LSU, ITS, BT and RPB2 sequence data of the genus *Phoma*. Only the topology generated from the ML analysis is shown. ML values greater than the 90% are indicated near the node. BI greater than 0.90 are indicated with thick branch. Taxa isolated in this study are in blue

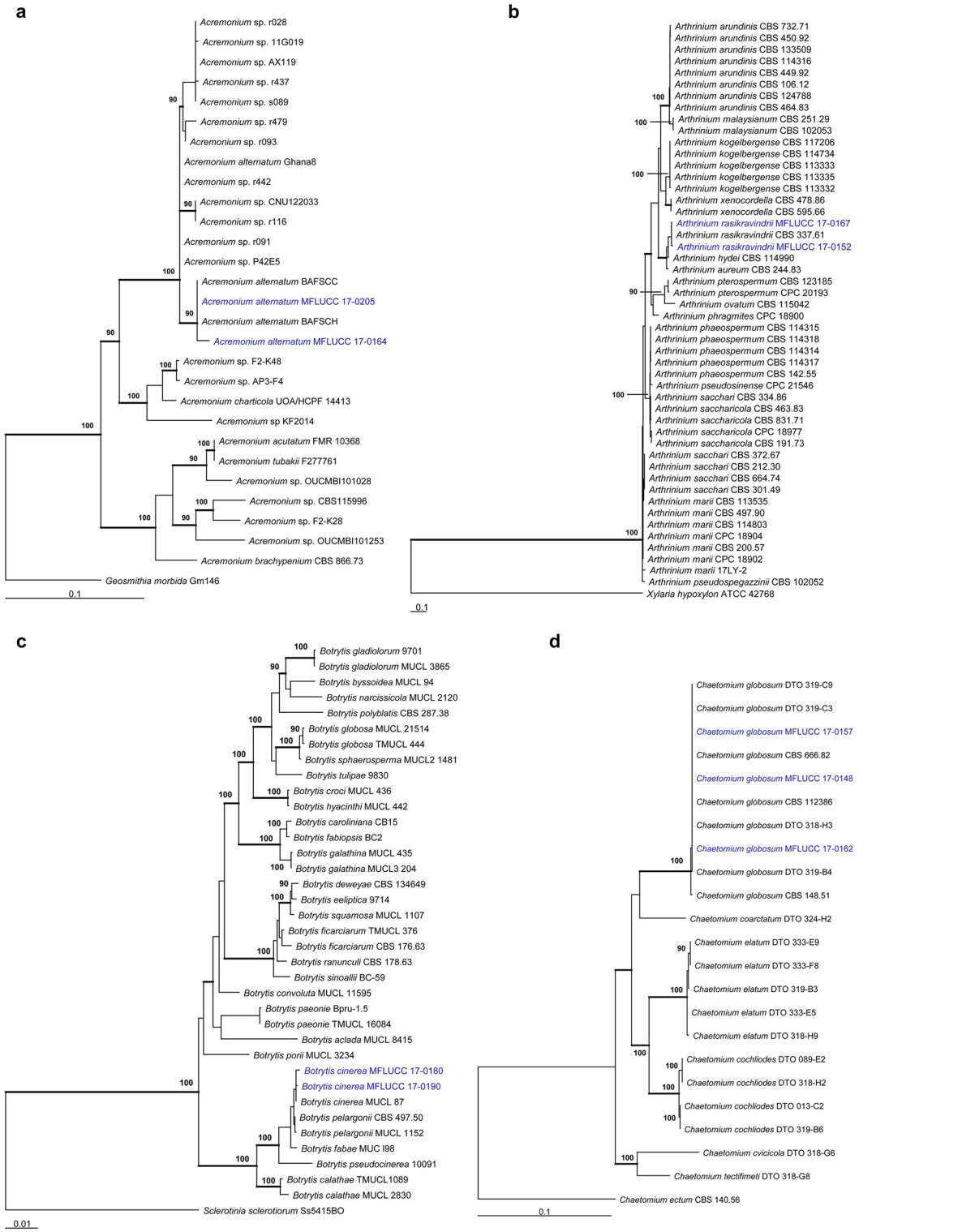

Fig. 7 Phylogenetic trees inferred from maximum likelihood (ML) and Bayesian inference (BI). **a** ITS sequence data of the genus *Acremonium*. **b** ITS sequence data of the genus *Arthrinium*. **c** Combined G3PDH, HSP60 and RPB2 sequence data of the genus Botrytis. **d** Combined ITS, TUB2, RPB2 and LSU sequence data of the genus *Chaetomium*. Only the topology generated from the ML analysis is shown. ML values greater than the 90% are indicated near the node. BI greater than 0.90 are indicated with thick branch. Taxa isolated in this study are in blue

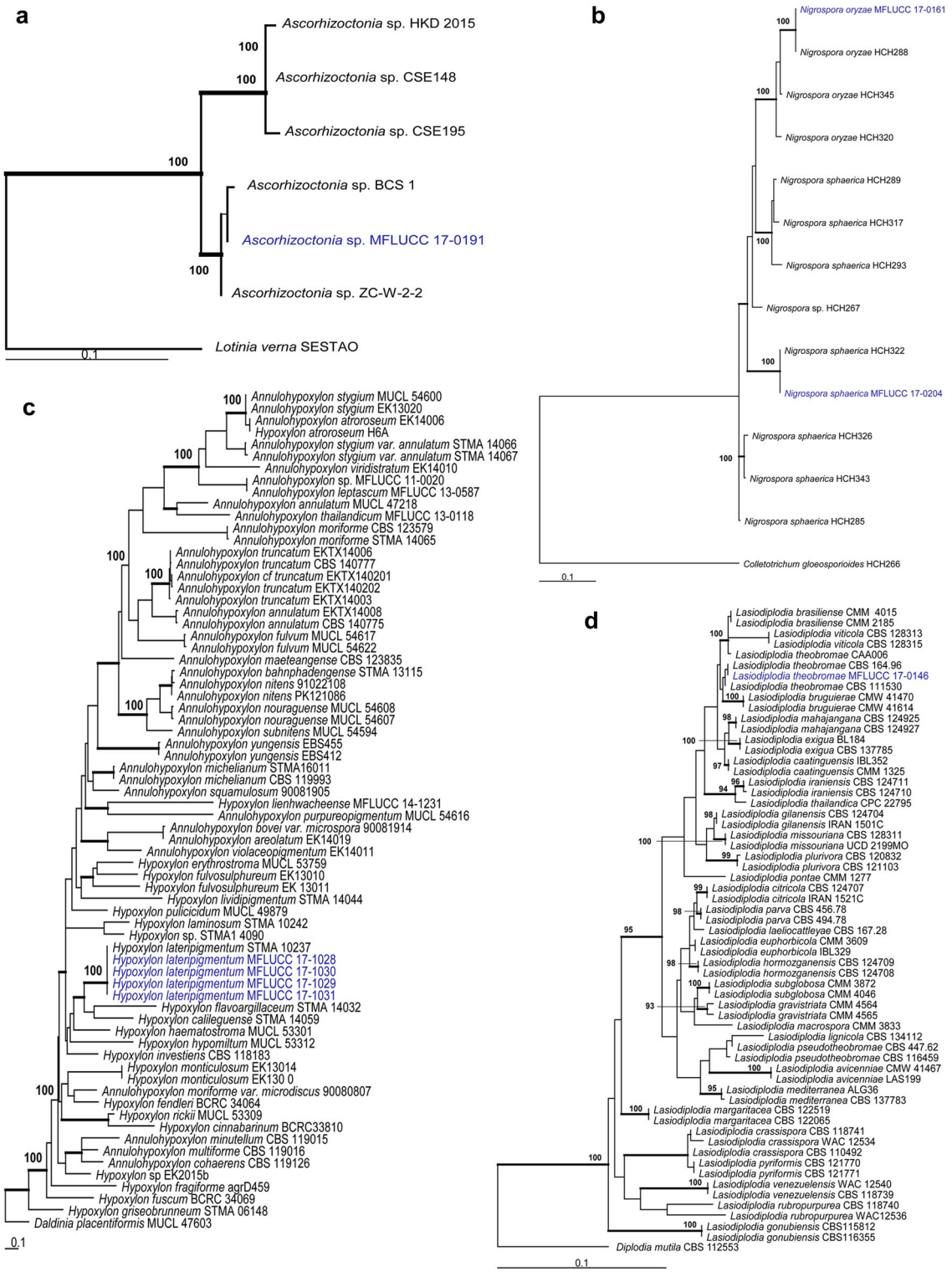

Fig. 8 Phylogenetic trees inferred from maximum likelihood (ML) and Bayesian inference (BI). **a** ITS sequence data of the genus *Ascorhizoctonia*, **b** Combined ITS and EF sequence data of the genus *Lasiodiplodia*, **c** ITS sequence data of the genus *Nigrospora*. **d** Combined ITS and BT sequence data of the genus *Hypoxylon*. Only the topology generated from the ML analysis is shown. ML values greater than the 90% are indicated near the node. BI greater than 0.90 are indicated with thick branch. Taxa isolated in this study are in blue

as abundant, representing 5.3% of the total strains characterized (Table 1; Fig. 4).

Culture-independent approach: mycobiome with low diversity and strong effect of grapevine age

Despite the high number of sequences per sample (55, 822 reads), low fungal richness was detected (59 OTUs in total, Table 2) ranging from 23 (three year-old samples) to 43 (13 year-old samples) OTUs (Fig. 9). The richness of endophytic fungi from different age levels were significantly different ($P < 0.05$), being highest at 13 years followed by eight and three years (Fig. 9). The majority of fungal OTUs were rare: 51 out of 59 had relative abundance lower than 0.1% (Table 2). Fungi identified by the culture-independent approach belonged to three phyla, Ascomycota (93.6%), Basidiomycota (4.2%) and Zygomycota (2.1%). Ascomycetous taxa were distributed among five classes: Dothideomycetes (34%), Eurotiomycetes (40.9%), Leotiomycetes (4.5%), Pezizomycetes (4.5%) and Sordariomycetes (15.9%). The frequently detected OTUs were *Cladosporium*OTU_1 (39.03%), *Pleosporaceae*OTU_2 (33.53%), *Cladosporium*OTU_4 (12.54%), AscomycotaOTU_15 (11.60%), *Cadophora*OTU_5 (1.60%), *Cladosporium*OTU_6 (0.56%), *Botryosphaeria*OTU_7 (0.45%) and AscomycotaOTU_3 (0.29%). Non-metric multidimensional scaling (NMDS) ordination and cluster analysis showed different fungal endophytic community composition among different ages of grapevine (Fig. 10). In three year-old stems, we frequently detected four OTUs: *Pleosporaceae*OTU_2 (56.30%), AscomycotaOTU_15 (24.75%), *Cladosporium*OTU_1 (13.05%) and *Cadophora*OTU_5 (4.74%). In eight year-old stems, *Pleosporaceae*OTU_2 (43.66%), *Cladosporium*OTU_1 (31.14%), *Cladosporium*OTU_4 (14.76%) and AscomycotaOTU_15 (9.50%) were often detected. *Cladosporium*OTU_1 (72.92), *Cladosporium*OTU_4 (22.77%), *Botryosphaeria*OTU_7 (1.32%) and *Cladosporium*OTU_6 (1.02%) were commonly associated with 13 year-old grapevines (Table 2).

Comparison between culture-dependent and culture-independent approaches

The results regarding the influence of age of grapevine plants on endophytic fungal communities were similar (Fig. 10). Detected all fungal genera were *Acremonium*, *Alternaria*, *Arthrinium*, *Ascorhizoctonia*, *Aspergillus*, *Aureobasidium*, *Bipolaris*, *Botryosphaeria*, *Botrytis*, *Cadophora*, *Chaetomium*, *Chaetothyriales*, *Cladosporium*, *Cryptococcus*, *Curvularia*, *Eupenicillium*, *Exophiala*, *Hypoxylon*, *Kernia*, *Lasiodiplodia*, *Lophiostoma*, *Mortierella*, *Mycosphaerella*, *Nigrospora*, *Oidiodendron*, *Penicillium*, *Phialosimplex*, *Phoma*, *Pyrenochaeta*, *Scopulariopsis*, *Tomentella* and *Toxicocladosporium* (Tables 1, 2). However, only *Acremonium*, *Aspergillus*, *Botryosphaeria*, *Botrytis*, *Cladosporium*, *Lasiodiplodia* and *Phoma* were detected in both approaches. The results from both approaches showed that members of ascomycetes were dominant in the endophytic fungal community inhabiting grapevine stems. However, we identified members of Basidiomycetes (*Tomentella*OTU_29, *Cryptococcus*OTU_41 and AgaricomycetesOTU_46) in the culture-independent approach. Direct matching of the ITS sequences of fungal endophytes detected from these two approaches confirmed that the results obtained in most cases are consistent, except for *Curvularia*, where the sequence similarity was lower than 90% and did not cluster together and for *Penicillium digitatum* that clustered with its sexual morph state *Eupenicillium*. In total, direct matching of ITS sequences of fungal endophytes detected by the two approaches matched 13/28 fungal species from the culture-dependent approach and 16/59 fungal OTUs from the culture-independent approach at genera (90% similarity) or species (97% similarity or higher) levels (Table 3). We were able to assign 9/16 and 7/16 OTUs from NGS at genus and species levels, respectively (Table 3). Five fungal OTUs that were identified only at the phylum, order or family level in the culture-independent approach were identified at genus level by direct matching (91–95% similarity). These include AscomycotaOTU_3 (overall relative abundance = 0.29) and AscomycotaOTU_15 (overall relative abundance = 11.60; *Cladosporium*), AscomycotaOTU_22 (overall relative abundance = 0.01%; *Chaetomium*), EurotialesOTU_25 (overall relative abundance = 0.01%; *Aspergillus*) and *Pleosporaceae*OTU_2 (overall relative abundance = 33.53%; *Alternaria*) (Table 3). All frequently detected genera in the culture-dependent approach (i.e. with relative abundance higher than 5%; *Alternaria*, *Aspergillus*, *Botryosphaeria* and *Cladosporium*) were also detected in culture-independent approach. *Alternaria* (detected as *Pleosporaceae*OTU_2 in culture-independent approach), *Cladosporium* sp. and *Botryosphaeria* were the frequently detected endophytes in both approaches. *Aspergillus* sp. was frequently detected in the culture-dependent approach, but exhibited low relative abundances in the culture-independent approach. On the other hand, one highly detected fungal taxon from culture-independent approach (*Cadophora* sp.) was not isolated in the culture-dependent approach.

Fungal guilds and endophytic fungal functional groups

Fungal guild analysis showed that endophytes from the culture-independent approach potentially comprised

Table 2 Mycobiome analysis data show potential functions and the relative abundances (in each sample and average across samples) of all fungal OTUs detected from the next generation sequencing

Final Taxon	Function	Confidence ranking	3 Years	8 Years	13 Years	Abundance
*Cryptococcus*OTU_41	Animal pathogen/saprotroph	Highly probable	0.000	0.004	0.002	0.002
*Tomentella*OTU_29	Ectomycorrhizal	Highly probable	0.000	0.000	0.013	0.004
*Cadophora*OTU_5	Endophyte	Highly probable	4.740	0.014	0.018	1.591
*Cladosporium*OTU_1	Endophyte/plant pathogen	Possible	13.052	31.135	72.916	39.034
*Cladosporium*OTU_17	Endophyte/plant pathogen	Possible	0.004	0.007	0.011	0.007
*Cladosporium*OTU_4	Endophyte/Plant pathogen	Possible	0.075	14.761	22.772	12.536
*Cladosporium*OTU_6	Endophyte/plant pathogen	Possible	0.138	0.516	1.021	0.558
*Oidiodendron*OTU_58	Ericoid Mycorrhizal	Probable	0.000	0.000	0.004	0.001
*Botryosphaeria*OTU_7	Plant Pathogen	Probable	0.005	0.014	1.322	0.447
*Curvularia*OTU_27	Plant pathogen	Probable	0.005	0.005	0.000	0.004
*Lasiodiplodia*OTU_9	Plant pathogen	Probable	0.038	0.011	0.149	0.066
*Penicillium_ citrinum*OTU_53	Plant pathogen	Probable	0.000	0.004	0.002	0.002
*Botrytis*OTU_31	Plant pathogen/saprotroph	Highly probable	0.000	0.000	0.014	0.005
*Phoma*OTU_28	Plant pathogen/saprotroph	Highly probable	0.000	0.016	0.004	0.007
*Phoma*OTU_34	Plant pathogen/saprotroph	Highly probable	0.000	0.009	0.000	0.003
*Acremonium alternatum*OTU_11	Saprotroph	Highly probable	0.000	0.018	0.099	0.039
*Aspergillus*OTU_10	Saprotroph	Probable	0.104	0.000	0.014	0.039
*Aspergillus*OTU_14	Saprotroph	Probable	0.000	0.018	0.005	0.008
*Aspergillus*OTU_51	Saprotroph	Probable	0.007	0.000	0.000	0.002
*Eupenicillium*OTU_16	Saprotroph	Probable	0.011	0.011	0.023	0.015
*Eupenicillium*OTU_52	Saprotroph	Probable	0.000	0.002	0.002	0.001
EurotialesOTU_18	Saprotroph	Possible	0.000	0.013	0.011	0.008
EurotialesOTU_19	Saprotroph	Possible	0.016	0.000	0.000	0.005
EurotialesOTU_24	Saprotroph	Possible	0.034	0.000	0.000	0.011
EurotialesOTU_25	Saprotroph	Possible	0.016	0.002	0.004	0.007
EurotialesOTU_36	Saprotroph	Possible	0.005	0.000	0.000	0.002
EurotialesOTU_50	Saprotroph	Possible	0.000	0.004	0.002	0.002
*Exophiala*OTU_54	Saprotroph	Probable	0.000	0.004	0.000	0.001
HypocrealesOTU_13	Saprotroph	Possible	0.000	0.000	0.048	0.016
*Kernia*OTU_30	Saprotroph	Highly probable	0.000	0.005	0.004	0.003
*Lophiostoma*OTU_56	Saprotroph	Probable	0.000	0.000	0.005	0.002
*Mortierella*OTU_48	Saprotroph	Probable	0.000	0.002	0.002	0.001
*Penicillium*OTU_49	Saprotroph	Highly probable	0.000	0.000	0.004	0.001
*Penicillium*OTU_55	Saprotroph	Highly probable	0.000	0.007	0.000	0.002
*Penicillium*OTU_8	Saprotroph	Highly probable	0.113	0.005	0.095	0.071
*Phialosimplex*OTU_59	Saprotroph	Probable	0.000	0.000	0.004	0.001
*Pyrenochaeta*OTU_42	Saprotroph	Highly probable	0.000	0.004	0.000	0.001
AgaricomycetesOTU_46	Unknown	–	0.000	0.000	0.004	0.001
*Ascobolaceae*OTU_32	Unknown	–	0.000	0.000	0.009	0.003
AscomycotaOTU_15	Unknown	–	24.745	9.450	0.595	11.596
AscomycotaOTU_20	Unknown	–	0.000	0.000	0.023	0.008
AscomycotaOTU_22	Unknown	–	0.000	0.000	0.018	0.006
AscomycotaOTU_3	Unknown	–	0.557	0.195	0.106	0.286
AscomycotaOTU_37	Unknown	–	0.000	0.000	0.004	0.001
AscomycotaOTU_43	Unknown	–	0.013	0.000	0.000	0.004
ChaetothyrialesOTU_40	Unknown	–	0.002	0.005	0.000	0.002
*Diaporthaceae*OTU_23	Unknown	–	0.000	0.013	0.000	0.004
FungalOTU_21	Unknown	–	0.000	0.013	0.014	0.009

Table 2 (continued)

Final Taxon	Function	Confidence ranking	3 Years	8 Years	13 Years	Abundance
FungalOTU_33	Unknown	–	0.000	0.000	0.009	0.003
FungalOTU_39	Unknown	–	0.007	0.000	0.000	0.002
FungalOTU_44	Unknown	–	0.000	0.000	0.004	0.001
FungalOTU_60	Unknown	–	0.000	0.002	0.002	0.001
*Pleosporaceae*OTU_2	Unknown	–	56.304	43.661	0.636	33.534
PleosporalesOTU_12	Unknown	–	0.000	0.038	0.000	0.013
*Pyronemataceae*OTU_45	Unknown	–	0.000	0.009	0.002	0.004
SordariomycetesOTU_35	Unknown	–	0.000	0.004	0.005	0.003
SordariomycetesOTU_38	Unknown	–	0.000	0.000	0.004	0.001
SordariomycetesOTU_47	Unknown	–	0.000	0.004	0.000	0.001
ToxicocladosporiumOTU_26	Unknown	–	0.009	0.018	0.000	0.009

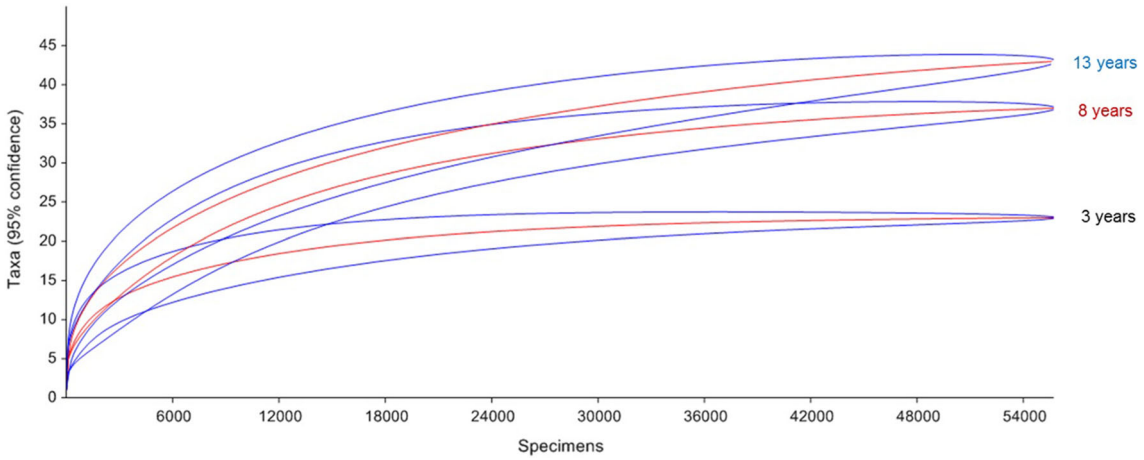

Fig. 9 Individual rarefaction curves of endophytic fungi detected in each age level. Blue lines indicate 95% confidence

various functional groups. This study indicates that the symptomless endophytes living in grapevine stems, can also exist in numerous life modes, such as being saprotrophs, pathogens or symbionts. *Cadophora* and *Cladosporium* sp. were frequently detected endophytes. The prevailing pathogen was *Botryosphaeria dothidea*. All saprotrophs were detected at low relative abundances (less than 0.1%) (Table 2). A list of all fungal endophytes detected in this study, with their possible functions, are listed in Table 2. The culture-independent approach revealed that fungal endophytes comprised endophytes, saprotrophs and pathogens.

Discussion

Comparison of culture-dependent versus culture-independent approaches

This is the first study conducted to compare the diversity and community composition of fungal endophytes in stems of grapevine (*Vitis vinifera*) using a culture-dependent approach, incorporating multigene phylogenetic analysis and a culture-independent approach using meta-barcoding and paired-end Illumina sequencing. The traditional approach for studying the diversity of endophytic fungi is the culture-dependent approach. Many studies have used sequence data from the ITS region to identify and evaluate endophytic fungi (Guo et al. 2000, 2001, 2003; Arnold 2002; Lacap et al. 2003; Promputtha et al. 2005, 2007, 2010; Tejesvi et al. 2011; Jeewon et al. 2003; Haghighi and Shahdoust 2015). In the present study we used subgroups of combined ITS, LSU, SSU, TEF, GPDH, RPB2, HSP60, TUB and ACT sequence data to identify the endophytes obtained from grapevine stems. Two isolates of *Acremonium* belong to *A. alternatum* were identified using analysis of ITS sequence data (Fig. 7a). Twenty-seven *Alternaria* isolates were subjected to combined analysis of ITS, LSU, GPDH, TEF and RPB2 sequence data and all *Alternaria* isolates were identified as *A. alternata* sensu stricto (Fig. 2). Two *Arthrinium* isolates were identified as *A. rasikravindrii* by using ITS sequence data (Fig. 7b).

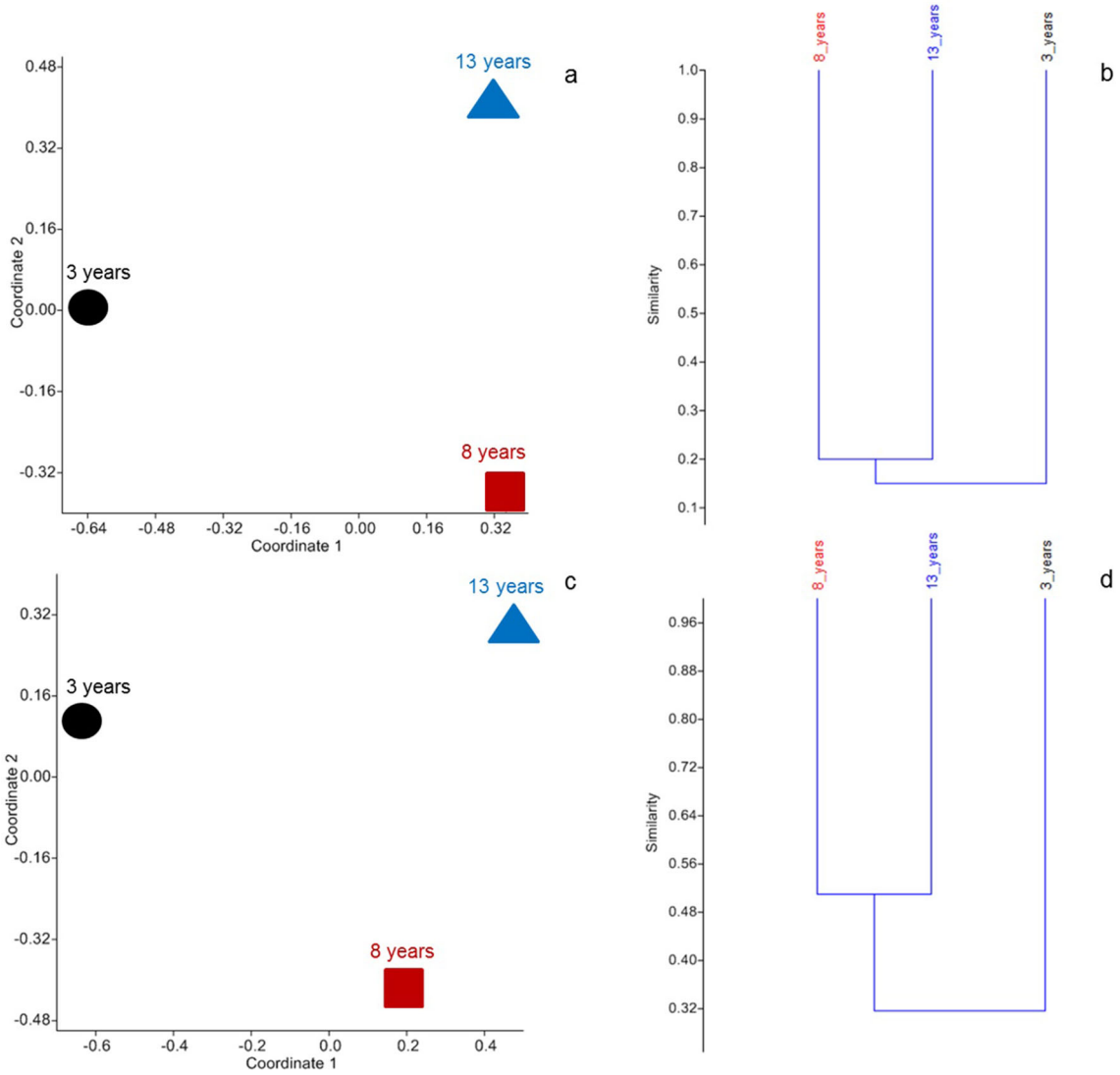

Fig. 10 Non-metric multidimensional scaling (NMDS) ordinations and cluster analysis of fungal community composition derived from culture-dependent (**a**, **b**) and high resolution culture-independent (**c**, **d**) approaches. Similarity from cluster analysis is ranged from 0 (completely different) to 1 (completely overlap)

Analysis of ITS sequence data, showed that one isolate was *Ascorhizoctonia* sp. (Fig. 8a) and confirmed the identification of four *Aspergillus* species as *A. japonicas*, *A. niger*, *A. pseudodeflectus* and *A. pseudoglaucus* (Fig. 3). Analysis of combined LSU, SSU and ITS sequence data identified three *Aureobasidium* taxa to be *A. pullulans* (Fig. 6a). The phylogeny inferred from combined ITS, GDPH, TEF and LSU sequence data resolved three *Bipolaris* isolates as *B. sorokiniana* (Fig. 6b). Combined ITS and TEF sequence data identified five *Botryosphaeria* taxa to be *B. dothidea* (Fig. 4) and resolved one isolate as *Lasiodiplodia theobromae* (Fig. 8d). Two *Botrytis* isolates were subjected to combined G3PDH, HSP60 and RPB2 sequence data and were identified as *B. cinerea* (Fig. 7c). Analysis of combined ITS, TUB2, RPB2 and LSU sequence data, resolved three isolates as *Chaetomium globosum* (Fig. 7d). The phylogenetic tree inferred from combined ITS, TEF and ACT sequence data resolved 20 *Cladosporium* strains as *C. cladosporioides*, *C. ramotenellum*, *C. silenes*, *C. sphaerospermum*, *C. tenellum* and *C. tenuissimum* (Fig. 5). Analysis of combined ITS, GDPH, TEF and LSU sequence data from four *Curvularia* isolates resolved *C. americana* (Fig. 6c). One isolate of *Hypoxylon lateripigmentum* was identified by combined ITS and BT sequence data (Fig. 8c). An isolate of *Mycosphaerella* was identified as *M. graminicola* by the combined analysis of ITS, ACT and TEF sequence data. Two *Nigrospora* isolates were identified as *N. oryzae* and *N. sphaerica* using ITS sequence data (Fig. 8b). Analysis of combined ITS, LSU and SSU sequence data resolved the two *Penicillium* isolates as *P.*

Table 3 Direct matching of ITS sequences of fungal endophytes inhabiting healthy stems of grapevines using culture-dependent and culture-independent approaches

Fungal taxon (culture)	Relative abundance in culture (%)	Fungal taxa (mycobiome)	Relative abundance in mycobiome (%)	Cluster identification (coverage) %	Number of OTUs in cluster
Cladosporium cladosporioides	7.38	*Cladosporium*OTU_1 AscomycotaOTU_3 AscomycotaOTU_15	50.92	91–92 (98–99)	3
Cladosporium sphaerospermum	1.85	*Cladosporium*OTU_6, *Cladosporium*OTU_17	0.57	93–98 (98)	2
Lasiodiplodia theobromae	1.11	*Lasiodiplodia*OTU_9	0.07	100 (99)	1
Aspergillus pseudoglaucus	4.2	*Aspergillus*OTU_10	0.04	100 (99)	1
Acremonium alternatum	1.48	*Acremonium*OTU_11	0.04	100 (99)	1
Aspergillus japonicus	1.1	*Aspergillus*OTU_51	< 0.01	99 (99)	1
Botryosphaeria dothidea	4.06	*Botryosphaeria*OTU_7	0.45	99 (99)	1
Aspergillus pseudodeflectus	4.2	EurotialesOTU_25	0.01	95 (99)	1
Alternaria alternata	16.24	*Pleosporaceae*OTU_2	33.53	91 (99)	1
Penicillium digitatum	0.37	*Eupenicillium*OTU_16	0.01	90 (98)	1
Chaetomium globosum	1.11	AscomycotaOTU_22	0.01	91 (100)	1
Botrytis cinerea	0.74	*Botrytis*OTU_31	<0.01	99 (100)	1
Phoma herbarum	1.48	*Phoma*OTU_28	0.01	92 (98)	1

Relative abundances of fungal taxa and OTUs in culture culture-dependent and culture-independent approaches are showed. A fungal taxon from culture-dependent approach was matched to one to three fungal OTUs from culture-independent approach with different matching (cluster identification; 90–100%) and coverage (98–100%) percentages

digitatum. A phylogeny inferred from combined LSU, ITS, BT and RPB2 sequence data resolved the three *Phoma* isolates as *P. herbarum* (Fig. 6d). An isolate of *Scopulariopsis* was analysed with combined ITS, LSU and SSU sequence data and was identified as *Scopulariopsis brevicaulis*. The current study identified all isolated taxa from culture-dependent method to species level with strong support in multi-gene phylogenetic analysis, setting a robust goal for future fungal community studies. Some endophytes such as *Acremonium*, *Alternaria*, *Aureobasidium*, *Penicillium* and *Phoma* have been used as biocontrol agents against pathogens in grapevines (Tables 1, 4).

Traditional taxonomy and nomenclature is unable to accurately document the vast number of unrecognized taxa, regardless of any doubts one might have to formally describe fungi based on DNA sequence data only (Lücking and Moncada 2017). The aim of environmental sequence nomenclature is to place names on hundreds of thousands of species of fungi that would otherwise be left undescribed (Lücking and Moncada 2017). Environmental high throughput sequencing reads almost 1000 times more than Sanger sequences for the fungal barcoding marker (Jayawardena et al. 2018). A recent study conducted by Lücking and Moncada (2017) showed that a formally recognized unnamed lichenicolous basidiomycete (in *Agonimia* and *Normandina* thalli) is a new genus, with seven new species, although no physical type specimens could be preserved. Lücking and Moncada (2017) suggested that this opens the door to the formal recognition of thousands of species of voucher less taxa detected through environmental sequencing techniques.

The main limitation of the culture-dependent approach is that unculturable species and some slow growing or weakly competitive species may not be isolated. To overcome the shortcomings of the culture-dependent approach, culture-independent approaches have been suggested as an alternative (Peršoh 2015; Hoppe et al. 2016; Gomez et al. 2017; Zapka et al. 2017). However, culture-dependent approaches do not seem to resolve species accurately, since taxa are not resolved to species level. Some studies have shown that endophytic fungi recovered by culture-dependent approaches are different from those detected by

Table 4 Functions of endophytic fungi reported in grapevine

Fungus	Function/activity	References
Acremonium byssoides	Antagonistic endophyte of the downy mildew agent *Plasmopara viticola*	Burruano et al. (2008)
Acremonium sp.	Inhibit the germination of sporangia of *Plasmopara viticola*	Assante et al. (2005)
Acremonium, Phoma, Chaetomium	Antagonistic activity against vine diseases	González and Tello (2011)
Alternaria alternata	Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew	Musetti et al. (2006)
Aureobasidium pullulans	Against post-harvest diseases by *Botrytis cinerea* and *Penicillium expansum*	Martini et al. (2009)
Aureobasidium pullulans	Inhibit various grapevine pathogens	de Felice et al. (2008), Schmid et al. (2011)
Aureobasidium pullulans and *Epicoccum nigrum*	Plant growth promoters, biological control agents against grapevine pathogens	Martini et al. (2009)
Penicillium expansum	Biocontrol agents against postharvest pathogens	Schena et al. (1999)
Colletotrichum gloeosporioides, Flavodon flavus	Antagonistic activity against *Fusarium oxysporum*	Brum et al. (2012)
Epicoccum nigrum	Control agent of *Plasmopara viticola*	Kortekamp (1997)
Epicoccum nigrum	Inhibition of the grapevine pathogens *Plasmopara viticola* and *Botrytis cinerea*	Elmer and Reglinski (2006)
Penicillium sp.	Potential biocontrol agent of *Botrytis cinerea*	Garoé et al. (2012)

culture-independent approaches, and some isolated strains were never found in the culture-independent methods (Campisano 2012; Kraková et al. 2017; Mendoza et al. 2017). This fact was also experienced in the current study as we obtained 9 fungal genera from the culture-dependent approach, which were absent in culture-independent approach. Nevertheless, our study demonstrates that all frequently detected fungal genera from the culture-dependent approach can be detected in culture-independent approach when we used a high resolution technique with a high quality dataset. In this study, we used paired-end Illumina sequencing that provided a minimum of 55, 822 high quality sequences per sample (saturated rarefaction curves for all samples) and revealed all frequently detected fungal genera from culture-dependent approach that have relative abundance higher than 5%. The most frequently detected genera in culture-dependent and culture-independent approaches are also mostly consistent, except some OTUs, such as *Aspergillus* (frequently detected in culture-dependent approach, but become less frequent in culture-independent approach) and *Cadophora* sp. (commonly detected fungal taxon in the culture-independent approach, but not detected in the culture-dependent approach). One reason for this observation might be that *Aspergillus* sp. have a fast growing ability, but possibly occurring in low amounts in the tissue samples. Thus, in the culture-dependent approach, they could grow fast and result in a high number of isolates, whereas they could not be detected in next generation sequencing. *Cadophora* sp. are classified as endophytes in this study based on FUNGUILD, but some members of this genus can be plant pathogens that may not be able to grow quickly on artificial media without their host (Travadon et al. 2015).

Although there are some differences in the fungal taxa and richness of the two approaches (culture-dependent < culture-independent approaches), the results regarding the effect of stem ages on fungal community composition and richness are consistent. Studies on the community of endophytes have often ignored the impact of plant age (Fuchs et al. 2017). To our knowledge, this is the first study showing endophytic fungal composition in grapevine in three different age levels. However, our results based on both culture-dependent and culture-independent approaches indicate that endophytic fungal community and richness is maximum at 13 years. Further studies are needed to confirm whether there is any correlation with age level and the number of endophytic taxa in various hosts. Despite the highest per sample sequence read coverage we detected low diversity of fungal endophytes in grapevine stem samples. The same outcome was perceived in culture-dependent approach, which comprises only 28 species from 94 isolates. The colonization rate was also very low, with only 58.3% of the studied stem fragments yielding endophytes, signifying that nearly half of the fragments may not support culturable endophytes.

Why was there no species overlap between the two approaches in the present study?

The current study indicates that NGS data are only accurate at the genus or family levels. Seven fungal genera obtained

by the culture-dependent approach (*Acremonium*, *Aspergillus*, *Botryosphaeria*, *Botrytis*, *Cladosporium*, *Lasiodiplodia* and *Phoma*) overlapped with those of the culture-independent approach (Table 3). NGS for fungal community analysis mostly acquires short sequence fragments of ITS (full, ITS1 or ITS2) that eventually is not adequate for species level identification at the currently agreed 97% sequence similarity for all fungi (Nilsson et al. 2008; Garnica et al. 2016). The ITS rDNA marker is not consistent due to their high variability, therefore not reliable for species level identification (Nilsson et al. 2008). In our study, we experienced that using NGS data to identify the taxa in a community is not accurate at the species level, as compared to the analyses using multigene sequence data using cultures from the culture-dependent method. ITS sequence data can be regarded as taxonomically less-informative for most of the fungal taxa belonging to Dothideomycetes and/or Sordariomycetes, which were identified through the culture-dependent approach which might account to the variable inter- and intra-specific variation of the ITS fragment.

Matching of endophytic fungi detected from the two approaches: opportunity to assign more correct taxonomic information to NGS datasets and reference databases

In this study, we were able to match sequencing and taxonomic data from culture-dependent techniques to assign taxonomic information at genera and species level to 9 and 7 fungal OTUs from the culture-independent approach. This matching allows us to assign better correct taxonomic information and functions to the fungal OTUs detected in culture-independent approach. Next generation sequencing often results in sequences that are associated with taxa which have not been reported in previous studies, as well as sequences that are not linked with any fungal sequences in GenBank (Tejesvi et al. 2010; Ko et al. 2011; Taylor et al. 2016). One of the major reasons for this might be the insufficient number of cultures based on reference sequences in GenBank or other databases, since early fungal identifications did not provide genetic data. In this study, we demonstrate that some commonly detected endophytes from the culture-independent approach can only be identified to phylum, order or family levels. These include *Pleosporaceae*OTU_2 (overall relative abundance 33.53%), which was later found to match the most frequently isolated endophytic species *Alternaria alternata*. NGS technologies together with general culturing methods allow synchronized exploration of a more complete picture of endophytic communities in host plants (Hardoim et al. 2015). This concept applies when fungal genera are identified by sequencing the pure cultures obtained in culture-dependent method and then relate to OTUs obtained from the culture-independent method with a defined similarity threshold based on the intra- and inter-specific variation of the isolate.

Potential functions of the endophytic fungal communities inhabiting the grapevine stem

Taxonomy based functional assignment of fungi has been used to study potential roles of endophytes in plant community structure and ecosystem functioning (Green et al. 2008; Roe et al. 2010). In the present study, culture-dependent and culture-independent approach allows the identification of potential roles of identified fungal taxa as endophytes, saprotrophs, pathogens or symbionts in the grapevine fungal community. Although the grapevines look healthy, they were already colonized by pathogens that may be inactive until suitable conditions arise to cause diseases. In this study, we identified two important fungal pathogens (*Botryosphaeria dothidea* and *Botrytis cinerea*) from both culture-dependent and independent approaches. A number of endophytes revealed by the culture-independent approach are classified as potential saprotrophs. This fact has been confirmed by earlier studies implying endophytes can change their lifestyle to become saprotrophs (Purahong and Hyde 2011; Fesel and Zuccaro 2016; Szink et al. 2016) and they may play important role in plant litter decomposition, especially at early decomposition stage (Purahong et al. 2016).

In general this study has shown the potential of using both approaches in a given study to link the NGS datasets with culture-based fungal isolates that are morphologically and phylogenetically identified. Such a complementary approach enables to properly identify and give correct taxonomic information to the fungal endophytes identified with the NGS approach. NGS studies without reference cultures may be misleading and many of the data generated in the past must be seen as very critical.

Acknowledgements This work was financially supported by Beijing Talent Program for Dr. Jiye Yan, CARS-29, Beijing science and technology project D17110001617002. We thank Dr. Heng Gui for his support to submit Raw Illumina reads to the Sequence Read Archive (SRA) of National Center for Biotechnology Information (NCBI).

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