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A Richer Community of Botryosphaeriaceae Within a Less Diverse Community of Fungal Endophytes in Grapevines than in Adjacent Forest Trees Revealed by a Mixed Metabarcoding Strategy

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ABSTRACT

Botryosphaeriaceae are a diverse group of endophytic fungi colonizing the inner tissue of many woody species. As opportunist pathogens, they have been increasingly involved in diebacks worldwide. Nonetheless, the diversity of Botryosphaeriaceae, especially in asymptomatic plants, remains largely unknown. Using an innovative and mixed strategy of metabarcoding, this study aims to investigate the diversity of the fungal endophyte community, with a focus on Botryosphaeriaceae, which colonize grapevine and adjacent oak and pine trees in a French landscape. These data were used to test if the differentiation between hosts is more important than geographical effects for shaping the Botryosphaeriaceae communities and whether that similarity is higher between communities of grapevine and oak (both Angiosperms) than between oak and pine trees. We revealed a high level of diversity in Botryosphaeriaceae fungi, in both grapevines and forest trees, with a greater richness for grapevines. Contrasting results were obtained for the endophytic community, which was more diverse in forest trees. Our results support the hypothesis that host factors prevail on geographic effects to explain the diversity of Botryosphaeriaceae at the studied spatial scale. However, the features of the agroecosystem, such as management practices, were suggested to be more important than phylogeny to structure the fungal community. This highlights the importance of management practices for the microbiome of plants.

Keywords: agronomy, *Botryosphaeria*, *Diplodia*, ecology, endophytes, heterogeneous landscape, fungal community, latent pathogens, management practice, microbiome, mycology, *Neofusicoccum*, plant pathology

The fungal family of the Botryosphaeriaceae (Ascomycota, Dothideomycetes, Botryosphaeriales) includes common and widespread pathogens of woody hosts, most of which belong to the *Lasiodiplodia*, *Neofusicoccum*, *Dothiorella*, *Diplodia*, and *Botryosphaeria* genera (Slippers et al. 2017). Recent surveys define 23 different genera and more than 180 species from culture (Slippers et al. 2017; Yang et al. 2017). The majority of these species, if not all, share the ability to live endophytically and asymptomatically in plant tissues for long periods of time (Slippers and Wingfield 2007). They can also switch to pathogenic "behavior" usually following a period of stress experienced by the host, provoking various symptoms, such as leaf spots, fruit and root rot, dieback, and cankers (Slippers and Wingfield 2007). Hence, members of the Botryosphaeriaceae are often described as latent or opportunistic pathogens (Slippers and Wingfield 2007; Yang et al. 2017).

Botryosphaeriaceae fungi are being increasingly reported to damage woody hosts, and several new species and pathogen–host associations have been steadily described (Alves et al. 2013; Brodde et al. 2018; Díaz et al. 2018; Li et al. 2018; Slippers et al. 2017; Zhou 2017; Zlatković et al. 2018). For example, the frequency of grapevine trunk disease has been on the rise worldwide (Bruez et al. 2012; Fontaine et al. 2016), with *Diplodia seriata*, *Diplodia mutila*, *Neofusicoccum parvum*, *N. australe*, *N. luteum*, *Lasiodiplodia theobromae*, and *Botryosphaeria dothidea* being the most frequent and pathogenic Botryosphaeriaceae species associated with this disease (Chethana et al. 2016; Urbez-Torres 2011). Interestingly, some of the species are not restricted to grapevine. *B. dothidea* has an important impact on pistachio production in China and the United States, and also affects native trees,

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such as acacia in Africa (Marsberg et al. 2016). Similarly, *Diplodia seriata* and *Diplodia mutila* have been isolated from fruit rot and canker of apple trees in North and South America and are becoming new threats for apple production (Crespo et al. 2018; Díaz et al. 2019; Úrbez-Torres et al. 2016). These two *Diplodia* species have also been described as causal agents of olive and nut tree dieback in the United States (Moral et al. 2019; Úrbez-Torres et al. 2013) and Europe (Kaliterna et al. 2012), as well as many other native and nonnative angiosperm and gymnosperm trees (Alves et al. 2013; Phillips et al. 2013; Zlatković et al. 2018). *Neofusiccocum luteum*, *N. parvum*, and *N. australe* have been frequently isolated from coniferous trees (Alves et al. 2013), and *L. theobromae* has been isolated from 252 different host genera, including both angiosperms and gymnosperms (U.S. National Fungus Collections Fungus-Host Database, checked 3 April 2019). *Diplodia sapinea* has never been found on grapevine, but is probably one of the most damaging pathogens of conifers. First reported as an agent of pine damage in the southern hemisphere in the early 20th century, it has been more recently associated with many outbreaks in Europe and worldwide (Bihon et al. 2011; Brodde et al. 2018; Decourcelle et al. 2015; Fabre et al. 2011; Luchi et al. 2014; Paez and Smith 2018; Stanosz et al. 2002). In only rare cases, *Diplodia sapinea* has been isolated from angiosperm species (Zlatković et al. 2017).

Climate change is likely to be a major factor linked to the emergence of diseases associated with Botryosphaeriaceae fungi, because it increases the intensity and frequency of stress experienced by trees, and it creates better growing conditions for thermophilic species (Desprez-Loustau et al. 2006; Sturrock et al. 2011). Additionally, the occurrence of Botryosphaeriaceae fungi in asymptomatic plants (including seeds) explain why these pathogens may be unnoticed, favoring their worldwide dissemination and diffusion through nursery trade (Decourcelle et al. 2015; Marsberg et al. 2016). The opportunistic "behavior" of the Botryosphaeriaceae may also explain why the role of Botryosphaeriaceae has been overlooked in several decline syndromes, especially in grapevine, since the same species were detected or isolated from both healthy and declining plants (Bruez et al. 2014; Úrbez-Torres et al. 2006). The broad host range of at least some Botryosphaeriaceae species (although host range is limited to the level of knowledge about the species) suggests that there is no barrier to disease transmission between native and nonnative plant species, or cultivated/urban and natural settings (Begoude Boyogueno et al. 2012; Burgess et al. 2006; Marsberg et al. 2016; Mehl and Roux 2017; Sakalidis et al. 2011; Stanosz et al. 2007; Zlatković et al. 2018). For example, no gene flow restriction was observed between *B. australis* populations from *Eucalyptus globulus* plantations and from native eucalypt forests in Australia (Burgess et al. 2006); nor was it found between *Lasiodiplodia* populations in Congo from *Theobroma cacao*, the cultivated cacao tree, and from the native *Terminalia* spp. timber trees (Begoude Boyogueno et al. 2012).

This ability to perform host shifts or to infect many different host species may explain the invasive success of Botryosphaeriaceae fungi, enabling them to spread in heterogeneous landscapes. In this study, we focus on a mosaic landscape formed by vineyards and forests in Nouvelle-Aquitaine, France. This region is the second largest wine-growing region in France with 216,000 ha of vineyards (especially Bordeaux vineyards), and it is among the most forested in Europe with 2.8 million ha of forest, almost 80% of which comprises maritime pine (*Pinus pinaster*) plantations. Several surveys have reported Botryosphaeriaceae fungi on various hosts in the region, especially *Diplodia sapinea* from pine trees; *Diplodia corticola*, *Diplodia mutila*, *N. parvum*, and *B. dothidea* in rarer cases from diverse hardwoods (Fabre et al. 2011, French Forest Health service database); and *N. parvum*, *B. dothidea*, *Diplodia mutila*, *Diplodia seriata*, *Diplodia intermedia*, *L. viticola*, and *Spencermartinsia viticola* from grapevine (Comont et al. 2016; Larignon et al. 2001; Nivault 2017).

A previous study in the same region used metabarcoding to investigate the fungal communities present in the air and leaves within vineyards and adjacent forests, showing strong differentiation between grapevine and forest tree fungal communities. Some results have further suggested that the major driver of differentiation between vineyards and forest patches was not dispersal but rather selective pressures associated with host plant, microclimate, and management practices (Fort et al. 2016). How these findings, obtained from three neighboring sites and for the total foliar fungal communities, can be generalized to a larger spatial scale and all groups of fungi is questionable. Our study aims to address this question by analyzing Botryosphaeriaceae diversity in comparison with the whole fungal community within a heterogeneous landscape consisting of vineyard and forest patches in South West France.

For the purpose of this study, we developed and tested an innovative DNA metabarcoding strategy that produced one dataset using Botryosphaeriaceae-specific primers and a second dataset using the "universal" fungal barcode (Schoch et al. 2012). The diversity of Botryosphaeriaceae fungi was investigated in adjacent vineyards and forest plots by sampling both healthy and symptomatic twigs from grapevines, pines, and oaks, the three main species in the ecosystems, with high environmental and economic significance. Our first objective was to assess the diversity of Botryosphaeriaceae in grapevines and forests trees at the regional

TABLE 1
Summary of the site and the sample numbers according to the appellations, the hosts, and the presence or absence of symptoms

		Number of samples (asymptomatic/symptomatic)			
Appellations	Site number	*Pinus*	*Quercus*	*Vitis*	Total
Entre deux mers	3	2/0	9/4	4/8	27 (15/12)
Graves	5	16/5	17/5	6/16	65 (41/24)
Libournais	5	16/1	19/9	7/14	66 (42/24)
Médoc	4	12/1	11/8	7/11	50 (30/20)
Pessac-Léognan	5	17/3	11/4	9/14	58 (37/21)
Tursan	3	9/0	15/4	2/16	46 (26/20)
Vin des sables	3	15/1	5/4	5/10	40 (25/15)
Total	28	87/11	87/38	40/89	352 (214/138)

scale, in terms of species richness and species composition. These data could then be used to test several hypotheses: (i) differentiation between hosts is more important than geographic effects, both for the whole fungal community and for Botryosphaeriaceae, as observed by Fort et al. (2016), and (ii) in this case, similarity is higher between fungal communities of grapevine and oak (both Angiosperms) than between oak and pine trees, according to a phylogenetic signal in the host range.

MATERIALS AND METHODS

Sampling. Samples were collected from 28 sites between 20 July and 4 August 2017, in an area in South West France spanning ~14,000 km^2 (Table 1, Supplementary File S1), with a distance between sites ranging from ~5 to ~220 km and belonging to different appellations. Appellations are delimitated geographical regions with specific geological, climate and grapevine growing conditions. Between three to five sites were sampled for each appellation, for a total of seven different appellations (Supplementary File S1). Sites were selected based on the occurrence of adjacent forests and vineyards. Sites with approximatively equivalent surface area of forests and vineyards were preferred, as well as those with forest plots composed of both pine and oak trees. Within each vineyard plot, lignified twigs from the previous growing season of about 0.5 to 2 cm in diameter were cut using sterilized pruning shears from three symptomatic and three asymptomatic grapevines, located randomly in the patch (distance between vines of 3 to ~50 m). Symptomatic grapevines were chosen on the basis of foliar symptoms linked to "grapevine trunk disease", i.e., dark red leaf discoloration causing eventual dry out of the tissues between the veins and the margin of the leaf or when the plant had undergone a severe form of the disease and was dying (Larignon et al. 2001). Lignified twigs from 0.5 to 1.5 cm in diameter from the previous growing season were cut using sterilized pruning shears for grapevine. Pine trees and oak trees were sampled from neighboring forest patches, at a distance ranging from ~10 m to a few hundred meters from the vineyard plot, either at the edge or in more central parts of the plot. Lignified twigs from 1 to 3 cm in diameter were cut from low lying branches of the crowns from 2 to 8 m high using sterilized pole pruner. Symptomatic trees were found at lower frequency than for grapevine and when found, symptomatic trees exhibited cankers, resinosis, shoot blight, and/or dieback (Supplementary File S1). Grapevine, oak, and pine twig samples were placed in individual and sterile plastic bags. In total, 352 twigs were collected and stored at –20°C until DNA extraction. For each sample, the presence of brownish/dark necrosis in the internal tissue of the living twig, and the symptomatic status of the sampled plant were noted.

DNA extraction and PCR protocols. Sample preparation was conducted inside a confined laboratory dedicated to environmental DNA preparation. Twigs were superficially sterilized using 70% ethanol. The dead and living bark of each sample was removed using a sterile scalpel and ~3-mm-long wood pieces of ~8 mm diameter were collected in 96-well plates using sterile secateurs. Wood pieces were then lyophilized overnight and ground with sterile metal beads (two per well) using Retsch MM400 until fine powder was obtained. Total DNA was then extracted from wood powder using Invisorb DNA Plant HTS 96 Kit/C (Stratec) following the manufacturer's instructions. The wood fungal community in these samples was studied by amplifying a partial sequence of the internal transcribed spacer 1 (ITS1) using the fungal specific ITS1F (Gardes and Bruns 1993) and ITS2 primers (White et al. 1990; Supplementary File S2a). The Botryosphaeriaceae community was specifically targeted by amplifying 348 bp of the 28s large subunit of the ribosomal RNA gene, referred hereafter as LSU, using the BotSp_LSU_F and BotSp_LSU_R primers, designed for the purpose of this study using Geneious 10.2.2 (Supplementary File S2a). These newly designed primers were tested against known Botryosphaeriaceae species, as well as other fungi isolated from grapevine, pine, and oak tissues in a preliminary experiment, in order to validate their specificity (Supplementary File S2a).

Library construction was done using a two-step PCR amplification. The first PCR amplification was carried out using locus specific primers, preceded by particular molecular sequences (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG for the forward primers and TCTCGTGGGCTCGGAGATGTGTATAA GAGACAG for the reverse primers). In order to limit the stochastic biases introduced by the PCR (Zinger et al. 2019), amplification was replicated twice in a final volume of 20 µl for each DNA sample. Negative controls for 96-well plate sample preparation, DNA extraction, and PCR were included. The PCR mix was composed of 4 µl of 5× Hot Firepol Blend Master Mix (Solis Biodyne), 0.4 µl of each primer at 10 µM, 5 µl of template DNA at ~20 ng/µl, and 10.2 µl of pure-grade water for a final reaction volume of 20 µl. PCR conditions consisted of a denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 20 s, primer hybridization at 55°C for 30 s, and elongation at 72°C for 30 s. Duplicates of PCR products from each primer set were pooled and a second amplification was performed to add the indexes (one different index per sample) and the Illumina adapters. This PCR amplification was carried out in a final volume of 20 µl. Each PCR mix included 4 µl of PCR1 products, 4 µl of 5× Hot Firepol Blend Master Mix (Solis Biodyne), 4 µl of S index 2 µM, and 4 µl of N index 2 µM (Nextera Set2 indexes, Illumina), and was supplemented with ultra-pure water. The PCR reaction conditions are as follows: denaturation step at 95°C for 15 min followed by 12 cycles comprising denaturation at 95°C for 15 s, hybridization of the primers at 60°C for 1 min, and elongation at 72°C for 1 min followed by a final extension at 72°C for 10 min. ITS1 and LSU libraries were then pooled separately in equivolume and purified with CLEAN NA (Proteigene) beads (1.8× ratio). The ITS1 and LSU final pools were then checked on Tapestation (Agilent Technologies) to verify the size of the libraries and quantified in qPCR with the Kapa libraries quantization kit (Roche). The ITS1 and LSU pools were sequenced in different Illumina MiSeq runs, using MiSeq Sequencing Reagent Kit v3 (600 cycles) and v2 (500 cycles), respectively. Reads produced in this analysis are retrievable from the NCBI database of the BioProject accession number PRJNA508496.

Mock community preparation and sequencing. In order to evaluate the family-targeted metabarcoding approach, DNA from two mock communities was tested. The first mock community was composed of six Botryosphaeriaceae species: *Diplodia mutila*, *Diplodia seriata*, *Diplodia corticola*, *Diplodia sapinea*, *Dothiorella viticola*, and *N. parvum*. The second included eight species, including two Botryosphaeriaceae: *Diplodia intermedia*, *B. dothidea*, *Epicoccum nigrum*, *Eutypa lata*, *Alternaria* sp., *Aureobasidium pullulans*, *Cladosporium* sp., and *Phoma* sp. (Supplementary File S2b). For each mock community, DNA extracted from all species in pure culture was mixed with 1 µl of DNA concentrated at 0.8 ng/µl for each species. Each DNA mixing was done in duplicate. All DNA mixtures were amplified following the procedure described above for both Botryosphaeriaceae family using BotSp_LSU_F and BotSp_LSU_R primers and for fungal community using ITS1F and ITS2 primers. Amplification duplicates of each DNA mixture were pooled prior to sequencing, giving a total of four datasets: DNA mixtures for the two mock communities, amplified either with the ITS

marker or with the Botryosphaeriaceae specific LSU marker. A first PCR was done using the same locus specific primers and using similar PCR conditions mentioned in "DNA extraction and PCR protocols" section. PCR products were purified with AMPure beads (1.8× ratio) and a second PCR was done with 4 µl of purified PCR1 products, 12 µl of 2× KAPA HiFi HotStart Ready Mix, 4 µl of S index, 4 µl of N index (Nextera Set2 index, Illumina) and 4.5 µl of ultra-pure water. The PCR conditions comprised: a denaturation step at 95°C for 3 min followed by 12 cycles comprising denaturation at 95°C for 30 s; hybridization of the primers at 60°C for 30 s; elongation at 72°C for 30 s followed by a final extension at 72°C for 5 min. The purification of the libraries was carried out using AMPUre magnetic beads (ratio of 0.9×). Libraries were quantified and standardized at 10 ng/µl before pooling in equivolume. The final pool was then checked on Tapestation to verify the size of the libraries and quantified in qPCR (LC480; Roche) with the KAPA Library Quantification Kit (Roche). Sequencing was conducted on Illumina MiSeq using Nano kit v2 producing paired 250 nucleotide long reads.

Database construction. A dedicated database was constructed composed of fungal ITS sequences retrieved from the general release for fungi of the UNITE Community database (UNITE general FASTA release for Fungi Version 18.11.2018. UNITE Community) including 71,042 "fungal species hypotheses" as defined by the UNITE community (i.e., terminal fungal taxa represented by at least one ITS sequence at a distance of 1.5% of any other sequences, among which 97% singletons). Of the 71,042 entries, only 66,647 that provide information at least at the phylum level were kept. Our database also included LSU sequences from

TABLE 2
Taxa identified from the mock sequencing[a]

Database sequence(s)	Read count	Taxonomy	Botryosphaeriaceae mock					
			Diplodia corticola	*Diplodia mutila*	*Diplodia sapinea*	*Diplodia seriata*	*Lasiodiplodia vitis*	*Neofusicoccum parvum*
LSU sequencing								*
Bot_LSU_TaxID_61	7,094	*N. australe*						*
Bot_LSU_TaxID_27	3,629	*Diplodia* sp.	*	*	*	*		
Bot_LSU_TaxID_1	2,591	*Diplodia mutila*, *Diplodia pyri*		**				
Bot_LSU_TaxID_15_48	2,213	*Diplodia* sp., *Dothiorella* sp.	*	*	*	*		
Bot_LSU_TaxID_39_42	1,249	*Diplodia* sp.[b]	**	*	**	**		
Bot_LSU_TaxID_59	947	*Neofusicoccum* sp.						*
Bot_LSU_TaxID_55	168	*L. vitis*					**	
Bot_LSU_TaxID_39_42	1,7184	*Diplodia* sp.[b]						
Bot_LSU_TaxID_2	2,359	*B. dothidea*						
HE820797	108	*Mycosphaerellaceae* sp.						
ITS sequencing								
Bot_ITS_TaxID_99	6,212	*Dichomera versiformis*						*
Bot_ITS_TaxID_83	5,272	*Diplodia sapinea*, *Diplodia seriata*			**	**		
Bot_ITS_TaxID_100	5,265	*Diplodia rosulata*, *Diplodia medicaginis*	*	*	*	*		
Bot_ITS_TaxID_9	2,460	*Diplodia mutila*, *Diplodia stevensii*		**				
MH716405	148	*L. pseudotheobromae*					*	
KX784262	1,1284	*Cladosporium* sp.						
KT895930, KX516021, MG818910, GU721359, EU479964	7,700	*Alternaria* sp.						
KT693730	796	*Aureobasidium namibiae*						
Bot_ITS_TaxID_83	311	*Diplodia sapinea*, *Diplodia seriata*						
KC843295_refs	70	*Phoma foeniculina*						
FJ426996_refs	66	*Epicoccum nigrum*						
Bot_ITS_TaxID_8	27	*B. dothidea*						

(*Continued on next page*)

[a] First mock community was composed of Botryosphaeriaceae species (*Diplodia corticola*, *Diplodia mutila*, *Diplodia sapinea*, *Diplodia seriata*, *L. vitis*, and *N. parvum*) and the second mock was composed of endophyte species including two Botryosphaeriaceae (*Diplodia intermedia*, *B. dothidea*, *Epicoccum nigrum*, *Eutypa lata*, *Alternaria* sp., *Aureobasidium pullulans*, *Cladosporium* sp., and *Phoma* sp.). ** indicates exact match, and * indicates genera match with wrong or imprecise species assignation.

[b] *Diplodia corticola*, *Diplodia gallae*, *Diplodia intermedia*, *Diplodia sapinea*, and *Diplodia seriata*.

diverse fungi, retrieved from the NCBI Reference Sequences (RefSeq) (O'Leary et al. 2016) using the search criteria txid4751 [Organism] (i.e., fungi) AND "large subunit ribosomal" [All Fields] NOT "whole genome" [All Fields] and only keeping sequences with sizes ranging from 400 to 5,000 bp (n = 611 sequences). Furthermore, LSU and ITS sequences issued from a recent phylogenetic reappraisal of the Botryosphaeriales order were added, including 18 genera from the Botryosphaeriaceae family and seven other genera from six other families (Yang et al. 2017). Of these, the 159 sequences corresponding to the LSU of Botryosphaeriales species were trimmed using Geneious 10.2.2 software in order to match the regions amplified by the Bot_sp LSU primer pair. Redundant sequences were removed (n = 84). When similar sequences were associated with different taxa, the different taxon names were kept in the sequence header. The prefix Bot_LSU_TaxID_[1:75] was also added to the header for tracking the sequence in the whole database. For the 472 ITS sequences retrieved from Yang et al. (2017), only the ITS2 primer was used for trimming, because the ITS1F primer was not systematically found in the sequences. Similarly, only the 145 sequences that were unique after trimming were added to the database with proper taxa in the header, including the prefix Bot_ITS_TaxID_[1:145]. Overall, the final fungal database constructed for this study is composed of 67,478 entries, later referred to as "Database Sequences". This fasta database was deposited at the INRAE data repository and is accessible at https://data.inrae.fr/citation?persistentId=doi:10.15454/JEOT4O.

Sequence processing and taxonomic assignment. The processing of metabarcoding data were inspired by the Si5 bioinformatic strategy as proposed by Pauvert et al. (2019), which they

TABLE 2 (*Continued from previous page*)

Endophytes mock							
Diplodia intermedia	*Botryosphaeria dothidea*	*Epicoccum nigrum*	*Eutypa lata*	*Alternaria* sp.	*Aureobasidium pullulans*	*Cladosporium* sp.	*Phoma* sp.
*							
	**						
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claim exhibits the best balance between sensitivity and accuracy parameters among the 360 bioinformatic pipelines that were tested. More precisely, only the forward reads produced by the sequencer were used and filtered using DADA2 and the filterAndTrim option (Callahan et al. 2016) with the following parameters: minimal read length=100 nucleotides, sequences with more than maximum number of N allowed in the reads = 0, maximal expected errors = 1, discard reads that match the phiX genome (viral genome used to test contamination) = TRUE. Sequences were dereplicated and amplified sequence variants (ASVs) were constructed using the dada option after error rate estimation. Chimeric sequences were filtered out and the remaining ASVs were blasted against our database using blastn (v2.6.0+, Camacho et al. 2009). For each ASV, the blast results were sorted according to bit-score and only the best hit, or several best hits if sharing the same bit-score, was/were kept. After manual inspections, ASVs with no hit or having a bit-score inferior to 300 for ITS and 350 for LSU were discarded. For the LSU dataset, ASVs showing similar bit-scores for the same database sequence(s) were pooled and their read counts were added. For the ITS dataset, ASVs assigned to the same genus after blast were pooled and their read counts were added together. The taxonomic units resulting from these operations will be later referred to as "clades" for both ITS and LSU analyses. Reads were occasionally obtained in the negative controls. These reads could arise from common laboratory contamination or minor cross contamination between samples. Because most reads were found in low numbers in negative samples and they were assigned to abundant and frequent taxa in the field samples (Supplementary File S2c), we suggest that those sequences were a result of minor instances of cross-contamination between samples of highly abundant species, and they were therefore not removed in downstream analysis (Supplementary File S2c). The online version of FUNGuild (Nguyen et al. 2016, http://www.stbates.org/guilds/app.php) was used to determine the trophic mode and the ecological guild of the ASVs identified after ITS amplification.

Diversity indices and statistical analysis. Two indicators describing fungal communities were used. The first one consisted in the number of clades, or clade richness, assessed with a resolution at the genus or upper taxonomic level for the ITS amplicons (i.e., the wood fungal community), and at the species or genus level for the LSU amplicons (i.e., the Botryosphaeriaceae community). The second indicator of diversity was the number of ASV, or ASV richness, which accounted for putative different species, populations or genotypes within a clade. Generalized linear mixed-effects models (GLMM) were used to test for the effects of small geographical region ('appellation'), host (grapevine, oak or pine) and wood symptomatic status (dead/necrosis or no necrosis) on clade richness and ASV richness (Poisson distribution), as well as on the frequency of the Botryosphaeriaceae clades (binomial distribution). In the clade and ASV richness models, we added the "host × wood symptomatic status" and "host × appellation" interactions as fixed effects to test whether the effects of symptomatic status and appellation on the fungal community depended on the host. The sampling site nested within the appellation was included in the models as a random factor to account for observations clustering. Significance of fixed effects was tested using Type II analysis of variance (ANOVA, in R environment) with alpha set at 0.01. We evaluated the fit of the model to the data by calculating the percentage of variance explained by fixed effects (marginal coefficient of determination R^2m calculated using the delta method) and by fixed plus random effects (conditional coefficient of determination R^2c calculated using the delta method) (Nakagawa and Schielzeth 2013). Finally, posthoc multiple comparisons of means were performed for each significant factor using Tukey's honestly significant difference method. All analyses were done using the R programming language (version 3.5.3; R Core Development Team 2013) and *lme4* (Bates et al. 2015), *MuMIn* (Barton 2018), and *multcomp* (Hothorn et al. 2008) packages.

RESULTS

Evaluation of the targeted metabarcoding approach with mock communities. The results of sequencing the two mock communities using the fungal ITS and Botryosphaeriaceae-targeted LSU are shown in Table 2. A total of 24 and 13 ASVs corresponding to seven and five clades (with more than four reads) and belonging to Botryosphaeriaceae were identified after ITS and LSU sequencing of mock 1, respectively. With the LSU marker, five among the six Botryosphaeriaceae species included in mock 1 had a match with at least one clade corresponding to closely related species in the same genus and including the target species (Table 2). *N. parvum* was assigned to *N. australe* and only three species had an exact match with ITS. Although the DNA of each species was set to equimolar concentration in the DNA mixture, important variations in the number of

TABLE 3
Data processing for internal transcribed spacer (ITS) and large subunit (LSU) sequencing[a]

	ITS sequencing				
	Step 1	Step 2	Step 3	Step 4	Step 5
Sample	Total number of reads after sequencing	Filtrated reads/clustered in ASVs	Reads/ASVs with no Blast results	Reads/ASVs with poor Blast results	Reads/ASVs kept for analysis
Pine twigs (n = 98)	6,577,463	3,267,399/2,710	5,273/127	1,068,410/1,181	2,193,716/1402
Oak twigs (n = 125)	6,659,029	3,423,599/3,055	1,455/31	952,336/1,141	2,469,808/1,883
Grapevine twigs (n = 129)	11,113,431	5,740,793/1,617	1,613/6	320,282/308	5,418,898/1,303
Negative control (n = 6)	8,833	1,641/24	0/0	16/5	1,625/19
Total	24,358,756	12,435,073/6,252	8,341/164	2,341,044/2,324	10,082,422/3,763

(*Continued on next page*)

[a] ASVs = amplified sequence variants.

reads between taxa were observed (e.g., 168 reads for *L. vitis* versus 7,094 for *Neofusicoccum* sp. for the LSU marker (Table 2).

Mock 2 was composed of eight fungal species with two Botryosphaeriaceae species (*Diplodia intermedia* and *B. dothidea*). A total of 9 and 12 ASVs corresponding to three and seven clades (with more than four reads) were identified after LSU and ITS sequencing, respectively (Table 2). Five of the species present in this mock were identified by the ITS marker. *Eutypa lata* had no match. A misidentification occurred at the species level for *Aureobasidium pullulans* and *Diplodia intermedia* (Table 2). With LSU, most reads were assigned to a clade of *Diplodia* species including the target species *Diplodia intermedia.* Many less reads corresponded to a second clade corresponding to Mycosphaerellaceae.

In view of these results, only the LSU marker was used to describe the Botryosphaeriaceae community in the subsequent analyses, using the defined clades as taxonomic units. The ITS marker was used to describe the whole fungal community, only taking into account the genus level to avoid any misidentifications at the species level. Moreover, read counts as an indicator of species abundance were not used for this study due to the inconsistency observed in the mock analysis, and indicators of diversity using abundancy were avoided.

Endophytes and Botryosphaeriaceae species identified from the metabarcoding sequencing. MiSeq sequencing of ITS and LSU amplicons from the 353 environmental samples and the six negative controls produced 24,358,756 and 14,657,024 reads, respectively. From these, 12,435,073 ITS reads passed the quality filter and were grouped in 6,252 ASVs (Table 3); similarly, 10,278,555 LSU reads passed the quality filter and were grouped into 2,500 ASVs (Table 3). Some ASVs gave no hit after blast against the custom database: 164 ASVs in 8,341 reads (0.07% of initial read number) for the ITS sequences (Table 3) and 1,310 ASVs in 1,106,150 reads (10.8% of initial read number) for the LSU sequences (Table 3). Among ITS sequences, no assignment was found after blasting the nucleotide collection of the NCBI for 122 out of 164 ASVs, possibly corresponding to chimeric sequences or unknown sequences. For the 42 remaining ASVs (for 3,440 reads) that matched the NCBI nucleotide collection, a total of 27 different sequences from the NCBI were identified corresponding to 11 different taxa: seven plant taxa, three fungal taxa, and one bacterial taxon. Sequences corresponding to *Vitis vinifera* were the most frequent and abundant, with 1,608 reads in 52 different samples, and were all found in grapevine twig samples. Regarding the LSU sequences giving no hit after blast against the custom database, only 557 out of 1,310 ASVs for 846,742 reads matched 183 different sequences and 36 taxa from the NCBI nucleotide collection. The 753 remaining ASVs gave no hit on the NCBI nucleotide collection, probably corresponding to chimeric ASVs or ASVs from uncharacterized species. The most frequent non-Botryosphaeriales fungal taxa amplified with LSU primers belonged to Pleosporales (76.0% of samples), Mycosphaerellaceae (51.1%), *Ciborinia* (48.6%), and *Hysterographium* (32.4%). Nonmatching LSU sequences in the Botryosphaeriales order were discarded (Table 3). Finally, 37.2% of ITS sequences for 18.8% of initial read number (Table 3), as well as 43.7% of LSU sequences for 49.7% of initial read number (Table 3) were filtered out based on low blast bit-score. Details of ITS and LSU ASVs obtained throughout the pipeline are available in Supplementary File S3.

The final dataset for the ITS amplicon sequencing is composed of 3,763 ASVs for 10,082,422 reads, grouped in 565 different taxonomic clades and corresponding to genus or upper taxonomic levels. Of these, 75.2% of ASVs belonged to Ascomycota and 24.6% belonged to Basidiomycota. Probable trophic mode could be determined for 394 out of the 565 clades, revealing a majority of pathotroph or saprotroph fungi (77.7% of taxon with determined probable trophic mode, Supplementary File S4). The most frequently identified genera were *Cladosporium* (78.1% of samples), *Alternaria* (68.2%), *Aureobasidium* (58.0%), *Devriesia* (56.3%), *Mycosphaerella* (54.5%), *Vishniacozyma* (54.3%), and *Epicoccum* (54.0%). The 20 most frequently identified clades, as well as their frequencies according to host tissue, are shown in Supplementary File S4.

The final dataset for the LSU amplicon sequencing is composed of 208 ASVs for 4,063,783 reads (Table 3). The 208 ASVs matched 22 clades, each of them corresponding to a group of species and generally in the same genus. Seven clades, encompassing four genera (*Neofusicoccum*, *Diplodia*, *Botryosphaeria*, and *Dothiorella*) were identified at a frequency greater than 5% of the samples (Table 4). The most frequent clades correspond to *Neofusicoccum* spp. (31.5% of the samples, Table 4), *Diplodia* sp. (29.8%, corresponding species: *Diplodia mutila*, *Diplodia scrobiculata*, *Diplodia seriata*, and *Diplodia conspersa*), *B. dothidea* (22.73%), another clade of *Diplodia* spp. (19.03%, corresponding species: *Diplodia corticola*, *Diplodia gallae*, *Diplodia intermedia*, *Diplodia*

TABLE 3 (*Continued from previous page*)

LSU sequencing					
Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
Total number of reads after sequencing	Filtrated reads/clustered in ASVs	Reads/ASVs with no Blast results	Reads/ASVs with no Botryosphaeriaceae match	Reads/ASVs with poor Blast results	Reads/ASVs kept for analysis
3,978,758	2,635,225/1,229	423,992/724	1,754,402/405	23,451/46	433,380/54
3,686,005	2,411,420/764	441,111/339	825,492/226	834,379/166	310,438/33
6,989,772	5,231,766/715	241,047/281	1,347,357/197	323,473/99	3,319,889/138
2,489	144/21	25/12	33/4	10/1	76/8
14,657,024	10,278,555/2,500	1,106,175/1,310	3,927,284/828	1,181,313/265	4,063,783/208

sapinea, and *Diplodia seriata*), and *Diplodia mutila* or *pyri* (11.65%). Fifteen rare clades in Botryosphaeriales were identified, with frequencies ranging from 0.28 to 0.85% of the samples (Table 4).

Factors influencing fungal richness. Average clade richness per sample and per site was of 23.5 and 133.6, respectively, for the total wood fungal community, and 1.4 and 6.8, respectively, for the Botryosphaeriaceae community (Table 5). Average ASV richness per sample and per site was of 36.3 and 327.6 respectively for all fungi and 1.9 and 14.0 respectively for the Botryosphaeriaceae community (Table 5). The total number of ASVs was higher in grapevine samples ($n = 138$) (Table 3) compared with pine and oak samples ($n = 54$ and 33, respectively) (Table 3). ANOVA revealed a significant effect of host on the clade richness for both the wood fungal community and the Botryosphaeriaceae (P value < 2.2e-16 and P value < 1.02e-05, respectively) (Table 6). A significantly greater fungal clade richness was observed in *Quercus* and *Pinus* twigs than in *Vitis* twigs (P value < 1e-05 for both comparisons, Fig. 1B), and inversely, a greater richness of Botryosphaeriaceae was observed within grapevine twigs compared with pine and oak twigs (P value = 0.18 and P value < 1e-04) (Fig. 1A). No significant effect was detected for symptomatic status (presence/absence of wood necrosis) nor vineyard appellation on the Botryosphaeriaceae clade richness. Additionally, significant effects on the total fungal clade richness were observed for vineyard appellations (P value = 1.28e-04), symptoms (P value = 1.85e-03), and for the interaction of host and appellations. More precisely, a greater richness in asymptomatic samples than symptomatic ones was observed, as well as a reduced richness in Graves and Vin des Sables compared with other regions. Similar patterns than those observed for clade richness were observed for ASV richness (Table 6). However, a significant effect of the interaction of host and appellations was demonstrated on the Botryosphaeriaceae ASV richness (P value = 3.88e-13). The variance explained by the models was of 12.9% for

TABLE 4
List of the seven most frequently identified Botryosphaeriaceae clades

Database sequence(s)	Clade ID	Potential assignations within the clade	Frequency in samples (%)
Bot_LSU_TaxID_59	Clade 1	*Neofusicoccum algeriense*, *N. andinum*, *N. arbuti*, *N. batangarum*, *N. cryptoaustrale*, *N. kwambonambiense*, *N. luteum*, *N. macroclavatum*, *N. mangiferae*, *N. nonquaesitum*, *N. occulatum*, *N. parvum*, *N. pistaciae*, *N. ribis*, *N. umdonicola*, *N. ursorum*, *N. viticlavatum*	31.53
Bot_LSU_TaxID_27	Clade 2	*Diplodia mutila*, *Diplodia scrobiculata*, *Diplodia seriata*, *Diplodia conspersa*	29.83
Bot_LSU_TaxID_2	Clade 3	*Botryosphaeria dothidea*	22.73
Bot_LSU_TaxID_39, Bot_LSU_TaxID_42	Clade 4	*Diplodia corticola*, *Diplodia gallae*, *Diplodia intermedia*, *Diplodia sapinea*, *Diplodia seriata*	19.03
Bot_LSU_TaxID_1	Clade 5	*Diplodia mutila*, *Diplodia pyri*	11.65
Bot_LSU_TaxID_61	Clade 6	*N. australe*	7.10
Bot_LSU_TaxID_15, Bot_LSU_TaxID_48	Clade 7	*Diplodia coryli*, *Diplodia juglandis*, *Diplodia medicaginis*, *Diplodia seriata*, *Diplodia spegazziniana*; *Dothiorella citricola*, *Dothiorella iberica*, *Dothiorella mangifericola*, *Dothiorella omnivora*, *Dothiorella parva*, *Dothiorella plurivora*, *Dothiorella rosulata*, *Dothiorella sarmentorum*, *Dothiorella sempervirentis*, *Dothiorella vidmadera*, *Dothiorella viticola*, *Dothiorella westralis*, *Dothiorella rosulata*	5.97

TABLE 5
Mean clade richness per sample and per site and mean amplified sequence variant (ASV) richness per sample and per site[a]

	Clade richness				ASV richness			
	Total	*Vitis vinifera*	*Quercus* sp.	*Pinus* sp.	Total	*Vitis vinifera*	*Quercus* sp.	*Pinus* sp.
Sample	n.sa = 352; n.si = 27	n.sa = 129; n.si = 27	n.sa = 125; n.si = 26	n.sa = 95; n.si = 18	n.sa = 352; n.si = 27	n.sa = 129; n.si = 27	n.sa = 125; n.si = 26	n.sa = 95; n.si = 18
Per sample								
Endophytes	23.5 (±12.5)	20.0 (±8.9)	26.5 (±12.1)	24.2 (±15.6)	36.3 (±22.5)	30.4 (±14.5)	39.5 (±21.3)	39.9 (±30.0)
Botryo	1.4 (±1.0)	1.8 (±1.0)	1.0 (±0.9)	1.1 (±0.9)	1.9 (±2.3)	2.9 (±2.7)	1.1 (±1.2)	1.6 (±2.5)
Per site								
Endophytes	133.6 (±30.4)	52.2 (±14.1)	80.5 (±20.3)	74.9 (±26.5)	327.6 (±116.1)	104.4 (±35.8)	155.5 (±48.7)	165.0 (±81.8)
Botryo	6.8 (±1.7)	4.8 (±1.0)	3.8 (±1.9)	4.3 (±1.5)	14.0 (±6.9)	9.9 (±5.4)	4.3 (±2.5)	6.6 (±4.6)

[a] n.sa indicates the number of samples, n.si indicates the number of sites, and ± indicates the standard deviation.

the Botryosphaeriaceae clade richness and 47.2% for the fungal clade richness (Table 6), 31.6% for Botryosphaeriaceae ASV richness, and 69.9% for fungal ASV richness (Table 6).

Diversity of the Botryosphaeriaceae communities across host plants. Out of the 21 clades identified with the LSU marker targeting Botryosphaeriaceae, eight were found in the three different hosts, including the seven major clades identified at a frequency greater than 5% of the samples (Table 4), in addition to one rare clade (belonging to *Neofusicoccum* sp.) (Fig. 2). Twelve clades were host-specific: four to grapevine, four to oak, and four to pine. Plant-specific clades always corresponded to rare ones (identified in one to three samples); for example, *Melanops* sp. and *Dothiorella dulcispinae* or *Dothiorella oblonga* in oak twigs, *Pseudofusicoccum ardesiacum* or *Pseudofusicoccum kimberleyense* in grapevine twigs, and *Tiarosporella africana/tritici* in pine twigs (Fig. 2B). Another rare clade was shared between pine and oak (*Aplosporella/Bagnisiella* sp.). In contrast to LSU clade distribution, a large majority of the 208 ASV (n = 194, i.e., 94%) were host-specific, with more than 60% (n = 128) exclusively found in grapevine samples, 20% (n = 42) in pine samples, and 12% (n = 24) in oak samples (Fig. 2). Seven ASV were found in all three hosts, three others were shared between grapevine and pine, and two between pine and

TABLE 6
***P* value of analysis of variance from the model testing, effect of the host (genera), presence or absence of symptoms in the wood, appellation, and interaction of host and symptoms on clade and amplified sequence variant (ASV) richness[a]**

Factors/response	Botryo clade richness	Endophyte clade richness	Botryo ASV richness	Endophyte ASV richness
Host	**1.02E-05**	**<2.2E-16**	**<2.2E-16**	**5.02E-16**
Symptoms	0.297	**1.85E-03**	0.4634	**8.64E-15**
Appellations	0.991	**1.28E-04**	0.2285	**2.52E-05**
Host × symptoms	0.708	0.1318158	0.379	0.05632
Host × appellations	0.2853	**<2.2E-16**	**3.88E-13**	**<2.2E-16**
R^2m	0.129	0.472	0.316	0.616
R^2c	0.129	0.522	0.363	0.699

[a] Significant *P* values (<0.01) are indicated in bold. The estimate effects, using the marginal coefficient of determination (R^2m) or the conditional coefficient of determination (R^2c), are indicated at the bottom.

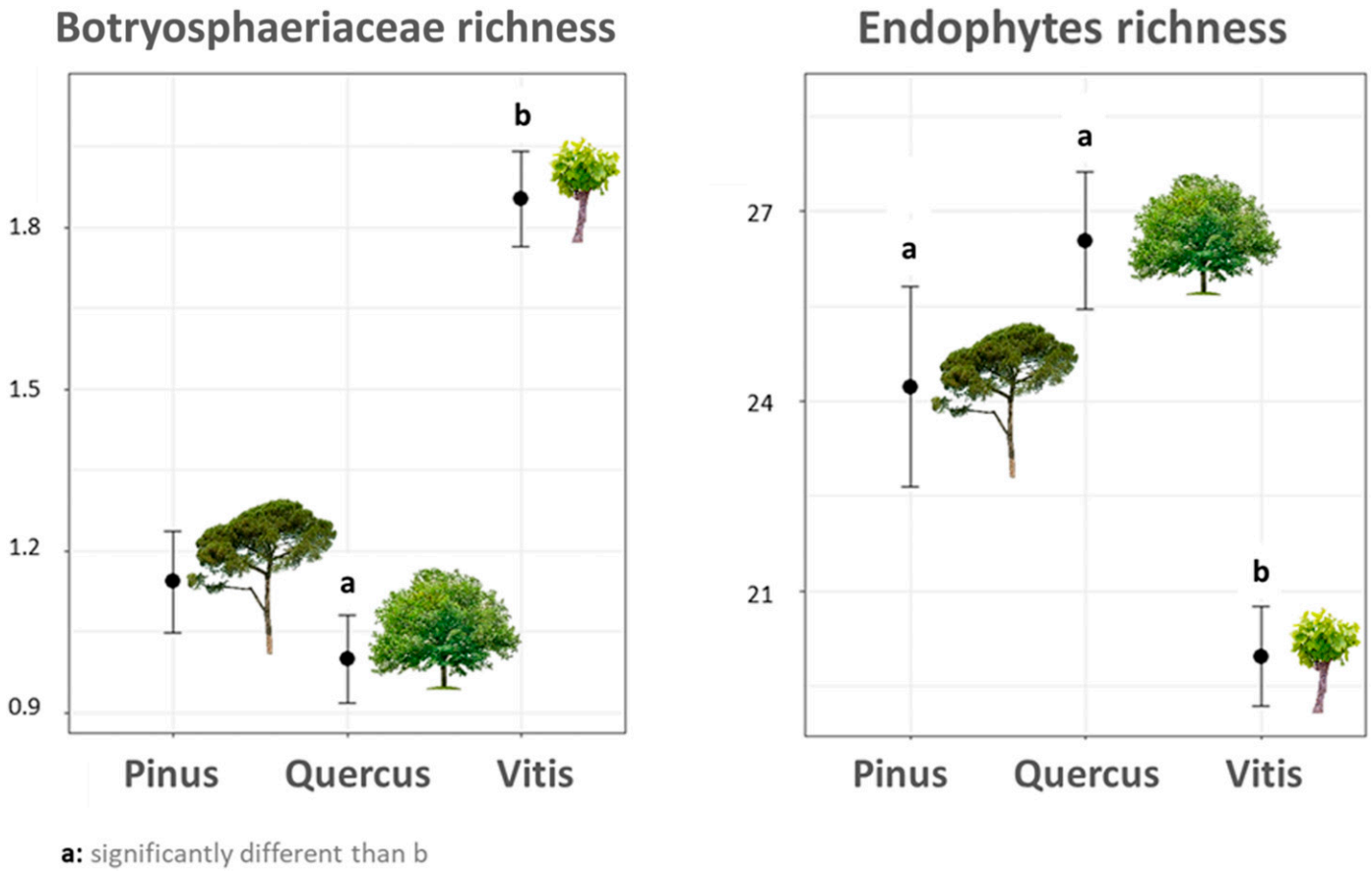

Fig. 1. Average clade diversity according to the host sampled for the Botryosphaeriaceae community and for the endophyte fungal community. Bars represent standard errors.

oak. All shared ASV, as well as the majority of plant-specific ASV (89% for grapevine samples, 58% for oak samples and 79% for pine samples) belonged to the seven major LSU clades (Fig. 2).

Effect of host and geographic location on Botryosphaeriaceae frequency. Quantitative differences of occurrence between hosts were investigated for the seven major Botryosphaeriaceae LSU clades, revealing a significant host effect for four of the seven clades: *Diplodia* sp. in clade 2, *Neofusicoccum* sp. in clade 1, and *B. dothidea* and *Diplodia* sp. in clade 4. More precisely, significant higher frequencies were observed in grapevine samples for *Diplodia* sp. in clade 2 (58.2% on average per site) compared with oak samples (17.1%, P value < 1e-6) and pine samples (18.3%, P value = 1.01e-5). Similar patterns were observed for *Neofusicoccum* sp. in clade 1 (an average of 50.6% of grapevine samples per site compared with 21.7 and 26.9% for oak and pine, respectively, significant with oak only) and for *B. dothidea* (an average of 34.5% of grapevine samples per site compared with 19.2 and 14.2% for oak and pine, respectively). The reverse was observed for the *Diplodia* sp. in clade 4, with higher frequencies in oak and pine twigs (23.2 and 26.5%, respectively) compared with grapevine twigs (6.5%, significant with oak only). Overall, *Diplodia* sp. in clade 2 (96% of the sites), *Neofusicoccum* sp. in clade 1 (93% of the sites), and *B. dothidea* (93% of the sites) were the most frequent Botryosphaeriaceae. *Diplodia* sp. in clade 4 was found in 89% of the forest patches where pine trees were sampled and 77% of the forest patches where oak trees were sampled (Fig. 3B). In contrast to the host effect, no geographic effect could be shown in the frequency of the major Botryosphaeriaceae clades and similar frequencies were obtained in the different appellations (Fig. 4).

DISCUSSION

Botryosphaeriaceae fungi have been increasingly recognized as causal agents of diseases in many different hosts and environments (Slippers et al. 2017). The wide host range of at least some species in this group has been emphasized (De Wet et al. 2008; Phillips et al. 2013; Slippers et al. 2017). Several species also show very wide geographic distributions (Phillips et al. 2013), although the natural dispersal of Botryosphaeriaceae fungi is generally assumed to mainly occur via rain-splashing of conidia (thus at rather short distances) at least for species with no sexual form (Baskarathevan et al. 2013; Silva et al. 2019; Slippers and Wingfield 2007; Úrbez-Torres et al. 2010). However, the frequent movement of these pathogens as a result of trade of plant products at both global and regional scales has been emphasized (Bihon et al. 2011; Sakalidis et al. 2013; Slippers et al. 2017). The endophytic behavior of these fungi, i.e., their ability to colonize plant tissues without causing symptoms, is probably an important factor explaining their unintentional transport in contaminated commodities and, more generally, the fact that their biogeographic distribution is still not well known.

Our study aimed to give a better understanding of the factors shaping the Botryosphaeriaceae communities at the landscape level. A striking result of our study is that the diversity patterns of Botryosphaeriaceae fungi were much more structured by the ecological compartment (forest versus vineyard) than by geographic factors in southwestern France, at a scale of several tens of kilometers. Indeed, contrary to expectations based on total fungal diversity, the richness of Botryosphaeriaceae fungi was higher in grapevine twigs than in forest trees and the patterns of richness and frequency of the major clades were more similar between *Pinus* and *Quercus* than between *Vitis* and *Quercus* (both Angiosperms). This result does not follow the phylogenetic signal hypothesis, stating that the likelihood that a pathogen can infect two plant species decreases continuously with the phylogenetic distance between the plants (Gilbert and Webb 2007).

However, a main limitation of the metabarcoding approach was the difficulty in obtaining a resolution at species level, despite some improvements having been made to the method. Finally, our study

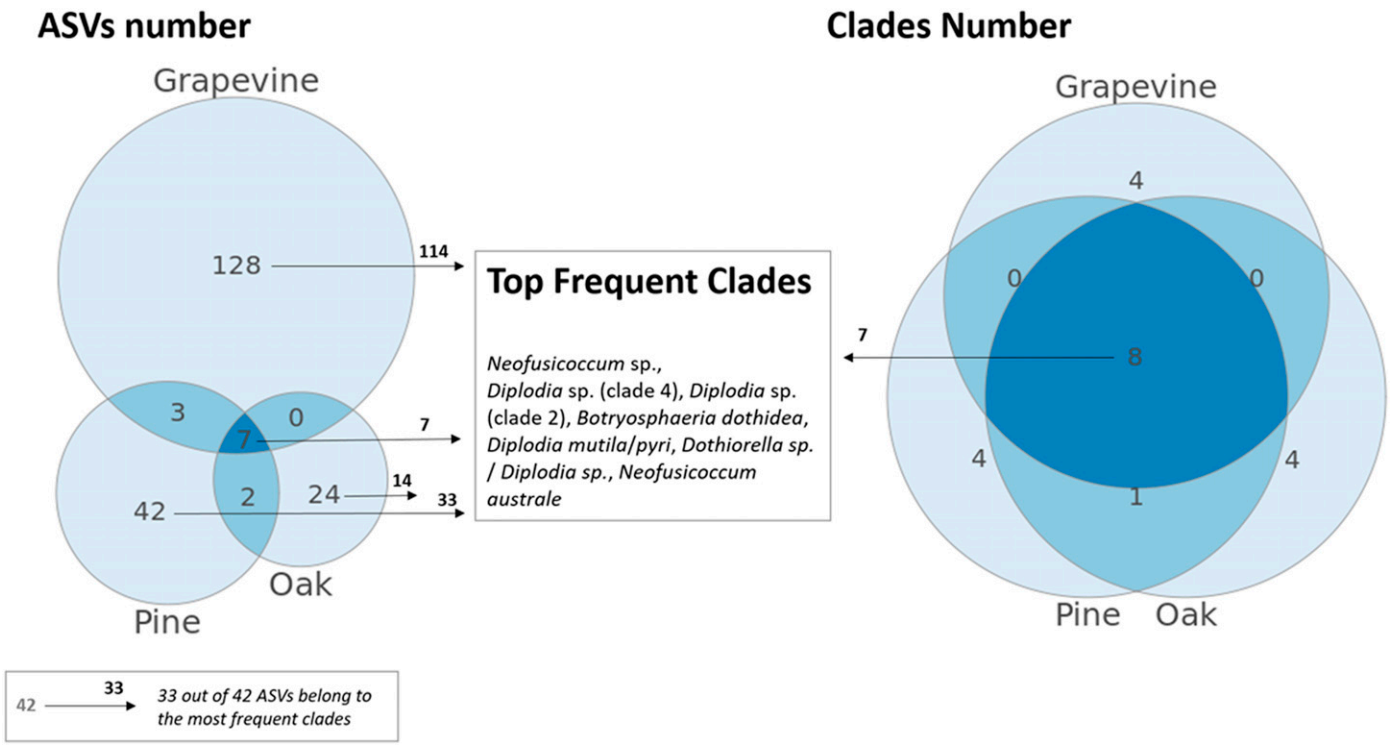

Fig. 2. Distribution of amplified sequence variants (ASVs) and clade according to hosts.

provides a first insight into Botryosphaeriaceae diversity in pines and oaks in France, showing their widespread occurrence as endophytic fungi in twigs. We discuss these main findings below.

A critical overview of the metabarcoding approach targeting Botryosphaeriaceae. Generally, metabarcoding analyses take advantage of the "universality" of the method to describe the most exhaustive snapshots of large microbial communities and to study how these communities vary in space and time. Additionally, recent examples suggest a new and increasing use of the metabarcoding approach to obtain occurrence data on targeted groups or species (Zinger et al. 2019). For example, by mining datasets constructed from the universal fungal ITS barcode sequencing, Da Lio et al. (2018) were able to identify several *Colletotrichum* species associated with the walnut anthracnose in France, and Bérubé et al. (2018) were able to detect the presence of *Diplodia corticola* in Canada by studying air samples. It is also possible to directly target the group of interest using dedicated primer pairs, giving access to more sensitive and reliable detection, as recently done by Cobo-Díaz et al. (2019) when investigating a *Fusarium* community in soil and plant samples.

Our goal was to develop such primer pairs to target the Botryosphaeriaceae family. In 2017, Yang et al. (2017) suggested that multilocus phylogeny using the LSU and RPB2 regions was effective at delineating taxa of Botryosphaeriaceae at the generic level, and RPB2 in addition to other markers like ITS were effective for species delimitation. In view of the difficulty in obtaining primer pairs specific to the Botryosphaeriaceae family for the RPB2 and ITS loci, only the LSU locus could be targeted. This marker was used as a complement to the fungal ITS marker in our study, advantageously giving valuable information about the whole fungal community. In a first attempt, the ITS dataset was constructed after assembly of both sequence pairs produced by Illumina sequencer (R1 and R2, data not shown) revealing very few Botryosphaeriaceae. We observed that the ITS1F forward primer showed mismatches with some Botryosphaeriaceae species, as previously shown with other fungal taxa (Bellemain et al. 2010; Op De Beeck et al. 2014). The second attempt at amplicon construction (inspired by Pauvert et al. (2019) and mentioned herein) used only the R1 read pair produced by Illumina sequencing and bypassed potential misassembly of the ITS sequence. This strategy drastically improved the performance of the analysis for the detection of wood fungi, including Botryosphaeriaceae. Our experience confirms that despite the ITS region being considered as the universal barcode for fungi (Schoch et al. 2012), its use can be problematic when dealing with some groups (Op De Beeck et al. 2014); moreover, the choice of bioinformatic pipeline will greatly impact the overall analysis (Pauvert et al. 2019).

In general, the designed LSU primers showed good specificity for amplifying the Botryosphaeriaceae community, but the taxonomical resolution of the amplified barcode showed only slight improvement over ITS for some groups of species, especially within *Diplodia*; this confirms the difficulty in identifying Botryosphaeriaceae with only one molecular marker, due to their close phylogenetic proximity (Slippers et al. 2013; Yang et al. 2017). Some authors have proposed methods to develop new molecular markers with high species resolution using other genes than those traditionally used in phylogeny (Feau et al. 2011), but their use in metabarcoding is currently hampered by the lack of DNA sequences in databases. Finally, our assignations were most frequently at a so-called clade-level, corresponding to several potential species. As a result, diversity was probably underestimated and host-taxa associations could not be fully described. Overall, the metabarcoding strategy developed herein is a powerful tool for giving a preliminary insight into Botryosphaeriaceae diversity in internal twig tissues, although more focused diagnostic tools will be needed to confirm the taxonomy of the species of interest.

A rich fungal community in twigs, including Botryosphaeriaceae species among other known pathogens. Knowledge about the microbiome of the plant internal tissue (the endosphere) is more limited than for the microbiome of the rhizosphere and phyllosphere, especially for trees (Terhonen et al. 2019). Nonetheless, it seems that Ascomycota species are predominant within plant tissue as observed in our study, unlike in soil environments, where

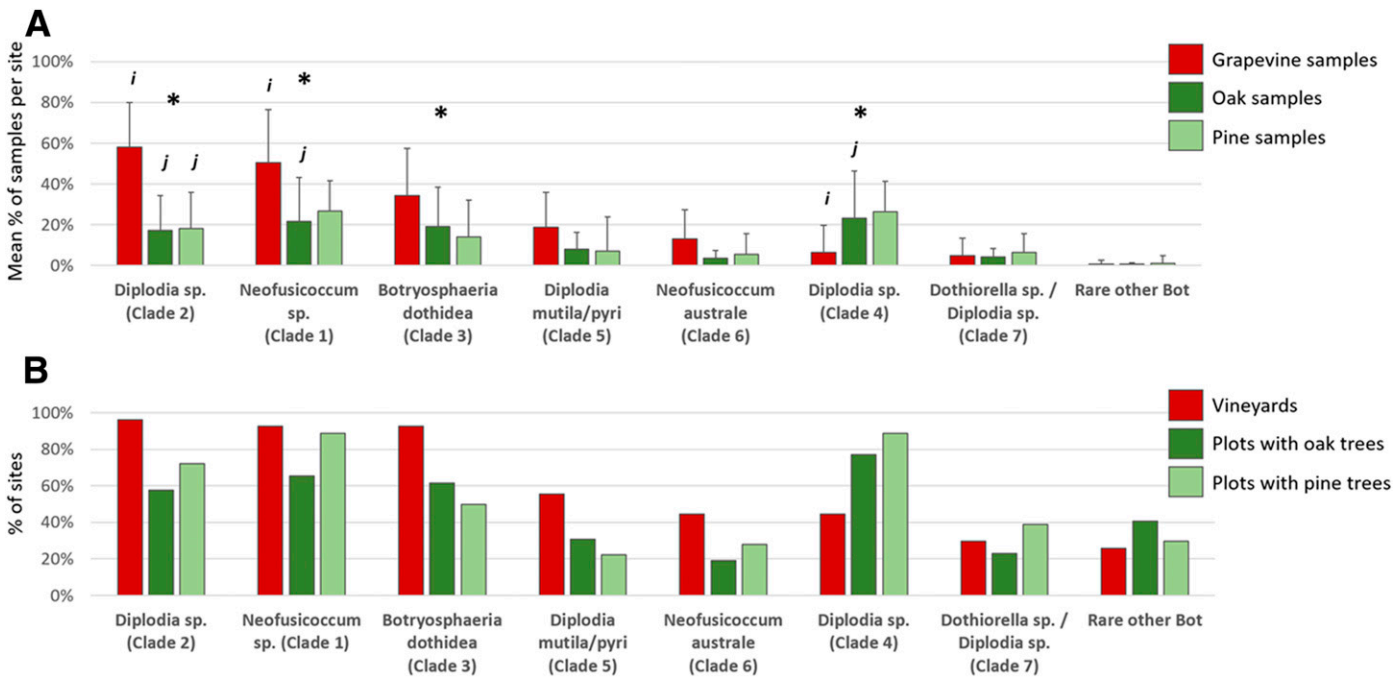

Fig. 3. Histogram of clade frequency. **A,** Average clade frequency per site. **B,** Total clade frequency across sites. * indicates significant host effect and letters (*i* and *j*) indicate significant difference between hosts.

Basidiomycota seem to be more abundant (Bruez et al. 2014; Kemler et al. 2013; Kovalchuk et al. 2018; Terhonen et al. 2019). Several taxa frequently identified in the wood tissue in this study, such as *Cladosporium* sp., *Alternaria* sp., *Aureobasidium* sp., *Mycosphaerella* sp., or *Epicoccum* sp., are well known ubiquitous fungi of the phyllosphere (Fort et al. 2016) and of the endosphere (Bensch et al. 2012; Bruez et al. 2014; Kemler et al. 2013; Pinto and Gomes 2016). Most of these frequent taxa have species members known to be plant pathogens, but this does not necessarily mean that they are pathogenic in the sampled plants (Caruso et al. 2012; Ganley et al. 2004); therefore, determining a clear ecological role for these taxa is not yet possible (Baldrian 2016). More interestingly, the large representation of these ubiquitous fungal taxa can hamper the detection of less abundant taxa, which may explain why Botryosphaeriaceae were not as frequently identified using the ITS marker as with the LSU marker.

The state of the Botryosphaeriaceae diversity in vineyards and adjacent forests in South West of France. Using the LSU marker, it was possible to identify Botryosphaeriaceae in all sampled sites and to confirm their high frequency in French vineyards as previously reported (Bruez et al. 2014). In French vineyards, seven species of Botryosphaeriaceae have been described in association with "black dead arm" disease in the past: *B. dothidea*, *Diplodia intermedia*, *Diplodia mutila*, *Diplodia seriata*, *Dothiorella viticola*, *N. parvum*, and *L. viticola* (Nivault 2017). All of these species are included in clades detected in our study, except *L. viticola.* The high frequency of *Diplodia* clade 2 and of *Neofusicoccum* clade 1 in our study is likely related to that of *Diplodia mutila*, *Diplodia seriata*, and *N. parvum*, often considered to be the most frequent and aggressive Botryosphaeriaceae species on grapevine (Bellée et al. 2016; Nivault 2017). Furthermore, we identified *N. australe*, a species originally thought to be native to the southern hemisphere and not previously reported in France, but recently reported on a variety of hosts—including on grapevine worldwide, especially in Europe, such as in Italy (Baskarathevan et al. 2017; Besoain et al. 2013; Linaldeddu et al. 2010; Pitt et al. 2010). Nonetheless, this taxonomic assignment will need to be confirmed using other genetic markers because *N. australe* could be confounded with genetically close species, like *N. luteum* which is broadly distributed in Europe (Alves et al. 2013; Barradas et al. 2013).

In French forests, a main source of information about the Botryosphaeriaceae diversity is the Forest Health Service ("DSF") database, which includes all disease reports, with suspected causative agents, made by forest health technicians. In this database (queried on 13 November 2019 for the period from 2007 to 2019), 87.6% of the 756 reports related to Botryosphaeriaceae fungi and validated by molecular methods referred to *Diplodia sapinea* on different pine species, confirming the serious impact of this pathogen on pines in France and elsewhere (Brodde et al. 2018; Fabre et al. 2011; Kaya et al. 2019; Luchi et al. 2014; Zlatković et al. 2019). However, the frequency of reports is relatively low for maritime pine compared with black pines (*Pinus laricio* and *Pinus nigra*), as also observed by Fabre et al. (2011). In oaks, a few reports from the DSF identified *Diplodia corticola/B. corticola* (n = 8), *Diplodia mutila/B. stevensii* (n = 6), *B. dothidea* (n = 1), *Diplodia seriata* (n = 1), *Dothiorella iberica* (n = 2), and *N. parvum* (n = 1) and were generally associated with observations of cankers or dieback. Linaldeddu et al. (2017) also reported *Diplodia corticola* as a cause of canker and dieback on *Quercus ilex*, *Q. petraea*, and *Q. suber* in Corsica. Hence, the high frequency of diverse Botryosphaeriaceae species on maritime pine and oak reported during this study was rather unexpected. Our finding can be explained by the high

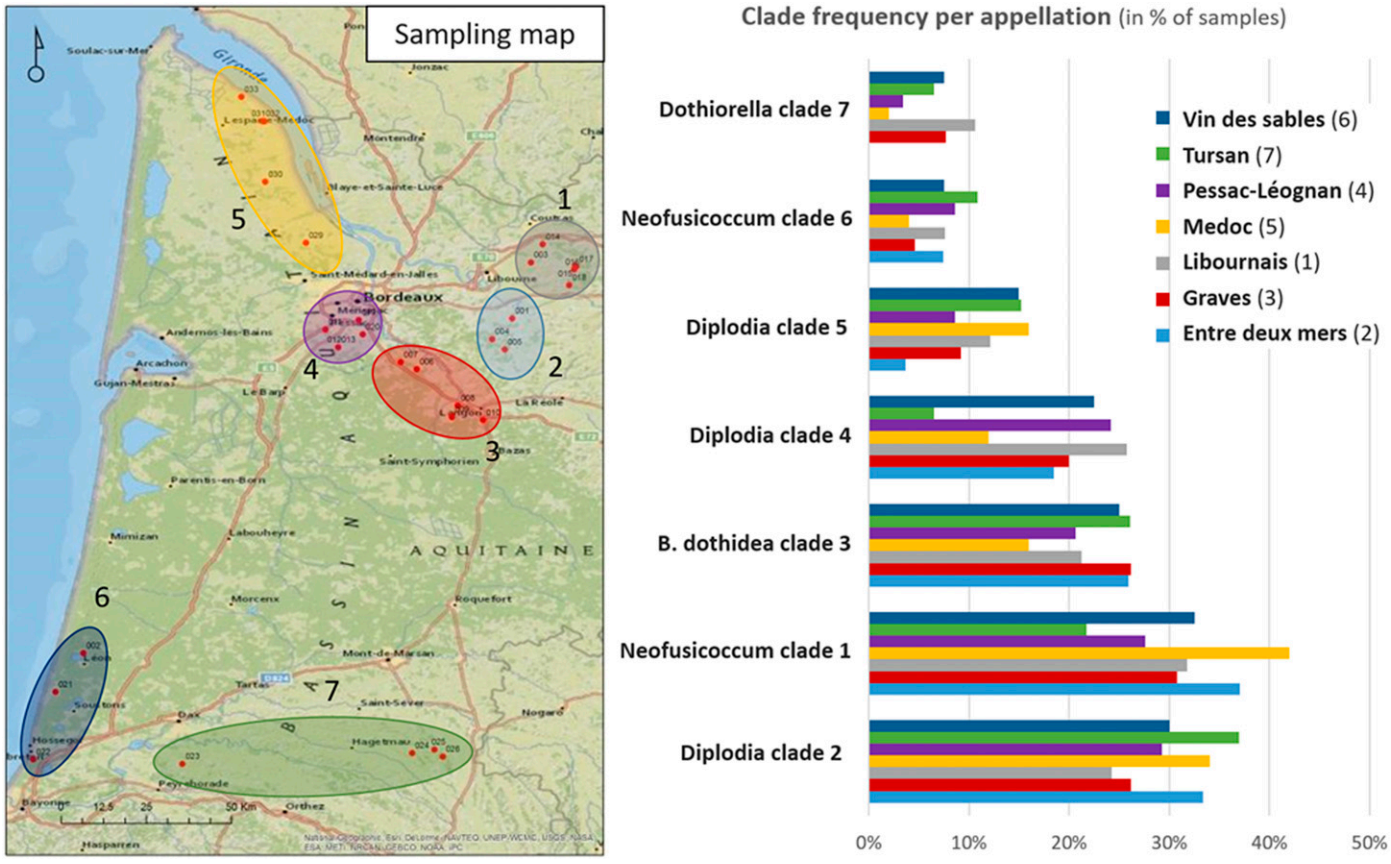

Fig. 4. Aquitaine map introducing the different appellations sampled and the histogram showing clade frequencies in these appellations.

sensitivity of the metabarcoding strategy compared with standard isolation. This may be due to the proximity of vineyards, but most importantly, it may result from the fact that asymptomatic tissues were also taken into account, in contrast to the French Forest Health Service reports that aim to identify the causal agent(s) of damaged trees.

Moreover, no association between symptoms and Botryosphaeriaceae diversity was revealed during this study. This is not really surprising since other agents of necrosis may be involved in the observed symptoms, and Botryosphaeriaceae fungi are well known endophytes, i.e., they can develop within plant tissues without causing symptoms (Bruez et al. 2014; Jami et al. 2013; Slippers and Wingfield 2007). Our results therefore support the importance of Botryosphaeriaceae species as prevalent endophytes in pines and oaks as previously suggested for other hosts and regions (Jami et al. 2013; Slippers and Wingfield 2007). Most of the species reported in the DSF database and elsewhere (Decourcelle et al. 2015; Fabre et al. 2011; Linaldeddu et al. 2017) are included in the most frequent clades identified in our study: *Diplodia* clade 4 (*Diplodia corticola*, *Diplodia gallae*, *Diplodia intermedia*, *Diplodia sapinea*, and/or *Diplodia seriata*), *Diplodia* clade 2 (*Diplodia mutila*, *Diplodia scrobiculata*, *Diplodia seriata*, and/or *Diplodia conspersa*), *Neofusicoccum* sp., and *B. dothidea*. It can be hypothesized that the high frequency of *Diplodia* clade 4 is associated with *Diplodia sapinea* (Decourcelle et al. 2015) in pine twigs, and with *Diplodia corticola* in oak twigs (Linaldeddu et al. 2017). On the other hand, the prevalence of *Neofusicoccum* species in forests is more intriguing. Recently, *N. parvum* was isolated at high frequency from declining oak stands in Italy, as well as from the insects present in the stands (Panzavolta et al. 2017), and *N. australe* was reported to be pathogenic to oaks in Portugal (Barradas et al. 2013). Severe damage by *Neofusicoccum* species have not yet been reported in French forests, and our study did not correlate *Neofusicoccum* occurrence with symptoms either. However, like the other Botryosphaeriaceae species identified during this analysis, *Neofusicoccum* species may become serious threats if the conditions become more favorable for these opportunistic pathogens especially favored by water stress (Slippers and Wingfield 2007); they should therefore be monitored more closely.

Do Botryosphaeriaceae spread from vineyard to forest and vice versa? The importance of fluxes across wild and cultivated compartments in agricultural landscapes for the epidemiology and evolution of plant diseases has only recently been emphasized and studied (Burdon and Thrall 2008; Papaïx et al. 2015). Like forest trees, grapevines are perennial plants which facilitate fungal exchanges over time. For example, it was shown that the emergence of a virulent isolate of *Venturia inaequalis* in European domestic apple orchards had its origin in a nonagricultural host (Lemaire et al. 2016) and that gene flow was high between the agricultural and nonagricultural compartments (Leroy et al. 2016). The generalist behavior of some Botryosphaeriaceae species further increases their chance of finding host plants in different compartments. For example, common pathogenic Botryosphaeriaceae species were frequently isolated from apple and pear orchards and nearby vineyards in South Africa (Cloete 2010), as well as from marula trees and adjacent mango trees (Mehl et al. 2017). For the latter case, gene flow between *N. parvum* populations colonizing both trees was demonstrated (Mehl et al. 2017). In this study, we report the existence of common Botryosphaeriaceae species between vineyard and forest, such as *B. dothidea* and *N. australe*. The identification of other common Botryosphaeriaceae species was hampered by the inability to better separate the taxonomy into more robust clades. Nonetheless, common ASVs, which are more likely to represent species and even populations within species (Callahan et al. 2016), were identified in all three host tissues; this suggests that vineyards and forest frequently share Botryosphaeriaceae species. Therefore, the risk of one pathogen spillover from one compartment to another under new selective pressures probably exists. More specific population genetic studies will help to discover the potential gene flow occurring between the populations of Botryosphaeriaceae that inhabit forests and vineyards in Nouvelle-Aquitaine.

Fungal richness and host associations are better explained by the nature of the agroecosystem than by phylogeny, including for Botryosphaeriaceae. The different indicators of richness and species frequencies used in our study consistently suggested that the Botryosphaeriaceae communities share similar patterns between oaks and pines as opposed to grapevine, despite there being a closer phylogenetic relationship between oaks and grapevines (Kumar et al. 2017). In 2016, Fort et al. (2016) showed that host species was the main factor explaining the composition and seasonal changes of foliar fungal communities in grapevines and adjacent oaks, hornbeams, and chestnuts. This host effect was suggested to include microclimatic conditions and agricultural practices that differed between grapevine and the adjacent forest patches. Consistently, our results may indicate that features of the compartment or agroecosystem, such as management practices that largely differ between the vineyards and the adjacent forests, are more likely to structure the fungal community than the host itself. The influence of human activity on Botryosphaeriaceae species composition was previously reported by Pavlic-Zupanc et al. (2015). In this study, *N. parvum* isolates was predominantly found on *Syzygium cordatum* in human-disturbed environment and was absent from natural stands, whereas other *Neofusicoccum* species predominate in these areas. The high occurrence of Botryosphaeriaceae in vineyards has already been highlighted (Jayawardena et al. 2018) and could be explained by some cultural practices over grapevine lifetime, such as pruning, which probably favor the entry and the development of such opportunist pathogens over unmanaged or less managed forest trees (Urbez-Torres 2011). Pruning practices were also suggested as an important factor explaining frequency of Botryosphaeriaceae and related cankers in various orchard crops such as avocado (McDonald and Eskalen 2011), mango (Sakalidis et al. 2011), or almond trees and other nut crops (Moral et al. 2019).

The observed lower richness of endophytic fungi in grapevines compared with forest trees could also be linked to management practices (Compant et al. 2019). Whether increased Botryosphaeriaceae richness is a cause or a consequence of an alteration of the plant microbiome through management practices requires further investigation. Nonetheless, considering the importance of microbial equilibrium for plant health (Compant et al. 2019; Hartman et al. 2018; Sharma et al. 2018; Wallenstein 2017), we believe that adapting management practices in order to maintain or enhance microbial diversity will be key to the development of durable and resilient systems for agriculture and forestry.

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