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Defining the twig fungal communities of *Fraxinus* species and *Fraxinus excelsior* genotypes with differences in susceptibility to ash dieback

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Abstract

Ash dieback disease (caused by *Hymenoscyphus fraxineus*) has affected European ash species (*Fraxinus* spp.) in recent decades. However, some Asian and American species of *Fraxinus* and certain genotypes of *F. excelsior* are less affected by the disease. We used ITS1-metabacoding to explore the drivers influencing diversity and composition of the twig fungal communities of *Fraxinus* species and *F. excelsior* genotypes. Our results revealed that fungi in the classes Eurothiomycetes and Dothideomycetes were among the most prevalent taxa in both *Fraxinus* species and *F. excelsior* genotypes. The diversity of the fungal communities differed significantly among *Fraxinus* species and could be explained by seed origin. Neither host genotype nor season had a significant effect on the community diversity of *F. excelsior* genotypes. On the other hand, the composition of twig fungal communities differed significantly among host species and among *F. excelsior* genotypes, and in *F. excelsior* there was also a significant effect of season on the composition of the fungal community. We did not find a clear effect of ash dieback susceptibility on either diversity or composition of fungal communities in twigs of *Fraxinus* species, although the effect was significant on the composition of fungal communities among *F. excelsior* genotypes. Our results demonstrated differences in fungal communities among species of *Fraxinus* and of *F. excelsior* genotypes, suggesting specific relationship between individual host genotypes and endophytic fungi.

Keywords: *Fraxinus*, *Fraxinus excelsior* genotypes, ash dieback, ITS1 rDNA, twig fungal communities

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Introduction

In recent decades, ash dieback has spread across Europe severely affecting ash stands across the continent. This devastating disease is caused by an alien fungus, *Hymenoscyphus fraxineus* (formerly known as *H. pseudoalbidus* and *Chalara fraxinea*). The fungus is believed to be indigenous in East Asia, where it occupies a specific niche as a foliar pathogen of Mandchurian ash (*F. mandshurica*) (Cleary et al., 2016; Cross et al., 2017). To date, 23 European countries, ranging from Russia to the UK, have reported the presence of ash dieback-infected and dead trees (CABI, 2018). The impact on local populations of ash and

its associated species is immense, and infection in many cases has led to increased felling.

Despite many ongoing efforts, the role of fungal endophytes in relation to ash dieback is not yet settled. Fungal endophytes live in plants for at least a certain period in their lifecycle (Rodriguez et al., 2009; Saikkonen et al., 1998) and in some cases they may improve host resistance to both biotic and abiotic stresses. For instance, *Serendipita indica* manipulated host defense responses to protect *Arabidopsis thaliana* from *Verticillium dahlia* (Sun et al., 2014). In forest trees, a contribution of foliar endophytes to quantitative resistance to *Melampsora* rust in *Populus* has been shown (Raghavendra and Newcombe, 2013), while an inoculation of endophyte-free leaves of *Theobroma cacao* with endophytes isolated from naturally infected asymptomatic hosts boosted disease tolerance to a soil-borne pathogen *Phytophthora* sp. (Arnold et al., 2003). More recently, Christian et al. (2017) demonstrated the ability of leaf litter microbiome from healthy cacao adults to protect young cacao seedlings from pathogen damage.

The genus *Fraxinus* comprises a group of deciduous trees, which are commonly distributed in the Northern Hemisphere from Eurasia to North America. The genus consists of at least 43 species, phylogenetically divided into seven clades (Hinsinger et al., 2013). Recent findings show that European species of *Fraxinus* – especially *F. angustifolia* and *F. excelsior* – are susceptible to ash dieback, while certain American species, i.e. *F. pennsylvanica*, and Asian, i.e. *F. mandshurica* and *F. lanuginosa*, seem to be less affected (Kowalski et al., 2015; Hauptman et al., 2016; Nielsen et al., 2017). Interestingly, many of the very susceptible species are members of the same clade (*Fraxinus*) as the native host, *F. mandshurica*, but are geographically distributed outside the natural distribution of *H. fraxineus*, which suggests that lack of susceptibility to ash dieback may depend on coevolution between host and fungus (Nielsen et al., 2017). Furthermore, a small proportion of *F. excelsior* and *F. angustifolia* individuals exhibit tolerance to the disease, which has been shown, to some degree, to be genetically controlled (McKinney et al., 2011; Lobo et al., 2015; Hauptman et al., 2016). The long generation time in forest trees compared to their pathogens poses a challenge to the adaptation and evolution of defence mechanisms in the hosts to the pathogens in their environment. However, the apparent tolerance observed in different species, and among genotypes within species, suggests the presence of additive factors providing host tolerance. The presence of these factors deserves further study and identification.

Only a few studies on endophytic fungal communities (endophytic mycobiomes) associated with species in the genus *Fraxinus* have been published based on isolation and cultivation of fungi (Kowalski T,

1992; Kowalski et al., 2016; Schlegel et al., 2016; Kosawang et al., 2018). In other studies, next generation sequencing was used to describe the endophytic fungal communities of *F. mandshurica* (Cleary et al., 2016), *F. excelsior* (Cross et al., 2017) and recently *F. ornus* (Schlegel et al., 2018). By describing the communities by cultivation, several uncultivable species are overlooked, and the method, therefore, does not provide a realistic characterization of the communities. In this study, ITS1-based amplicon sequencing was applied to explore twig fungal communities of eight *Fraxinus* species from different evolutionary clades and seed origin (America, Asia and Europe), and of seven Danish genotypes of *F. excelsior*. These *Fraxinus* species and *F. excelsior* genotypes were previously found to have different levels of tolerance to ash dieback (McKinney et al., 2011; Lobo et al., 2015; Nielsen et al., 2017). Based on this material, we raised the question whether the fungal communities associated with different *Fraxinus* species and *F. excelsior* genotypes differed significantly from each other. We additionally questioned whether environmental and evolutionary drivers (season, host clade, seed origin, and susceptibility to ash dieback) could explain potential differences among fungal communities.

Materials and Methods

Sample collections and preparations

A total of 20 individuals from eight *Fraxinus* species from different evolutionary clades, distribution ranges and levels of susceptibility to ash dieback (Nielsen et al., 2017) were selected from the arboretum in Hørsholm (hereafter Hørsholm study – 55° 52' 11.141" N, 12° 30' 8.902" E). Each species was represented by two or three individuals originating from the same seed lot (same accession; Supplementary Table 1). To deepen our understanding of twig fungal community in *F. excelsior* and resistance to ash dieback, seven additional Danish genotypes of *F. excelsior* with 2-3 ramets per genotype (a total of 20 ramets) were sampled from a clonal seed orchard in Tuse Næs (hereafter the Tuse Næs study – 55° 52' 11.141" N; 11° 42' 47.077" E) (Supplementary table 2). Susceptibility to ash dieback of these selected genotypes was monitored as described in (McKinney et al., 2011; Lobo et al., 2015). Samples were collected in 2015 in May (before sporulation of *H. fraxineus*) and September (after sporulation of *H. fraxineus* which peaks in July-August in Denmark). Diseased leaves in ash tree crowns were clearly visible at both sites during the collection in September.

Only healthy-looking twigs were collected and used in this study. Three terminal twigs (ca. 30 cm)

were taken from 3 different locations (preferably northern, eastern and southern axes of the trees) of the lower half of the crowns of each individual/ramet. For each season, a total of 6 or 9 twigs per *Fraxinus* species (3 twigs per tree) and 6 or 9 twigs for the *F. excelsior* genotypes (3 twigs per ramet) were sampled. In total, 240 twigs from the two study sites were collected. The samples were stored in plastic bags and kept at 4°C before processing within 48 h. Ten-cm-long twig segments were taken from each sample for surface sterilization as follows: 1 min of 95% ethanol, 5 min of 4% sodium hypochlorite, 1 min of 95% ethanol, and subsequent washed with sterile water twice. The sterilized twig segments were air-dried under laminar flow for 10-15 min before being transferred to a 50-ml Falcon tube and stored at -70°C.

DNA purification, PCR and amplicon sequencing

Wood discs of 1-2 mm thickness containing mixed cell types (bark, sapwood, xylem, phloem and cambium) were cut from the middle of the wood segments. Approximately 40 mg of wood discs from each axial twig from the same individual were pooled and freeze-dried for 72 h before being ground for a minute at 30 Hz twice using a Mixer Mill MM400 (Retsch, Germany). DNA was extracted from the pulverized samples using Nucleospin® Soil (Macherey-Nagel, Germany) following the manufacturer's instructions. The DNA was quantitated using Nanodrop 2000 (ThermoFisher, USA) and stored at -20°C.

The Internal Transcribed Spacer 1 (ITS1) region of fungal endophytes from the *Fraxinus* species and *F. excelsior* genotypes was amplified using the primer pair BITS/B58S3 (Bokulich and Mills, 2013) containing appropriate Illumina Nextera i7 and i5 adapters. The 25-µl PCR reaction mixtures contained 100 ng of DNA, Fidelity™ PCR master mix (Affymetrix, USA) and 0.2 µM each of the primers. PCR was set up on a BioRad C1000 thermal cycler (Bio-Rad, USA) with the following conditions: 94°C for 2 min followed by 32 cycles of 94°C for 30 s, 54°C for 30 s, and 68°C for 30 s with final extension at 68°C for 3 min. Each sample was amplified in triplicate. The triplicates were pooled, checked on a 1% agarose gel and purified using GenElute™ PCR Clean-Up kit (Sigma-Aldrich, USA). The purified samples were sent for further processing, including barcode annexing and sequencing with Illumina MiSeq V3 chemistry at Genome Analysis and Sequencing Center at the University of Texas in Austin.

Bioinformatic workflows

Only forward reads were used in this study. Primers, adapters and low-quality bases (q < 20) were

removed from raw reads using BBduk version 35.82 (<http://jgi.doe.gov/data-and-tools/bbtools/>). High quality reads were subsequently parsed to QIIME version 1.9.1 (Caporaso et al., 2010), where chimeric sequences were identified with USEARCH version 6.1 (Edgar, 2010). The identified chimeric sequences and low frequency reads (<5 reads) were dislodged from further processes. Operational Taxonomic Units (OTUs) were clustered at 97% similarity using USEARCH 6.1 implemented through the pick open reference OTU workflow of QIIME 1.9.1 without the final *de novo* OTU picking step. Taxonomy assignment was also carried out using BLAST algorithm (Altschul et al., 1990) with default settings through the pick open reference OTU workflow in QIIME 1.9.1 against the UNITE fungal ITS database version 7.1 (released 2016-08-22) (Kõljalg et al., 2013). Reads with either no blast hit or assigned to plant sequences were removed from the analysis. To identify the presence of the ash dieback pathogen *Hymenoscyphus fraxineus* in the samples, we performed a pairwise sequence alignment (e-value > e-50 and coverage > 97%) of an OTU previously identified as *Hymenoscyphus* sp. against the curated reference databases of Mycobank (www.mycobank.org). OTU binning of samples from the Hørsholm and Tuse Næs studies were performed separately.

For the Hørsholm data set, we rarefied all samples to 19,900 reads and for the Tuse Næs data we rarefied the samples to 6,900 reads/sample. The rarefied data sets were used for the statistical analyses. The full dataset containing read 1 DNA sequences was deposited in the DNA DataBank of Japan (DDBJ) database under BioProject PRJDB7986.

Statistical analyses

To explore effects of host clade, seed origin (America, Asia and Europe) and susceptibility to ash dieback on the *Fraxinus* endophytic twig fungal communities in the Hørsholm study, we assigned the *Fraxinus* species into different groups used for subsequent statistical analyses (Supplementary table 1). Three classes of disease susceptibility (tolerant, moderately tolerant and susceptible) were used following Nielsen et al. (2017). Shannon (alpha) diversity was computed for each sample. A Linear Mixed Model (LMM) was performed with fixed effects of species and season, and random effect of tree to examine season and overall species effects on Shannon diversity. Since species were nested in each variable (origin, clade and susceptibility), the effects of these variables were further examined in separate LMMs with the variable in question and season as fixed effects, and tree and species as random effects. Tests were carried out as

likelihood ratio (LR) tests. Dissimilarity matrices based on Bray-Curtis distances were used as input for non-metric multidimensional scaling (NMDS) ordinations to explore variation in species composition. Generalized Linear Models (GLMs) were used to identify possible OTUs responsible for differences in compositions of twig fungal communities. Negative binomial (NB) models with effects of season and species were fitted for each OTU separately, and the LR test statistics were added to give overall tests across OTUs for season and species effects (Warton et al., 2012). Test significances were evaluated by 7500 permutations, obeying that species were nested in trees and that the same trees were tested both in spring and autumn. P-values were also computed for each OTU marginally, and OTUs with Bonferroni corrected P-values (Bonferroni, 1936) smaller than 0.1 were analyzed for host clade, seed origin and susceptibility effects with an LMM as was described for the Shannon diversity index, but with log-transformed counts as outcome. For the Tuse Næs study, the same types of analyses were carried out as mentioned above to examine effects of season, genotype and susceptibility to ash dieback. The clonal susceptibility was estimated from all the biological replications in the Tuse Næs trial as the average crown damage score (PDS in 2015 and 2016; See Lobo et al., 2015). Finally, core fungi for each *Fraxinus* species and *F. excelsior* genotypes were defined as OTUs present in all individuals within the *Fraxinus* species or the genotypes of *F. excelsior* in both seasons using the compute core microbiome workflow of QIIME.

Unless otherwise stated, all statistical analyses mentioned above were carried out in R version 3.3.3 (R Core Team, 2017) using the following packages: lme4 (Bates, 2015) for LMM analyses, vegan version 2.4-3 (Oksanen et al., 2017) for computation of Shannon diversity and NMDS analyses, and mvabund version 3.12.3 (Wang et al., 2017) for the NB models with custom made code for permutation tests.

Results

Four classes of fungi dominated twig fungal communities of the *Fraxinus* species in Hørsholm

Sequencing of the ITS1 region resulted in a total of 2.02 million high-quality reads, representing a mean of 50,907 reads per sample in both seasons. The observed OTU curves (after rarefaction) of all *Fraxinus* species tended to incline toward the saturation plateau, indicating that only rare species may have escaped from the survey (Supplementary Figure 1A and 1B). At 97% similarity, a total of 343 OTUs were assigned to 12 fungal classes, corresponding to 28 orders and 48 families. Most of the OTUs belonged to ascomycetes (290 OTUs; 84.54%), 31 OTUs belonged to basidiomycetes (9.03%) and only 22 OTUs

(6.41%) were assigned to unknown taxonomic groups. We did not observe OTUs belonging to other phyla of the fungal kingdom. OTU richness was similar across seasons (291 OTUs in spring and 288 OTUs in autumn) with 236 OTUs shared between the two seasons. OTU richness was highest in the twig fungal community of *F. mandshurica* (139 and 165 OTUs in spring and autumn, respectively) and lowest in the twig fungal community of *F. angustifolia* (97 and 72 OTUs in spring and autumn, respectively) (Table 1).

The *Fraxinus* fungal communities were dominated by fungal taxa within four classes – Eurotiomycetes, Pezizomycotina (*incertae sedis*), Dothideomycetes and unidentified ascomycetes. The contribution of fungal taxa in the four classes across the twig fungal communities of the *Fraxinus* species corresponded to $\leq 32.5\%$, 21.7%, 18.8% and 14.3% of total reads in spring, and $\leq 30.3\%$, 17.5%, 19.4% and 14.3% of total reads in autumn, respectively (Figure 1A and 1B). The frequencies of these fungal taxa varied among the twig fungal communities. For example, Eurotiomycetes was most predominant in the fungal communities of the European *Fraxinus* species (*F. angustifolia* and *F. excelsior*), while Dothideomycetes were most common in the fungal communities in twigs of the Asian ash species (*F. mandshurica* and *F. chinensis* subsp. *rhynchophylla*). At genus level, *Xenocylindrosporium*, *Anhellia* and *Diaporthe* were among the prevalent genera in both spring and autumn. We were able to identify an OTU representing the ash dieback pathogen *Hymenoscyphus fraxineus* in most ash species in this study and in both seasons although we examined only healthy-looking twigs.

Fraxinus core fungi differed slightly among species and were composed mainly of the genera within Dothideales and Pleosporales (Supplementary Table 3). Some genera, including *Alternaria* and *Boeremia*, were common to the fungal communities of the *Fraxinus* species (Supplementary Table 3). Several species within the genera *Alternaria* and *Boeremia* are known to function as opportunistic plant pathogens and saprophytic fungi in their natural hosts.

Host species, but not season, affected diversity and composition of twig fungal communities of *Fraxinus* species in the Hørsholm arboretum

The levels of fungal diversity, as measured by Shannon diversity index, differed significantly among the twig fungal communities of different *Fraxinus* species ($P < 0.0001$), but not between seasons ($P = 0.81$) (Table 1). The twig fungal communities of American and Asian *Fraxinus* species had marginally higher Shannon diversity than those associated with European *Fraxinus* species (Table 1; Figure 2A). The highest and lowest diversity in spring were found in the twig fungal communities of *F. chinensis* f.sp. *rhynchophylla*

and *F. angustifolia*, respectively, similar to the results of OTU richness. On the other hand, in autumn, the twig fungal community of *F. mandshurica* had the highest diversity, while the lowest diversity remained in the twig fungal community of *F. angustifolia*. The LMM analysis indicated a significant effect of seed origin on the species differences ($P=0.0013$), but not by host clade or susceptibility to ash dieback ($P=0.38$ and $P=0.28$, respectively) (Figure 2A-C).

Non-metric multidimensional scaling (NMDS) on Bray-Curtis dissimilarities resolved no clear clusters determined by either species or season (Figure 3A and 3B). Observations from the same species tended to cluster together, and so did species from the *Fraxinus* clade, but within-species variations were also large. Although the Figures 3A and 3B showed only weak patterns, the GLM analyses indicated an overall significant effect of host species ($P<0.00013$), but not an effect of season ($P=0.27$), seed origin ($P=0.3$), host clade ($P=0.55$) and susceptibility to ash dieback ($P=0.47$) on the twig fungal communities of *Fraxinus* species (Table 4).

The subsequent GLM analyses indicated eight OTUs with significant effects of host species with Bonferroni corrected marginal P-values below 0.1 (data not shown). We assessed these OTUs separately using the log-transformed count-based LMM whether the observed species effect can be explained in part by one of the three variables – host clade, seed origin and susceptibility to ADB. While the LMM analysis was not appropriate for two of the OTUs, the remaining six OTUs can be explained in part by one of the three variables – i.e., host clade, seed origin and susceptibility to ADB. While two OTUs showed a significant effect of host clade, one OTU exhibited a significant effect of seed origin. A further two OTUs exhibited significant effects of susceptibility to ADB. Of the five OTUs, it was possible to identify only one OTU at genus level, whereas the remaining four OTUs could be identified only at higher levels of biological classifications. The only distinguishable OTU, OTU183 with a significant effect of seed origin, belonged to the genus *Alternaria*.

Eurotiomycetes and Dothideomycetes also dominated the twig fungal communities of *F. excelsior* genotypes in Tuse Næs

The sequencing of the seven *F. excelsior* genotypes sampled in the clonal seed orchard in Tuse Næs generated roughly 2 million raw reads for all samples in the two seasons, resulting in 6,900 reads/sample after rarefaction. At this depth, the observed number of OTUs of the samples tended to

approach the saturation plateau, suggesting that the depth was sufficient for exploration of the *F. excelsior* twig fungal communities (Supplementary Figure 1C and 1D).

In total, 214 and 201 OTUs were observed in spring and in autumn, respectively; 131 of which were shared between the two seasons, whereas 83 and 70 OTUs were specific to spring and autumn, respectively. OTU richness of the twig fungal communities differed among the genotypes of *F. excelsior* in both seasons (Table 2). The highest and lowest OTU richness were not limited to certain genotypes, suggesting that the twig fungal communities responded to temporal variables differently. It was interesting that although the Tuse Næs field trial is approximately 80 km from the arboretum in Hørsholm, dominant fungal taxa remained within Eurotiomycetes and Dothideomycetes, which contributed 61.3% and 22.5% of total reads in spring and 57.1% and 20.6% of total reads in autumn, respectively (Figure 1C and 1D). In addition, the abundance of fungal taxa from the Tremellomycetes was higher in Tuse Næs than in Hørsholm. *Alternaria*, *Anhellia*, *Aureobasidium*, *Cryptococcus*, and *Phoma* were among the most frequently observed genera and were documented across the *F. excelsior* genotypes in both seasons.

Several core fungi of the genotypes of *F. excelsior* were also classified as frequently observed taxa (Supplementary table 4). These core fungi included taxa in the genera *Anhellia*, *Aureobasidium* and *Phoma* that were present in most of the genotypes. In both seasons, the dieback pathogen *H. fraxineus* was detected in all genotypes even though only seemingly non-affected tissue samples were included in the sampling. *Hymenoscyphus fraxineus* was even recognized as a core fungal species of the genotypes 34 and 35 that are known to have low susceptibility to ash dieback disease.

Diversity and composition of the twig fungal communities of *F. excelsior* genotypes in Tuse Næs were not influenced by the same drivers

Shannon diversity of the twig fungal communities associated with *F. excelsior* genotypes was found in a range of 1.14-2.46 in spring and 0.92-2.21 in autumn, respectively (Table 2). The diversity was generally higher in spring than autumn, and there was no correlation between alpha diversity and level of resistance to ash dieback. LMM analyses of the Shannon diversity indices resulted in neither significant effect of season ($P=0.35$) nor genotype ($P=0.71$). Despite the insignificant effect of genotype, we fitted a LMM with fixed effect of the most recent disease scores with individual genotype as random effects, and found that the P-value for susceptibility to ash dieback (PDS) was insignificant ($P=0.18$) (Table 3).

The NMDS plot based on Bray-Curtis dissimilarities showed a tendency for susceptible genotypes to be separated from the moderate resistant and resistant ones, but the pattern was not confirmed when observations were plotted according to either genotype or season (data not shown). However, the subsequent GLM tests revealed that both season ($P=0.012$) and host genotype ($P=0.0077$) had significant impacts on the composition of *F. excelsior* twig fungal communities (Table 4). We additionally observed a significant effect of susceptibility to ash dieback (PDS) on the composition of *F. excelsior* twig fungal communities ($P=0.031$) (Table 4). However, none of the OTUs had either a marginal significant effect of season or genotype even though the overall test was significant. Furthermore, abundance tests with linear regression of the 75 most promising OTUs against PDS revealed no significant OTUs after Bonferroni correction.

Discussion

In recent decades, ash dieback has devastated European forests causing extensive economic and ecological losses (Schumacher, 2011). In this study, we aimed to further our understanding of the relationship between twig fungal community and susceptibility to ash dieback in *Fraxinus* species and *F. excelsior* genotypes. We concluded that the twig fungal communities of the *Fraxinus* species and *F. excelsior* genotypes were composed primarily of Ascomycota, especially those in Eurotiomycetes, Pezizomycotina (*incertae sedis*) and Dothideomycetes. Our results were uniform with the previous studies in *F. excelsior*, *F. mandshurica* and *F. ornus* that used ITS-targeted next generation sequencing although the distribution of dominant species may differ due to geographical discrimination (Cleary et al., 2016; Schlegel et al., 2018). Influence of spatial variation on fungal community compositions in trees has previously been described (Zimmerman and Vitousek, 2012; Ek-Ramos et al., 2013; Materatski et al., 2019). Comparison of the twig fungal communities of *Fraxinus* species or genotypes of *F. excelsior* maintained under homogenous environmental set-ups, as in this study, can therefore minimize spatial effects on fungal communities and allow us to investigate spontaneous fungal communities associated with such species in detail.

The diversity of *Fraxinus* twig communities was significantly influenced by host species. However, we could not find significant support to conclude that the diversity of twig fungal communities of *Fraxinus* species were also affected by either level of susceptibility to the disease (tolerant/moderately resistant/susceptible) or seasonal variables (spring/autumn) as previously shown in *Quercus marcocarpa*,

Pinus pinaster and *Ulmus* spp. (Jumpponen and Jones, 2010; Martin et al., 2013; Perez-Izquierdo et al., 2017). This may indicate that the *Fraxinus* twig fungal communities were not very dynamic in time and that only particular local fungal species, not a whole community, within the twig fungal communities may interact and be involved in determination of disease outcomes. The hypothesis is in line with our previous study on culturable endophytic fungi of ash dieback-resistant *Fraxinus* species, which suggested that only certain endophytic fungi were capable of suppressing growth of *H. fraxineus* *in vitro* (Kosawang et al., 2018). On the other hand, leaves are a main route of infection for *H. fraxineus* although the pathogen can occasionally gain access through lenticels (Husson et al., 2012; Nemesio-Gorriz et al., 2019). As endophytic fungi can be tissue- and organ-specific, it could be of interest to examine foliar endophytic fungi in such species of *Fraxinus*.

Our findings showed that the twig fungal communities of the European ash species were less diverse than that of the non-native ones. It is interesting as the studied trees were all derived from imported seeds and have been grown under similar environmental conditions (Hørsholm Arboretum) in Denmark. These differences among species with different origins (America, Asia and Europe) suggested that indigenous fungal endophytes may have been introduced with the seeds. As seeds are known to accommodate diverse fungal endophytes (Ganley and Newcombe, 2006), we can only speculate that certain fungal species were imported with the seeds and have later become associated with the seed-grown trees in the Arboretum.

A previous study in elm (*Ulmus* spp.) showed reduced diversity of fungal endophytes in resistant genotypes (Martin et al., 2013), and a similar trend, despite not being significant, was noted in this study where *F. excelsior* genotypes with less resistance to ADB had higher Shannon diversity. In both cases, a relationship between high disease resistance and reduced diversity of endophytic fungal communities is far from being fully understood and is clearly needed to be advanced. While neither host genotype nor seasonal variables had significant impact on diversity of the twig fungal communities associated with genotypes of *F. excelsior*, the composition of the communities was significantly influenced by both variables. This suggests that the twig fungal communities of *F. excelsior* genotypes responded differently to the seasonal variables, which could be due to certain fungal taxa governing resistance to ash dieback in the resistant genotypes or *vice versa*. This is similar to previous findings in other tree species, for example, aspen (*Populus tremula*) (Albrechtsen et al., 2018), narrowleaf cottonwood (*P. angustifolia*) (Lamit et al., 2014) and coffee (*Coffea arabica*) (Saucedo-Garcia et al., 2014). Furthermore, we also detected a significant effect of susceptibility to

ash dieback on the twig fungal communities of *F. excelsior* genotypes. A shift in associated microbiota and tree health has been observed in other host-pathogen systems (Gomes et al., 2019). Presence of specific fungal taxa in disease-resistant trees may possibly be considered as a signature for tree health and may benefit a breeding program for disease resistance.

A total of 13 OTUs were most abundant in the ash dieback-resistant *F. excelsior* genotypes, but none of these candidate OTUs remained significant when corrected for multiple testing. It is possible that some of the candidates may actually represent 'protective' fungal endophytes, as documented earlier in a variety of plant-pathogen systems (Arnold et al., 2003, Gonzalez-Teuber, 2016). However, with the statistical power of our study, it is not possible to conclude whether any of the identified OTUs are associated with enhanced resistance in the *F. excelsior* genotypes. The key suppressive species can vary among host-pathogen systems with many of the suppressive species being potentially pathogenic. Adame-Alvarez et al. (2017) thus showed that fungal endophytes could act as either resistance inducers or disease facilitators depending on order of arrival in relation to plant pathogens. Intriguingly, some of the candidate OTUs belong to the genus *Cryptococcus* and *Sporobolomyces*, where certain species members are known to exhibit biological control potentials (Qin & Tian, 2005; Ferraz et al., 2016). Further *in vivo* investigation is needed to conclude whether these fungal taxa play roles in promoting resistance to ash dieback.

Diaporthe and *Fusarium* are likely to be common taxa among *Fraxinus* species (Kowalski, 1992; Scholtysik et al., 2013; Cleary et al., 2016; Kowalski et al., 2016; Schlegel et al., 2016; Cross et al., 2017; Kosawang et al., 2018). Previously, several species of *Diaporthe* and *Fusarium* were associated with twigs and stems in initial and advanced stages of dieback of *F. excelsior* (Kowalski et al., 2016), and certain taxa were later shown to be pathogenic towards *F. excelsior* (Kowalski et al., 2017). So far, it remains unknown whether the core fungal taxa identified in the studied *Fraxinus* species may become pathogenic to their hosts or if they sustain their endophytic lifestyle in the individual hosts. The presence of *H. fraxineus* reads in the surface-sterilized healthy materials suggested that *H. fraxineus* establishes a latent infection and lives as an endophyte in the trees. There is currently no evidence of an endophytic behavior of *H. fraxineus* (Cleary et al., 2016; Drenkhan et al., 2017), and a recent study may suggest that *H. fraxineus* can produce asexual spores at low temperature, which may disperse and cause an early infection (Fones et al., 2016). Dvorak et al. (2016) also detected occurrence of *H. fraxineus* DNA from spore trap samples as early as 1st April, when the apothecia were still absent. To our knowledge, the production of *H. fraxineus* apothecia in Denmark and

other Nordic countries begins from early July and intensifies over time to a peak in August (Hietala et al., 2013; Cross et al., 2017). This suggests that the fungus may potentially settle in the hosts from the previous season or early spring and lie dormant in the hosts prior to becoming active in spring/summer and causing visible symptoms. Therefore, it is essential to determine whether *H. fraxineus* can live endophytically in *F. excelsior* and other *Fraxinus* species to provide a better understanding of the biology of the fungus and the disease, and to develop effective disease management strategies.

In conclusion, our results suggested that: (I) host species and seed origin have an impact on the level of diversity of twig fungal communities of *Fraxinus* species, (II) Eurothiomycetes and Dothideomycetes are core fungal taxa of the genus *Fraxinus*, and (III) host species influence the twig community compositions of *Fraxinus* species, and both host species and season affect the twig community compositions of *F. excelsior* genotypes. We found no clear effect of susceptibility to ash dieback on composition or diversity of twig fungal communities of *Fraxinus* species although the effect was significant for the composition of the twig fungal communities of *F. excelsior* genotypes. In addition to host species and temporal variables, plant compartment and tissue type have previously been shown to influence diversity and composition of endophytic fungal communities (Mishra et al., 2012; Coleman-Derr et al., 2016). Future investigations of plant compartment- and tissue-specific fungal communities of *Fraxinus* species and *F. excelsior* genotypes with more individuals may be needful to better understand the interactions that can affect resistance in *Fraxinus*; information that may prove valuable in the development of a sustainable breeding program.

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Figure legends

Figure 1 Taxonomic distribution of fungal classes in the twig fungal communities of *Fraxinus* species (A, spring; B, autumn) and *F. excelsior* genotypes (C, spring; D, autumn). Each bar represents an average relative abundance across 2 or 3 individuals per *Fraxinus* species or *F. excelsior* genotypes. Host clade and susceptibility to ash dieback for *Fraxinus* species and *F. excelsior* genotypes were described in Supplementary Table 1.

Figure 2 Combined Shannon diversity index of the *Fraxinus* twig fungal communities in spring and autumn plotted according to (A) host clade, (B) seed origin and (C) level of susceptibility to ash dieback. Box plots show median (middle lines), inter-quartile ranges (IQR, boxes), lines with a length of 1.5 times the IQR (whiskers) and extreme points outside the whisker interval (points). AN = *F. angustifolia*; EX = *F. excelsior*; LN = *F. lanuginosa*; LT = *F. latifolia*; MA = *F. mandshurica*; OR = *F. ornus*; PN = *F. pennsylvanica*; RH = *F. chinensis* subsp. *rhynchophylla*.

Figure 3 Nonmetric multidimensional scaling (NMDS) analysis of the twig fungal communities of *Fraxinus* species according to species (A) and season (B). AN = *F. angustifolia* (Fraxinus; susceptible); EX = *F. excelsior* (Fraxinus; susceptible); LN = *F. lanuginosa* (Ornus; resistant); LT = *F. latifolia* (Melioides; susceptible); MA = *F. mandshurica* (Fraxinus; resistant); OR = *F. ornus* (Ornus; resistant); PN = *F. pennsylvanica* (Melioides; moderate resistant); RH = *F. chinensis* subsp. *rhynchophylla* (Ornus; resistant). Host clade and level of susceptibility were described in brackets.

Figure 1

Figure 2

Figure 3

Table 1 OTU richness and Shannon diversity index of different *Fraxinus* species (Hørsholm study) in spring and autumn. Shannon diversity is given as mean $\pm$ standard deviation

Fraxinus species	Biogeography	Total number of OTUs ¹		Shannon diversity	
		Spring	Autumn	Spring	Autumn
<i>F. angustifolia</i>	Asia	97	72	1.00 $\pm$ 0.34	0.99 $\pm$ 0.28
<i>F. excelsior</i>	Europe	106	93	1.46 $\pm$ 0.27	1.93 $\pm$ 0.50
<i>F. lanuginosa</i>	Asia	138	108	2.22 $\pm$ 0.26	2.33 $\pm$ 0.02
<i>F. latifolia</i>	America	126	114	2.18 $\pm$ 0.15	1.94 $\pm$ 0.42
<i>F. mandshurica</i>	Asia	139	165	2.33 $\pm$ 0.62	2.56 $\pm$ 0.11
<i>F. ornus</i>	Europe	102	120	1.36 $\pm$ 0.69	1.55 $\pm$ 0.56
<i>F. pennsylvanica</i>	America	107	122	2.34 $\pm$ 0.99	2.46 $\pm$ 0.70
<i>F. chinensis</i> subsp. <i>rhynchophylla</i>	Asia	131	105	2.87 $\pm$ 0.05	2.21 $\pm$ 0.62

¹Total number of OTUs was measured as the number of different OTUs present in at least one individual for the species.

Table 2 OTU richness and Shannon diversity index of the *F. excelsior* genotypes (Tuse Næs study) in spring and autumn. Shannon diversity is given as mean $\pm$ standard deviation. Susceptibility to ash dieback (PDS) of each *F. excelsior* genotype is shown in parenthesis.

Genotypes of <i>F. excelsior</i>	Total number of OTUs ¹		Shannon diversity	
	Spring	Autumn	Spring	Autumn
No. 15 (66.1)	89	61	1.52 $\pm$ 0.81	1.00 $\pm$ 0.77
No. 23 (64.6)	76	58	1.17 $\pm$ 0.46	0.64 $\pm$ 0.18
No. 25 (90.0)	87	59	1.71 $\pm$ 0.64	1.00 $\pm$ 0.74
No. 27 (88.7)	81	89	1.33 $\pm$ 0.52	1.53 $\pm$ 0.43
No. 28 (91.1)	87	104	1.27 $\pm$ 1.09	1.47 $\pm$ 0.46
No. 34 (55.4)	48	68	0.77 $\pm$ 0.21	1.50 $\pm$ 0.31
No. 35 (24.5)	91	72	1.20 $\pm$ 1.06	0.94 $\pm$ 0.18

¹Total number of OTUs was measured as the number of different OTUs present in at least one individuals of the genotype of *F. excelsior*

Table 3 LMM analysis of Shannon diversity of *Fraxinus* species (Hørsholm study) and *F. excelsior* genotypes (Tuse Næs study)

Factor	df	LR	P
<i>Hørsholm</i>			
Species	7	32.7	<0.0001
Season	1	0.061	0.81
Biogeography	2	13.3	0.0013
Host clade	2	1.91	0.38
Susceptibility to ash dieback ¹	2	2.56	0.28
<i>Tuse Næs</i>			
Genotype	6	3.72	0.71
Season	1	0.87	0.35
Susceptibility to ash dieback (PDS) ²	1	1.80	0.18

¹ Susceptibility to ash dieback as in Nielsen et al. (2017).

² Susceptibility score to ash dieback (PDS) as in supplementary table 1

Table 4 GLM analysis of community composition of *Fraxinus* species (Hørsholm study) and *F. excelsior* genotypes (Tuse Næs study)

Factor	Sum of LR	P
<i>Hørsholm</i>		
Species	4904	<0.00013
Season	616	0.27
Biogeography	1736	0.3
Host clade	662	0.55
Susceptibility to ash dieback ¹	1473	0.47
<i>Tuse Næs</i>		
Genotype	2242	0.0077
Season	555	0.012
Susceptibility to ash dieback (PDS) ²	629	0.031

¹ Susceptibility to ash dieback as in Nielsen et al. (2017).

² Susceptibility to ash dieback (PDS) as in supplementary table 1

Supplementary Table 1 List of different *Fraxinus* species used in the study.

Species	Origin	Age	Seed origin	Evolutionary clade ^a	Susceptibility to ash dieback ^b
<i>F. angustifolia</i>	Romania	1980	Europe	Fraxinus	Susceptible
<i>F. angustifolia</i>	Romania	1980	Europe	Fraxinus	Susceptible
<i>F. angustifolia</i>	Romania	1980	Europe	Fraxinus	Susceptible
<i>F. excelsior</i>	Georgia	1983	Europe	Fraxinus	Susceptible
<i>F. excelsior</i>	Georgia	1983	Europe	Fraxinus	Susceptible
<i>F. lanuginosa</i>	Japan	1977	Asia	Ornus	Resistant
<i>F. lanuginosa</i>	Japan	1977	Asia	Ornus	Resistant
<i>F. lanuginosa</i>	Japan	1977	Asia	Ornus	Resistant
<i>F. latifolia</i>	USA	1985	America	Melioides	Susceptible
<i>F. latifolia</i>	USA	1985	America	Melioides	Susceptible
<i>F. mandshurica</i>	China	1979	Asia	Fraxinus	Resistant
<i>F. mandshurica</i>	China	1979	Asia	Fraxinus	Resistant
<i>F. mandshurica</i>	China	1979	Asia	Fraxinus	Resistant
<i>F. ornus</i>	Croatia	1971	Europe	Ornus	Resistant
<i>F. ornus</i>	Croatia	1971	Europe	Ornus	Resistant
<i>F. ornus</i>	Croatia	1971	Europe	Ornus	Resistant
<i>F. pennsylvanica</i>	USA	1972	America	Melioides	Moderate resistant
<i>F. pennsylvanica</i>	USA	1972	America	Melioides	Moderate resistant
<i>F. chinensis subsp. rhynchophylla</i>	South Korea	1977	Asia	Ornus	Resistant
<i>F. chinensis subsp. rhynchophylla</i>	South Korea	1977	Asia	Ornus	Resistant

^a according to Hinsinger et al. (2013); ^b according to Nielsen et al. (2017)

Supplementary Table 2 List of *Fraxinus excelsior* genotypes used in the study.

Clone	Origin	Susceptibility to ash dieback (% damage)
15	Denmark	66.1
23	Denmark	64.6
25	Denmark	90.0
27	Denmark	88.7
28	Denmark	91.1
34	Denmark	55.4
35	Denmark	24.5

Supplementary Table 3 Core fungal species associated with *Fraxinus* species.

<i>F. angustifolia</i>	<i>F. excelsior</i>	<i>F. lanuginose</i>	<i>F. latifolia</i>
Diaporthe OTU11 (2827) Unidentified OTU53 (384) Devriesia OTU63 (405) Unidentified OTU100 (540) Diaporthe OTU225 (383) Xenocylindrosporium OTU278 (12418) Unidentified OTU280 (89138) Anhellia OTU281 (4605) Unidentified OTU306 (353) Unidentified OTU324 (2803)	Diaporthe OTU11 (221) Unidentified OTU19 (1417) Boeremia OTU47 (116) Unidentified OTU53 (388) Devriesia OTU63 (267) Unidentified OTU100 (466) Unidentified OTU130 (17) Unidentified OTU139 (232) Macroventuria OTU142 (44) Unidentified OTU165 (3402) Unidentified OTU226 (276) Leptosphaeria OTU251 (1725) Unidentified OTU263 (33) Xenocylindrosporium OTU278 (21945) Unidentified OTU279 (53) Unidentified OTU280 (30151) Anhellia OTU281 (1034) Unidentified OTU283 (305) Unidentified OTU306 (78) Phaeosclera OTU320 (57) Unidentified OTU324 (4346) Anhellia OTU336 (57)	Diaporthe OTU11 (7980) Unidentified OTU19 (8534) Unidentified OTU53 (4861) Devriesia OTU63 (1076) Unidentified OTU73 (2570) Unidentified OTU100 (8398) Unidentified OTU139 (1396) Devriesia OTU206 (337) Diaporthe OTU225 (1512) Unidentified OTU233 (130) Xenocylindrosporium OTU278 (19120) Unidentified OTU279 (34770) Unidentified OTU280 (666) Anhellia OTU281 (1207) Unidentified OTU293 (1339) Unidentified OTU318 (1085) Unidentified OTU319 (1086) Unidentified OTU324 (31) Unidentified OTU343 (27)	Diaporthe OTU11 (12029) Unidentified OTU19 (2908) Unidentified OTU53 (2820) Diaporthe OTU61 (15) Devriesia OTU63 (173) Diaporthe OTU84 (20) Unidentified OTU100 (3568) Unidentified OTU101 (4952) Unidentified OTU116 (1562) Unidentified OTU139 (136) Diaporthe OTU158 (1019) Alternaria OTU183 (47) Mycosphaerella OTU199 (117) Devriesia OTU206 (15) Diaporthe OTU224 (10) Diaporthe OTU225 (3060) Diaporthe OTU234 (9969) Aureobasidium OTU237 (25) Leptosphaeria OTU251 (165) Unidentified OTU254 (614) Phoma OTU257 (195) Unidentified OTU259 (97) Teratosphaeria OTU277 (53) Xenocylindrosporium OTU278 (9130) Unidentified OTU279 (62) Unidentified OTU280 (23427) Anhellia OTU281 (207) Trullula OTU282 (155) Unidentified OTU294 (229) Unidentified OTU306 (373) Unidentified OTU324 (384) Unidentified OTU329 (52)

Supplementary Table 3 Core fungal species associated with *Fraxinus* species (continued).

<i>F. mandshurica</i>	<i>F. ornus</i>	<i>F. pennsylvanica</i>	<i>F. chinensis</i> subsp. <i>rhynchophylla</i>
Diaporthe OTU11 (327) Unidentified OTU105 (1108) Alternaria OTU183 (1780) Mycosphaerella OTU199 (989) Unidentified OTU202 (11374) Leptosphaeria OTU251 (3448) Teratosphaeria OTU277 (6354) Xenocylinndrosporium OTU278 (601) Unidentified OTU279 (560) Unidentified OTU280 (2249) Anhellia OTU281 (25769) Unidentified OTU317 (1232) Unidentified OTU331 (588) Anhellia OTU336 (1292)	Diaporthe OTU11 (4249) Unidentified OTU53 (13279) Devriesia OTU63 (201) Unidentified OTU100 (7268) Unidentified OTU139 (143) Devriesia OTU206 (721) Diaporthe OTU225 (697) Diaporthe OTU234 (806) Xenocylinndrosporium OTU278 (63258) Unidentified OTU279 (1805) Unidentified OTU280 (17034) Anhellia OTU281 (427) Unidentified OTU283 (215) Unidentified OTU294 (183) Unidentified OTU306 (54) Unidentified OTU324 (657) Unidentified OTU331 (158)	Unidentified OTU8 (8) Diaporthe OTU11 (4205) Unidentified OTU19 (5318) Unidentified OTU53 (889) Devriesia OTU63 (191) Unidentified OTU100 (1442) Unidentified OTU139 (71) Lophiostoma OTU177 (123) Alternaria OTU183 (22) Diaporthe OTU225 (1191) Unidentified OTU249 (106) Leptosphaeria OTU251 (5171) Unidentified OTU263 (114) Xenocylinndrosporium OTU278 (14695) Unidentified OTU279 (5974) Unidentified OTU280 (3055) Trullula OTU282 (3114) Unidentified OTU283 (5024)	Diaporthe OTU11 (219) Sclerostagonospora OTU36 (200) Unidentified OTU53 (1534) Devriesia OTU63 (895) Unidentified OTU73 (3881) Unidentified OTU100 (5322) Unidentified OTU139 (662) Sclerostagonospora OTU160 (1489) Lophiostoma OTU177 (685) Alternaria OTU183 (2551) Unidentified OTU200 (42) Devriesia OTU206 (610) Diaporthe OTU225 (43) Unidentified OTU226 (1442) Unidentified OTU241 (15) Leptosphaeria OTU251 (1668) Phoma OTU257 (625) Xenocylinndrosporium OTU278 (10159) Unidentified OTU279 (2846) Unidentified OTU280 (12517) Anhellia OTU281 (604) Trullula OTU282 (577) Unidentified OTU283 (121) Unidentified OTU293 (47) Unidentified OTU294 (19) Unidentified OTU306 (18) Unidentified OTU324 (280) Anhellia OTU336 (223)

Supplementary Table 4 Core fungal species associated with *Fraxinus excelsior* genotypes. Total reads from both spring and autumn for each OTU present in each genotype were shown in brackets.

Clone 15	Clone 23	Clone 25	Clone 27
Unidentified OTU93 (2041) Alternaria OTU171 (735) Aureobasidium OTU212 (3284) Phoma OTU230 (3143) Unidentified OTU262 (26081) Anhellia OTU266 (241) Anhellia OTU270 (35)	Unidentified OTU13 (64) Unidentified OTU40 (281) Unidentified OTU93 (644) Alternaria OTU171 (392) Cryptococcus OTU210 (12) Aureobasidium OTU212 (337) Phoma OTU230 (2631) Unidentified OTU262 (32266) Anhellia OTU266 (444) Anhellia OTU270 (115)	Diaporthe OTU7 (224) Alternaria OTU171 (321) Unidentified OTU262 (20233) Anhellia OTU266 (1164) Anhellia OTU270 (123)	Unidentified OTU2 (590) Kabatiella OTU161 (218) Alternaria OTU171 (7397) Aureobasidium OTU212 (1190) Phoma OTU230 (7760) Unidentified OTU262 (7489) Anhellia OTU266 (266)
Clone 28	Clone 34	Clone 35	
Diaporthe OTU7 (103) Unidentified OTU13 (116) Unidentified OTU40 (98) Unidentified OTU93 (361) Alternaria OTU171 (1381) Mycosphaerella OTU180 (1045) Aureobasidium OTU212 (1355) Phoma OTU230 (1263) Unidentified OTU262 (15212) Anhellia OTU266 (96) Anhellia OTU270 (20)	Unidentified OTU13 (526) Unidentified OTU40 (288) Unidentified OTU93 (583) Aureobasidium OTU212 (1009) Unidentified OTU262 (17227)	Alternaria OTU171 (2021) Phoma OTU230 (4459) Unidentified OTU262 (29145) Unidentified OTU264 (257) Anhellia OTU266 (220)	