Charles University

Faculty of Science

Study programme: Botany

Mgr. Zuzana Haňáčková

Infection pathway of *Hymenoscyphus fraxineus* and its interactions with ash mycobiota

Hymenoscyphus fraxineus – jeho infekční cesta a interakce s mykobiotou jasanu

Doctoral thesis

Supervisor: doc. Mgr. Ondřej Koukol, Ph.D.

Prague, 2023

Prohlášení:

Prohlašuji, že jsem předloženou disertační práci zpracovala samostatně a všechny použité zdroje jsem řádně ocitovala. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Declaration:

I hereby declare that I have written this thesis independently, using the listed references. I have not submitted this thesis, or any of its parts, to acquire any other or same academic degree.

In Prague/ V Praze ……

Zuzana Haňáčková

CONTENTS

ACKNOWLEDGEMENTS

My gratitudes goes to Ondra Koukol for guiding me responsibly through the entire study and for always trying to get the best out of me and never stop trying to motivate me. I thank him very much for numerous consultations and patience. I cannot forget the many other members of the mycology group and other colleagues and students from the Department of Botany, whom I thank for their valuable advice and help.

I thank to all my colleagues from VUKOZ for the wonderful time I was able to spend with them at this workplace. Namely, I would like to thank Karel Černý for his valuable ideas and Lída Havrdová for passing on many practical experiences with ash dieback.

I thank to prof. Ottmar Holdenrieder for the possibility of my short internship at ETH and Andrin Gross for his kind approach, many advices and a demonstration of Swiss professionalism in practice.

I would also like to acknowledge the many *H. albidus* "hunters" who was bringing us petioles samples.

A huge thank you belongs to my family for countless babysitting of my daughters when I needed to write.

ABSTRACT

Ash dieback is a disease that affected populations of native ash species throughout Europe at the beginning of the 21st century. It causes necrotic spots on the leaves, necrosis of shoots and branches and, especially in young individuals, the total death of the tree. In particular, *F. excelsior* is attacked by ash dieback. The causative agent of the disease is the invasive ascomycete fungus from Asia - *Hymenoscyphus fraxineus*.

The aims of this dissertation thesis were as follows: 1) to study population structure of *H. fraxineus* at different scales – at regional scale (Czech populations) and at the level of particular ash petioles, this latter was to serve as a confirmation of the pathogen infection pathway; 2) to elucidate the effect of *H. fraxineus* on saprotrophs in the litter (namely *H. albidus*) and 3) to compare endophytic mycobiota of tolerant and susceptible *F. excelsior* trees and to test antagonistic interactions of these fungi against the pathogen.

Analysis of Czech populations showed lower average gene diversity compared to other European populations with one Czech population differing significantly from the rest due to the lowest allelic richness and very low average gene diversity. The results indicate that despite minimal differences among European populations, local ecological or epidemiological factors may influence the structure of *H. fraxineus* populations. In a subsequent study, strains of *H. fraxineus* were isolated and genotyped from petioles and from lesions of adjacent shoots. In most cases, the genotype from the lesion was associated with the genotype from the proximal segment of the petiole, thus confirming the infection route of *H. fraxineus* into the shoot through the petiole-shoot junction. However, testing variables such as the number of colonized petiole segments and the number of other genotypes sharing the same segments did not show an effect on the successful entering of the pathogen into the shoot.

The possible negative effects of *H. fraxineus* on the saprotrophs of fallen petioles were monitored in the second part of the study, where we attempted to capture *H. albidus*. Fruiting bodies of *H. albidus* were not found in the Czech Republic, however ascospores of this species were detected in low concentration at four sites with spore traps. Based on this and other studies, it can be assumed that *H. albidus* is not threatened with extinction and its abundance was probably low even in the

past. Similarly, other species forming fruiting bodies on ash petioles seem to be unaffected by the expansion of *H. fraxineus*.

In the third part of the thesis, the members of classes Dothideomycetes and Sordariomycetes were isolated as the most common culturable endophytic fungal species. Although no significant differences in the overall composition of the fungal community were found between tolerant and susceptible individuals, susceptible individuals hosted more *Diaporthe* sp. Species of the genus *Diaporthe* are frequent pathogens and therefore it can be assumed that trees susceptible to ash dieback generally have a weakened ability to suppress pathogenic endophytic fungi. The inhibitory effect of 36 endophytes significantly reduced the growth of *H. fraxineus* by 43% to 83%. The highest growth reduction was achieved by the fast-growing species *Botrytis cinerea* and *Phoma macrostoma* var. *incolorata*. However, the results of the interaction with *H. fraxineus* in *planta* can be further influenced by the multiple antagonistic interactions and the ability of endophytes to grow in living tissue.

Keywords: antagonism; ash dieback; ash petioles; ash shoots; fungal endophytes; *Hymenoscyphus albidus;* population structure; saprotrophs

ABSTRAKT

Nekróza jasanu je choroba, která na začátku 21. století zasáhla populace původních druhů jasanů v celé Evropě. Způsobuje nekrotické skvrny na listech, nekrózy výhonů a větví a především u mladých jedinců celkové odumření stromu. Nekrózou jasanu je napadán obzvláště *F. excelsior*. Původcem onemocnění je nepůvodní vřeckovýtrusná houba z Asie – *Hymenoscyphus fraxineus.*

Cíle této dizertační práce byly následující: 1) studovat populační strukturu *H. fraxineus* na různých škálách – na regionální škále (české populace) a na úrovni jednotlivých řapíků, toto měřítko mělo sloužit k potvrzení infekční cesty patogenu; 2) objasnit efekt *H. fraxineus* na saprotrofy v opadu (jmenovitě *H. albidus*) a 3) porovnat endofytickou mykobiotu tolerantních a citlivých stromů a testovat antagonistické interakce těchto hub proti patogenu.

Analýza českých populací zjistila nižší průměrnou genovou diverzitu v porovnání s dalšími evropskými populacemi. Jedna česká populace se od zbytku signifikantně lišila kvůli nejnižší hodnotě allelic richness a velmi nízké průměrné genové diverzitě. Výsledky naznačují, že i přes minimální rozdíly mezi evropskými populacemi můžou lokální ekologické nebo epidemiologické faktory ovlivňovat strukturu populací *H. fraxineus*. V navazující studii byly izolovány a genotypovány kmeny *H. fraxineus* z řapíků a z lézí přilehlých výhonů. Ve většině případů byl genotyp z léze spojen s genotypem z proximálního segmentu řapíku, čímž byla potvrzena infekční cesta *H. fraxineus* do výhonu skrze spojení řapíku a výhonu. Růst patogenu skrze řapík a proniknutí do výhonu se zdá být silně filtrujícím prostředím, vzhledem k vysokému počtu genotypů v řapících jednoho výhonu, ale zároveň indukce léze výhonu nejčastěji jediným genotypem. Avšak, testování proměnných, jako je počet kolonizovaných segmentů řapíku a počet jiných genotypů sdílících stejné segmenty, neprokázalo vliv na úspěšný průnik patogenu do výhonu.

Možné negativní účinky *H. fraxineus* na saprotrofy řapíků v opadu pak byly sledovány v druhé části studie, kde jsme se pokoušeli zachytit *H. albidus*. Plodnice *H. albidus* nebyly v ČR nalezeny, avšak spory tohoto druhu byly detekovány v nízké koncentraci na čtyřech lokalitách s lapači spor. Na základě této a dalších studií se dá předpokládat, že *H. albidus* není ohrožen extinkcí a pravděpodobně jeho abundance byla i v minulosti nízká. Podobně i jiné druhy tvořící plodnice na jasanových řapících se zdají být neolivněny expanzí *H. fraxineus.*

Ve třetí části práce byly členové třídy Dothideomycetes a Sordariomycetes nejčastějšími druhy kultivovatelných endofytických hub. I když mezi tolerantními a citlivými jedinci nebyly zjištěny signifikantní rozdíly v celkovém složení společenstva hub, citliví jedinci hostili více *Diaporthe* sp. Druhy rodu *Diaporthe* jsou častými patogeny a proto se dá předpokládat, že stromy citlivé k nekróze jasanu mají obecně oslabenou schopnost potlačovat patogenní endofytické houby. Inhibiční účinek 36 endofytů signifikantně redukoval růst *H. fraxineus* o 43 % až 83 %. Nejvyšší redukce růstu dosáhly rychlerostoucí druhy *Botrytis cinerea* a *Phoma macrostoma* var. *incolorata*. Výsledky interakce s *H. fraxineus in planta* však mohou být dále ovlivněny i mnohonásobnou antagonistickou interakcí a schopností růstu endofytů v živém pletivu.

Klíčová slova: antagonismus; endofytické houby; *Hymenoscphus albidus;* jasanové výhony; jasanové řapíky; nekróza jasanu; populační struktura; saprotrofové

LIST OF PUBLICATIONS

This thesis is based on the following papers. The contribution of Zuzana Haňáčková to the individual publications is given after the title.

I. HAŇÁČKOVÁ, Z., KOUKOL, O., HAVRDOVÁ, L. & GROSS, A. (2015): Local population structure of *Hymenoscyphus fraxineus* surveyed by an enlarged set of microsatellite markers. Forest Pathology, 45/5: 400–407.
Molecular analysis, data analysis and manuscript preparation – **total contribution 60%**

II. KOUKOL, O., HAŇÁČKOVÁ, Z., DVOŘÁK, M. & HAVRDOVÁ, L. (2016): Unseen, but still present in Czechia: *Hymenoscyphus albidus* detected by real-time PCR, but not by intensive sampling. Mycological Progress, 15/1: 1-9.
Field sampling, molecular analysis and manuscript preparation – **total contribution 40%**

III. HAŇÁČKOVÁ, Z., HAVRDOVÁ, L., ČERNÝ, K., ZAHRADNÍK D. & KOUKOL, O. (2017): Fungal endophytes in ash shoots – diversity and inhibition of *Hymenoscyphus fraxineus*. Baltic Forestry, 23/1: 89-106.
Study design, field sampling, fungal isolations and identification, antagonistic tests, molecular analysis and manuscript preparation – **total contribution 90%**

IV. HAŇÁČKOVÁ, Z., KOUKOL, O., ČMOKOVÁ, A., ZAHRADNÍK, D., & HAVRDOVÁ, L. (2017): Direct evidence of *Hymenoscyphus fraxineus* infection pathway through the petiole-shoot junction. Forest Pathology, 47/6: 1-6.
Study design, field sampling, data analysis and manuscript preparation – **total contribution 70%**

On behalf of all the co-authors, I declare the keynote participation of Zuzana Haňáčková in completing the research and writing the papers, as described above.

…….

In Prague Ondřej Koukol

PART A - GENERAL INTRODUCTION

1 INTRODUCTION

1.1 ASH DIEBACK AND ITS CAUSAL AGENT

Ash dieback is a fungal disease of native European ash trees (*Fraxinus excelsior*, *F. angustifolia*); (Fig. 1). Infection occurs through natural leaf openings as leaf stomata (Cleary et al. 2013b), although lenticels on the bark and direct penetration of young shoots also allow the entry of the pathogen (Mansfield et al. 2019; Nemesio-Gorriz et al. 2019). First signs of infections include necrotic spots on leaves and petioles, wilting and premature falling of foliage. The spread of disease is characterised by ochre colored lesions appearing on the adjacent shoots and gradually growing into older branches. In response to the disease, the tree produces a number of epicormic shoots and creates a characteristic bushy appearance of the tree crown. If necrosis invades the main trunk (Fig. 1), the tree can rapidly die back (Kowalski 2006; Skovsgaard et al. 2010; Gross et al. 2012; Baral and Bemmann 2014). For this reason, the most endangered are seedlings, young trees and individuals forming epicormic shoots directly on the main trunk. Cases of root neck necrosis caused by the pathogen have been reported too (Husson et al. 2012; Chandelier et al. 2016; Skovsgaard et al. 2017). Unfortunately, trees suffering from the ash dieback are often susceptible to secondary diseases caused by honeycomb fungi (*Armillaria* spp.), bark beetles and other pathogens, which affect the main trunk and eventually kill the tree (Chandelier et al. 2016). Ash dieback is most devastating especially in young plantations and replacing dead trees with new individuals may only support further spread of the disease. Adult trees can successfully cope with dieback by rich shoot regeneration, especially in drier years when the infection pressure is lower.

Fig. 1: Plantation of European ash damaged by ash dieback (left) and necrosis caused by the disease invading main trunk of young tree via an epicormics shoot (right). Photo: Z. Haňáčková.

The first observations of dying ashes[1] in forests in Europe come from Poland and Lithuania in the early 1990s (Kowalski 2001; Juodvalkis and Vasiliauskas 2002; Przybył 2002; Kowalski and Łukomska 2005; Lygis et al. 2005) and the spread of this disease initiated the study of its cause. The putative causal agent of ash dieback was first isolated in Europe in 2006. A fulvous brown to orange brown asexual ascomycete (Fig. 2) was regularly observed among numerous isolates of endophytes and potential candidates for the pathogen. This fungus produced cylindrical conidia from phialides with a long tubular collarette (Fig. 2), typical for the genus *Chalara*. This fungus was supposed to represent a new species and was therefore described as *Chalara fraxinea* (Kowalski 2006; Baral et al. 2014). Further research is framed by several erroneous assumptions and rather surprising discoveries. *Chalara fraxinea* was first assumed to be an asexual state of the ascomycete *Hymenoscyphus albidus* (Helotiales) a common saprotroph on ash petioles (Kowalski and Holdenrieder 2009a). However, molecular analyses

[1] Ash is in the following text reffered as common European ash *Fraxinus exelsior* unless otherwise stated

subsequently refuted this opinion; multiple isolates of *C. fraxinea* formed a well supported clade sister to specimens originating from apothecia of *H. albidus.* This clade was named *Hymenoscyphus pseudoalbidus* and hybridogenous origin of this novel species that resulted in its pathogenic life style was supposed (Queloz et al. 2011). The correct origin of the pathogen was finally clarified when it was discovered that it had previously been reported by Japanese scientists from petioles of native Asian ash *Fraxinus mandshurica* under the name *Lambertella albida* (Hosoya et al. 1993). Not only was the morphology of apothecia matching, but Hosoya et al. (1993) also described production of "spermatia" that matched the *Chalara*-like anamorph (Zhao et al. 2012). The final and presently accepted name *Hymenoscyphus fraxineus* was assigned to the pathogen following nomenclature rules after the oldest basionym was combined into the phylogenetically correct genus (Baral et al. 2014). *Hymenoscyphus fraxineus* is morphologically almost indistinguishable from its sister species, but the presence of croziers on ascal basis and longer ascospores allow its identification even without the molecular data.

Together with correct name, also the life cycle of this fungus was elucidated. Fruiting bodies (apothecia) of *H. fraxineus* grow from petioles of ash leaves fallen in previous season and release ascospores infecting new leaves during the summer (Kowalski and Holdenrieder 2009a; Timmermann et al. 2011; Gross et al. 2012; Baral et al. 2014; Chandelier et al. 2014).

The pathogen is able to spread usually on shorter distances only - most ascospores fall within 50 m to hundreds of meters from the source (Chandelier et al. 2014). However, a small amount of spores can overcome even great distances. Models of airflows of University College Dublin have shown that 10 ascospores per billion can cross LaManche. By combining these short and long-range dispersions, the disease spread through Europe very quickly. In about 20 years since the first records in the Baltics, the United Kingdom was reached by the disease, and by this time, European ash has been affected also in other countries (Sansford 2013). The rate of spread in Europe was finally set at 75 km / year (Landolt et al. 2016).

Fig. 2: Colonies of *Hymenoscyphus fraxineus* growing from ash petioles cultivated on Malt Extract Agar (left). Asexual state produces phialides releasing unicellular conidia (right). Photo: Z. Haňáčková.

1.2 *HYMENOSCYPHUS FRAXINEUS* – ITS ORIGIN AND WAY OF ENTRY TO EUROPE

First observations have long escaped attention and it is thought that the introduction of *H. fraxineus* into Europe may have occurred ten to thirty years earlier than the fungus was isolated for the first time (Sønstebø et al. 2017). An example is the confirmed presence of *H. fraxineus* in Great Britain using annual ring counts as early as 7-8 years before its official recording in 2012 (Wylder et al. 2018). The first herbarium specimen of *H. fraxineus* comes from Estonia from 1997 (Drenkhan et al. 2016) and is supposed that the introduction is a result of the import of Manchurian ash (*F. mandshurica*) into the Baltic countries from Russia in 1960-1980 (Drenkhan et al. 2014; Drenkhan et al. 2017). *Hymenoscyphus fraxineus* has also been confirmed in eastern Russia as a saprotroph and an occasional pathogen of Asian ash trees (Drenkhan et al. 2017). It is estimated that the onset of the European invasion was triggered by only two haploid individuals that started the spread. These two individuals were genetically compatible and thus could reproduce sexually, which was crucial for further spread of this heterothallic species (Gross et al. 2014a; McMullan et al. 2018). However, the latest synthesis of phylogenetic, population-genetic and population genomic studies again favor the theory that the invasive behavior of *H. fraxineus* in Europe occurred after hybridization event with a closely related European ascomycete (Buschbom 2022).

1.3 *FRAXINUS EXCELSIOR* – DISTRIBUTION AND FUNCTION

European ash is widespread throughout the temperate zone of Europe from the British Isles to the Russian Volga from the lowlands to altitudes of 1800 m (Pliûra and Heuertz 2003). Its ecological niche is very wide and therefore can be found in many types of vegetation. A high proportion of this tree species in the species composition of forest stands can be found, for example, in the British Isles, where it makes up to 5% of forests and is the second most common tree species in local hedges. Similar proportion may be observed in Denmark (Vasaitis and Enderle 2017). Across whole Europe, it occurs in forests in less than 1% (Hemery 2008). It has excellent stabilizing abilities, tolerates high groundwater levels, regenerates well and is resistant to environmental stress. It is therefore used as a land reclamation tree and tree species into protective forests and belts, tree lines and windbreaks (Černý et al. 2016). There are distinguished three ecotypes of European ash in the Czech Republic. Floodplain ash in the vicinity of rivers, mountain ash at higher altitudes around streams and limestone ash in warmer areas (Gregorová et al. 2006). Floodplain and other ecosystems along rivers are currently at high risk from already present pathogens such as Dutch elm diseases (*Ophiostoma novo-ulmi*), alder decline (*Phytophthora alni*), polyphagous oomycete *Phytophthora plurivora* and other invasive species of this genus. At the same time, ash dieback prospers best in these humid conditions. The range of suitable tree species is thus very limited when replanting damaged riparian forests (Černý et al. 2016; Chumanová et al. 2021).

Another essential role of ash is in its habitat function for other organisms. Mitchell et al. 2014 assessed the potential environmental impact of ash dieback in the UK. They associated 952 species of organisms with ash, of which 44 used the ash exclusively as a habitat and 62 exceptionally occupied other hosts. They did not find any replacement tree that could alternate the ash tree. The closest similar species were pedunculate and sessile oaks (*Quercus robur, Q. petrea*), which could host 69% of the same species. Ash litter is also specific for its nutrient complexity and high rate of decomposition, which may result in the loss of more nutritionally demanding species from the undergrowth, reduced microbial diversity in the soil, overall slowdown in nutrient flow and thus also in wider ecosystem functions if it is replaced.

From an economic point of view, ash is important mainly for the production of high-quality wood material suitable for the production of furniture, sports equipment, musical instruments and other specialized products. Although ash does not belong to the most important forestry trees in the Czech Republic (in

forest stands it forms only 1.4%, Rozsypálek et al. 2017) and is often taken as a „weedy“ tree, its value is mainly in its technical and ecological functions, which are used rather outside the forests.

1.4 CURRENT SITUATION OF ASH DIEBACK

Promising findings were reported about differences in metabolomes of high and low susceptible ash genotypes. Low susceptible trees had increased levels of two types of coumarins (fraxetin and esculetin). These two substances as well as bark extract of these low susceptible trees inhibited growth of *H. fraxineus in vitro* (Nemesio-Gorriz et al. 2020). However as expected, the condition of ash forests is not developing favorably. A European-wide monitoring has revealed that the defoliation of ash crowns has almost doubled in the last thirty years and the overall probability of survival has reached a critical value of 0.51. The worst situation is in northern Europe, which faces an extreme risk of *F. excelsior* extinction, further in the Baltics, Belarus, north-eastern and south-western Germany, and also in eastern France (Fig. 3). Large scale data also did not confirm that areas with lower host densities show lower tree mortality (George et al. 2022).

Retention and propagation of resistant or tolerant trees and breeding for resistance seems the most important measures to maintain *F. excelsior* in Europe (Enderle et al. 2019). Unfortunately, breeding is challenged by the fact that emerald ash borer (*Agrilus planipennis*) is likely to spread in Europe. This Asian bark borer is damaging large areas of ashes in North America and Russia (Poland and McCullough 2006; Musolin et al. 2017; Volkovitsh et al. 2021). Therefore, breeding must avoid possible conflicting adaptations if resistance to these two pests is negatively correlated (George et al. 2022).

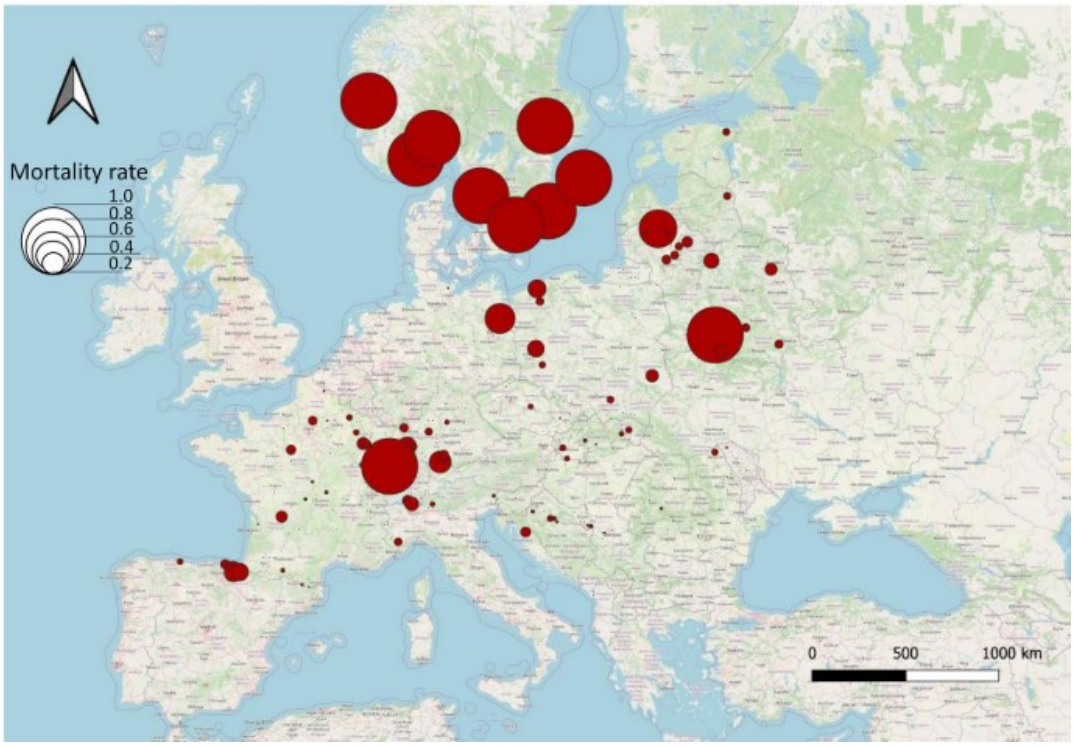

Fig. 3: Cumulative mortality rate of ash in Europe between 1987 and 2020 (adopted from George et al. 2022)

1.5 MEASURES AGAINTS ASH DIEBACK

At presence, there are no ways to effectively control the spread of *H. fraxineus*. Keeping a healthy seedling in the nursery does not guarantee their survival in nature (Halecker et al. 2020). There are only preventive and retarding measures that are applicable to individual trees rather than to whole stands, e. g. removal of necrotic branches. However, this treatment allows spread of the pathogen through the wood without external symptoms and also induces the deployment of new branches that are more susceptible to infection by ascospores (McKinney et al. 2012b). For individual trees, such as in urban plantations, it is possible to practice some of the more expensive measures such as fungicide treatment, leaf litter removal, removal of necrotic branches, or reducing moisture in the undergrowth by mowing the grass (Černý et al. 2016).

Only preventive measures to reduce the effects of the disease can be used to protect larger areas. In forests, it is recommended to remove only individuals with a high rate of crown damage in order to preserve naturally resistant genotypes in the population and to leave the habitat to natural regeneration, especially in sites of conservation importance (Skovsgaard et al. 2017). It is advisable to avoid new ash plantations in moist habitats, which support the fructification of the pathogen and subsequent infections. Whenever possible, nutrient-rich soils, which support

the fast growth of ash, are not suitable for growing in terms of higher susceptibility to infection. Some tree species as pine (*Pinus* spp.), fir (*Abies* spp.) or maple (*Acer* spp.) might be beneficial for admixture in ash stands. Thinning and reduction of shrub layer may be beneficial in already existing forest sites (Černý et al. 2016; Havrdová et al. 2017; Skovsgaard et al. 2017; Chumanová et al. 2019)

1.6 FUNGAL ENDOPHYTES OF TREES

Plants harbour an enormous microbial diversity inside and also on the surface of their living organs. It includes organisms such as bacteria, fungi, algae, archaea or protists (Baldrian 2017; Compant et al. 2019; Terhonen et al. 2019). Their effect ranges from pathogenic across neutral to beneficial (Lyu et al. 2021).

These microorganisms living within the plant tissues and even inside plant cells are called endophytes. From the very first meaning of endophytes as any organism living inside plant tissues including pathogens (de Bary 1879), the use of this term was narrowed to organisms living asymptomatically in plants throughout their life cycle or in some part of it, regardless of their life strategy outside the plant or in weakened/dead plant tissue (Petrini 1991; Schulz et al. 2002; Sieber 2007; Schulz et al. 2015). In addition to endophytes, epiphytic species are also considered a part of the microbiome of plants. These species colonize plant surfaces during the growing season and use plant exudates and diffusive substances released by the host. All these organisms together give the plant an advantage in its collective genome allowing increase in the life possibilities of the plant itself (Viterbo et al. 2007; Schlegel et al. 2016; Yadav et al. 2017; Latz et al. 2018; Agan et al. 2020).

Fungi, as one of the most common and important endophytic organisms, are divided into four main groups (Table 1). The aboveground parts of the trees host endophytic fungi from group 3. This group is characterized by multiple independent infections; when individual fungi consist of only a few cells and their growth through tissue is not systemic and strongly limited. The endophytic community of these fungi is a mosaic of many individuals of different species (Rodriguez et al. 2009). Another feature of this group is the predominantly horizontal transfer to new hosts after sporulation on the dead substrate. Only a limited number of species transfer vertically through tree seeds to the new seedling. However, after seed germination, the plant is quickly colonized by spores from the immediate area (Arnold 2007; Agan et al. 2020). The endophytes then accumulate in the tissues of the plant. In permanent tissues, such as wood,

endophytes survive to next years, although their number may decrease due to abiotic conditions such as low temperatures (Granath et al. 2007). In case of deciduous trees, first colonisation of leaves by endophytic fungi starts after the leaf flushing (Arnold et al. 2003; Arnold 2007).

The diversity of endophytic fungi is largely controlled by inoculum resources in the environment. Therefore, the composition of endophyte and also epiphyte communities of different tree species growing sympatrically may overlap considerably (Agan et al. 2020). Trees host both fungi specific to the given host species, generalists without preference for a particular tree, and even species functionally associated with non-plant substrates may be present among endophytes. Composition of endophytic assemblages in plant tissues can be affected by host features such as genotype, nutrition or stress and environmental variables as climate, season, microclimatic conditions, exposure to sunlight, distance of plant tissue from undergrowth, diversity of surrounding plant vegetation etc. (Unterseher et al. 2007; Scholtysik et al. 2013; Lamit et al. 2014; Rottstock et al. 2014; Nguyen et al. 2016a; Schmidt et al. 2018; Franić et al. 2020; Henning et al. 2021; Oita et al. 2021).

The diversity of endophytic fungal species has received serious attention especially in previous three decades. From a methodological point of view, culture-based methods with identification of isolated fungi based on phenotype represented the only approach for a long time. Our knowledge about diversity, factors influencing diversity and function of these organisms in plants was thus rather limited to culturable fungi (Saikkonen et al. 1998). Later, the accessibility of DNA analyses and routine use of molecular markers for fungal identification enabled to support the identification of isolated fungi (that frequently remain sterile) with molecular data. Also the beginning of the 21st century experienced revolutionary shift to our knowledge of the plant microbiome due to the use of the High Throughput Sequencing (also known as the Next Generation Sequencing) (Siddique et al. 2017). These approaches based solely on identification of fungi in a given substrate by sequencing of one or several molecular markers (mostly the ITS rDNA) enables not only to reveal also unculturable fungi, but also to measure multiple variables in a single experiment. From one sample we can also reveal the diversity of fungi and bacteria and we can monitor the qualitative and quantitative characteristics of the community (Lyu et al. 2021). We are even able to reveal the ecological properties of the studied community (Nguyen et al. 2016b). Due to the fact that studies of fungal communities usually focus on entire fungal

genome of plant tissues, the terms mycobiom or phytomycobiom are used more often today - without division into endophytes and epiphytes (Terhonen et al. 2019).

Table 1. Categories of fungal endophytes and their main characteristics (Rodriguez et al. 2009, adopted according to Terhonen et al. 2019 and adjusted).

Main Group	Class	Transmission type	Endophyte classification	Host range	Tissue type	Functional roles/ Characteristics of endophytes
C[1]	**1**	Vertical	Ascomycota	**Grasses**		Enhance drought tolerance, increase plant biomass, decrease herbivory
NC[2]	**2**	Most horizontal, few vertical	Dikarya (Ascomycota, Basidiomycota)	**Trees** (and all plants)	**Shoot, root and rhizome**	Enhance stress tolerance of host plants (salinity, pH, temperature), increase host root and shoot biomass
	3	Horizontal	Dikarya (Ascomycota, Basidiomycota)		**Aboveground tisues, especially leaves**	Localized infections, pioneer decomposers, protection against herbivores or pathogens,niche competition, induction of systemic resistance, inhibition of insect and pathogen growth
	4	Horizontal	Ascomycota and Basidiomycota; mainly DSEs[3]		**Roots**	Growth inhibition of pathogens, host plant growth promotion

[1]Clavicipitaceous endophytes

[2]Non-clavicipitaceous endophytes

[3]Dark septate endophytes

1.7 ASH MYCOBIOME AND ITS ANTAGONISTIC POTENTIAL AGAINST *H. FRAXINEUS*

There has been great progress in the field of ash mycobiome in recent years stimulated by the spread of ash dieback. Between 2017 and 2021 more than 10 studies dealing with this topic were published, of which the vast majority used molecular data to describe the structure of the mycobiome. The main focus of these studies was 1) diversity of fungi in plant tissues and 2) the influence of the microbial community on susceptibility to ash dieback.

Using High Throughput Sequencing, enormous diversity of fungi in ash mycobiome was recorded. Particular studies have recorded up to 2000 fungal operational taxonomic units (OTUs) in ash leaves, of which 815 was shared by leaves, bark and wood (Cross et al. 2017; Becker et al. 2020; Agan et al. 2020; Agostinelli et al. 2021). However, there were only about 20 of the most abundant species which can make up half to almost the entire community (Agan et al. 2020; Lahiri et al. 2021; Agostinelli et al. 2021). Becker et al. 2020 pointed out that even four species of fungi can represent more than 60% of the ash leaf fungal community. The most abundant classes of endophytic fungi in ash leaf mycobiome are Dothideomycetes and Tremellomycetes, followed by Leotiomycetes, Eurotiomycetes, Taphrinomycetes and Cystobasidiomycetes (Cross et al. 2017; Becker et al. 2020; Griffiths et al. 2020; Agostinelli et al. 2021). The epiphytic community is dominated by yeasts of genera *Cryptococcus, Bullera, Vishniacozyma, Papiliotrema* and *Taphrina*, dimorphic fungus *Aureobasidium pullulans* and further by species of the genus *Cladosporium, Phyllactinia fraxini, Venturia fraxini* and *H. fraxineus.* Many of these species inhabit also the inner parts of leaves as endophytes whereas *V. fraxini* is the most frequent (Bakys et al. 2009a; Cross et al. 2017; Schlegel et al. 2018; Becker et al. 2020; Griffiths et al. 2020; Agan et al. 2020). Ash leaves can share up to 60% of its endophytic community with other broadleaved trees on the site (Agan et al. 2020; Agostinelli et al. 2021).

Ash shoots, bark, wood, seeds and roots received less attention and also the diversity in these tissues was lower in contrast to leaves (Lahiri et al. 2021; Agostinelli et al. 2021). Only 214 fungal OTUs were found in ash shoots using ITS1-metabarcoding. Fungal community in the shoots were dominated by members of classes Eurotiomycetes and Dothideomycetes and the same was true for mycobiome of other species of the genus *Fraxinus*. Endophytic fungi that are typically isolated from shoots of *Fraxinus* species are *Boeremia exigua* and members of genera *Phoma, Diaporthe* and *Fusarium*. A meta-analysis of fungal sequences

isolated from twigs and leaves of various *Fraxinus* species has shown that the mycobiome of the genus *Fraxinus* has characteristic patterns across geographic areas (Schlegel 2018).

Ash seeds and roots host limited number of fungi, approximately in one order of magnitude. Seed endophytes include ubiquitous species as *Gibberella avenacea*, *Alternaria alternata* and members of the genera *Septoria*, *Cladosporium* or *Cryptococcus*. Rather surprisingly, also *H. fraxineus* was recorded as seed endophyte (Cleary et al. 2013a). The seed surface is covered mainly by epiphytic members of classes Eurotiomycetes and Mucoromycetes (Cleary et al. 2013a; Lahiri 2020; Lahiri et al. 2021). Endophytical assemblage of ash roots were distinctive from the above ground tree tissues by including also endophytic species from Class 4 (see Table 1) as members of the genera *Cadophora* together with species of the genera *Cordyceps*, *Dactylonectria*, *Ilyonectria*, *Nectria* and *Neonectria* (Lahiri 2020; Lahiri et al. 2021).

Some fungi present in fresh ash leaves can be found later as frequent saprotrophs in fallen petioles as *Nemania serpens*, *Epicoccum nigrum*, *Venturia fraxini*, *Diaporthe eres*, and members of the genera *Alternaria*, *Boeremia* and *Fusarium*. First year in litter Dothideomycetes and Leotiomycetes are still the most abundant group of fungi in petioles (Kowalski and Bilański 2021).

Besides description of the ash mycobiome, the effect of the composition of endophytic fungal communities as well as bacteria on the susceptibility of trees to ash dieback has been questioned in numerous studies (Schlegel et al. 2018; Kosawang et al. 2019; Griffiths et al. 2020; Ulrich et al. 2020; Agan et al. 2020). Interestingly, no difference in the overall community was observed in most of the studies and also fungal communities of other *Fraxinus* species probably are not responsible for their resistance to the disease (Schlegel et al. 2016; Kosawang et al. 2018). Only in the study of Griffiths et al. 2020, significant differences between trees with various disease intensity were observed. Higher infection levels were positively correlated with alpha-diversity of fungal community in leaves. Similarly a trend of higher fungal diversity in shoots of more susceptible ash genotypes was documented (Koawang et al. 2018; Kosawang et al. 2019). However this increase could be due to a tendency to host a larger number of species with pathogenic life style (Fig. 4) as was shown by Agostinelli et al. 2021 and (Schlegel et al. 2018), where e. g. *Diaporthe rudis, Boeremia* sp. and *Neofabraea vagabunda* were recorded in positive associations with *H. fraxineus*. An increase in the relative number of lichenized fungi in leaves of diseased trees was also noted (Griffiths et al. 2020).

Ash individuals with a high rate of *H. fraxineus* infection also had a higher number of other endophytic fungal species that were more associated with each other. This is probably a reflection of some physiological dysbiosis in trees with higher disease susceptibility or dysbiosis established after exceeding a certain pathogen infection threshold, such as increased leaching of nutrients on the leaves (Ragazzi et al. 2003; Giordano et al. 2009; Martín et al. 2013; Agostinelli et al. 2018; Griffiths et al. 2020; Agostinelli et al. 2021). However, contradictory data have been described to support the hypothesis that more diversified communities better prevent the entry of pathogens due to higher occupancy of available niches (Levine and D'Antonio 1999). Kosawang et al. 2019 noted, that despite the biodiversity hypotheses, high diversity can be an indicator of poor tree health and that a positive effect of a microbial community could be probably not due to a specific community, but to the accidental occurrence of antagonistic species.

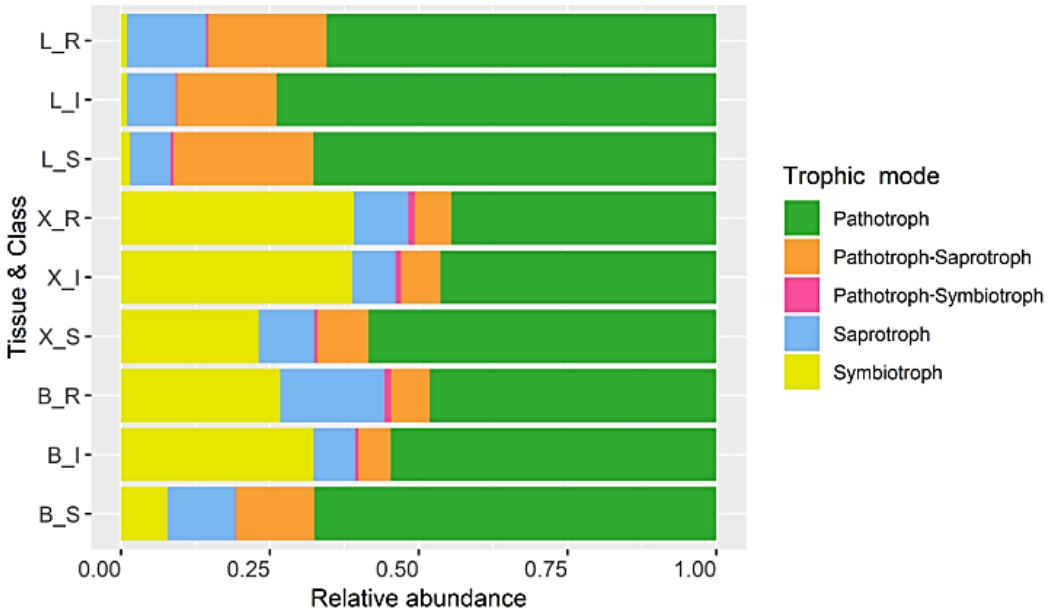

Fig. 4: Trophic modes of fungal OTUs and their relative read abundance in ash (*F. excelsior*) tissue types (L, leaves; X, xylem; B, bark) and their susceptibility class (R, resistant; I, intermediate; S, susceptible, Agostinelli et al. 2021).

Griffith et al. 2020 identified only three microbial genera significantly associated with leaves without *H. fraxineus* infection, out of which fungi of the genus *Neofabraea* seemed to be promising antagonists. Further the yeasts *Taphrina padi*, *Vishniacozyma laurentii* and *Rhodosporidiobolus colostri* were negatively associated with *H. fraxineus* (Agan et al. 2020). *Venturia fraxini* showed a trend of higher frequency in the leaves of healthy trees (Schlegel et al. 2018). Interestingly, two independent papers confirmed the higher presence of the yeast *Papiliotrema*

flavescens (syn. *Cryptococcus flavescens*) in tolerant individuals of ash, which is used as bioagent against post-harvest crop diseases (Rong et al. 2017; Kheireddine et al. 2018). Its significant effects were also confirmed in antagonistic tests with *H. fraxineus* (Becker et al. 2020; Agan et al. 2020). Members of the order Tremelalles, into which *P. flavescens* belongs, are common inhabitants of plant mycobiomes with predominant mycoparasitic and fungicolous ecology (Zugmaier et al. 1994; Kurtzman et al. 2011). They may have protective function, such as *Vishniacozyma* species that enhanced resistance of wheat to infection by smut fungi (Vujanovic 2021). Contrary effects of *H. fraxineus* infection were observed on the causative agent of ash powdery mildew *Phyllactinia fraxini*; infection by this species was once positively correlated with *H. fraxineus* (Cross et al. 2017), but in another case negatively (Griffiths et al. 2020).

Extensive screenings of ash endophytes for antagonistic interactions with *H. fraxineus* have been performed and many potential antagonists have been identified, however these species are often fast-growing plant pathogens and the antagonistic interaction with *H. fraxineus* is usually reciprocal (Kosawang et al. 2019; Becker et al. 2020; Lahiri 2020; Halecker et al. 2020). The effect of exudates of endophytic fungi was also investigated. Germination of *H. fraxineus* ascospores was inhibited by exudates produced by *Venturia fraxini*, *Paraconiothyrium* sp., *Boeremia exigua*, *Kretzschmaria deusta* and *Neofabraea alba* during *in vitro* experiments on water agar (Schlegel et al. 2016). Experimentally inoculated freshly flushed ash leaves with spores from leave litter showed fungal community dominated by members of the genus *Venturia*. Unfortunately, further infection test with seedlings exposed to *H. fraxineus* did not provide clear evidence of a protective effect. However, results of this study were not conclusive due to adverse climatic conditions during the experiment. Kowalski and Bilański 2021 also assumed that *V. fraxini* has competitive advantage of earlier sporulation; its niche is in a thin surface layer of petioles whereas *H. fraxineus* colonizes inner parts of a petiole so they can colonize the same substrate without direct contact.
Halecker et al. 2020 tested antagonistic fungi on ash seedlings in axenic cultures and selected *Hypoxylon rubiginosum* as the only suitable candidate for biological control due to its non-pathogenic behaviour towards the plant and at the same time no *H. fraxineus* inhibition to this fungus.

Although biological control of *H. fraxineus* is assumed to be most effective in living tissues, considering to role of protective pseudosclerotial plates[2] formed in litter petioles (Gross and Holdenrieder 2013), also saprotrophs of fallen ash petioles were tested as possible antagonists. Wide inhibition zones in dual cultures on agar plates were formed by *Malbranchea* sp. and *Pseudocoleophoma polygonicola*. Soil fungi with known strong antagonism such as *Clonostachys rosea* and *Trichoderma viride* were also successful in suppression of the pathogen. Overgrowing was further noted at xylariaceus taxa of genera *Nemania*, *Rosellinia* and *Xylaria* and basidiomycetes *Hypholoma fasciculare* and *Peniophora incarnata*. However, these results are based on tests on agar plates, where *H. fraxineus* competes as "nude" mycelium without the pseudoslerotium, whose effect is probably very strong *in situ*. Finally it is hypothesised that some fungal saprotrophs could have indirect biological effect against *H. fraxineus* inducing faster decomposition of petioles (Kowalski and Bilański 2021).

Although the positive effect of endophytes on tree health is expected, practical application in the biological protection of trees is so far unique or with an ambiguous effect. The basic problem is how to get the desired fungal strains into the plant. Colonisation of these tree endophytes is strongly localized, so we cannot, for example, inoculate a trunk or infest seeds and expect them to spread throughout the plant system. It is possible to spray the whole tree, but this can only be used on young plants, in addition, the degree of attachment is difficult to predict and in case of leaves it is only a one-year effect (if we do not calculate the possible multiplication of used bioagents in fallen leaves) (Christian et al. 2017). Another complication is the presumed specificity of microbial derived resistance, which is uniquely formed under specific environmental conditions together with interactions with many endophytic organisms and the plant host itself (Griffith 2020).

1.8 *HYMENOSCYPHUS ALBIDUS* AND OTHER SAPROTROPHS OF ASH PETIOLES

Saprotrophic microfungi colonizing ash petioles in the litter may also be affected by the spread of *H. fraxineus* due to an imbalance in the use of niches. *Hymenoscyphus albidus* was the most frequently mentioned species in this regard. It was described as early as 1851 as *Peziza albida* from France (Desmazières 1851; Husson et al. 2011) and was considered a common species in Europe in the 20th century (Velenovský

[2] Pseudosclerotium is a structure formed by the growth of melanized hyphae of the fungus through plant tissue and borders colonized area of a petiole.

1934; Baral and Bemmann 2014). It forms whitish stalked apothecia, which are quite conspicuous because they grow out of black pseudosclerotial plate in the petioles. The abundance of *H. albidus* in Europe prior to the onset of ash dieback is largely unknown.

Morphologically, this species is almost indistinguishable from *H. fraxineus* and the only differences are dimension of ascospores and presence of croziers at the basis of asci at the latter species (Baral and Bemmann 2014). Fruiting bodies found in nature in the last decades were mostly identified as belonging to *H. fraxineus*. Therefore, there were speculations that it could be displaced by the invasive sister species (Baral and Bemmann 2014). Based on spore traps and molecular analysis of DNA from fresh leaves, we know that *H. albidus* still occurs in the environment, even its abundances are low comparing to *H. fraxineus* (Hietala et al. 2013; Chandelier et al. 2014; Dvorak et al. 2016; Schlegel et al. 2018; Griffiths et al. 2020) and some recent studies did not reveal this species (Hietala et al. 2018a; Agan et al. 2020). The pseudosclerial layer which protect a thallus and in which it could survive for many years, similar to *H. fraxineus*, might be essential for the survival of *H. albidus* although the pseudoslerotia are rather insular in case of *H. albidus* (Gross and Holdenrieder 2013; Baral and Bemmann 2014; Hietala et al. 2018a)

The decrease in abundance of *H. albidus* after the invasion of *H. fraxineus* is explained by several theories, which can complement each other 1) huge propagule pressure of *H. fraxineus,* which can be amplified by sequence of several favorable seasons (Cross et al. 2017; Hietala et al. 2018a; Hietala et al. 2022), 2) earlier sporulation of *H. fraxineus* (Baral and Bemmann 2014), 3) competitive similarity of these fungi due to similar enzyme profiles and inhabiting same spatial niche, which support the stochastic niche theory of resource competition (Tilman 2004; Baral and Bemmann 2014; Stenlid et al. 2017; Agan et al. 2020), 4) faster growth of *H. fraxineus* in ash leaf tissue (Gross and Sieber 2016) and 5) insufficient genetic equipment of *H. albidus* to maintain its ecological niche, which also correspond with its homothallic reproduction and a lack of recombination (Wey et al. 2016; Elfstrand et al. 2021). It has been hypothetized that survival strategies of *H. fraxineus* reflect its wide range in Asia concerning various extreme weather conditions and conversely facilitate its dispersal in milder climate of Europe (Hietala et al. 2018a; Hietala et al. 2018b).

Except of *H. albidus*, there can be another more than one hundred fungal taxa inhabiting fallen ash petioles. These include *Venturia fraxini*, species that colonizes petioles as endophytes prior to their fruitbody formation in the litter, helotialean

species *Cyathicula fraxinophila, Cyathicula coronata, Hymenoscyphus caudatus*, *Hymenoscyphus scutula,* other ascomycetes *Hypoderma rubi, Pyrenopeziza petiolaris, Leptosphaeria sclerotioides* or basidiomycetes with minute basidiocarps from the genus *Typhula.* Although members of the order Helotiales are the most frequently fructifiing species on petioles, cultivation methods have still revealed Pleosporales as the most common colonizators (Kowalski and Bilański 2021).

2 OBJECTIVES OF THE STUDY

The main objectives of this thesis were to study spatial population structure of *H. fraxineus*, the causal agent of ash dieback disease, on a regional scale and also on a microscale to confirm its infection pathway into the tree, to characterize interactions of *H. fraxineus* with other fungi in the litter and with fungi in the healthy plant tissues. Specifically, I have addressed these particular aims and searched for response to following questions:

Part 1:

- What is the genetic structure in Czech populations of *H. fraxineus* i.e. region with presumably low genetic diversity of the pathogen?
- Can we track colonisation path of individual strains of *H. fraxineus* to confirm the infection of shoots through petioles?

Part 2:

- Did the invasive *H. fraxineus* outcompete its closely related *H. albidus* in its habitat?
- Did its spread and heavy colonisation of fallen petioles affect also other fungi colonizing petioles in the litter?

Part 3:

- Is there difference among endophytic communities in shoots of susceptible and tolerant trees?
- What is the effect of season on these communities?
- What type of interactions occur between *H. fraxineus* and endophytes of ash shoots?

My dissertation consists of four publications labelled P1-P4 that were dealing with the above mentioned aims and question.

3 KEY RESULTS AND DISCUSSION

3.1 SPATIAL STRUCTURE OF *H. FRAXINEUS* AT DIFFERENT SCALES

Microsatellite markers represent powerful tool to distinguish populations and even individuals. This is specifically needed for modular organisms, such as fungi, where individuals are difficult to delimitate. Results of population studies and above all allelic richness was essential to provide evidence of the origin of *H. fraxineus* in Asia (Gross et al. 2014). However, only eight microsatellite markers were available for comparison of European and Asian population at that time that had limited resolution.

In **Publication P1**, we tested newly developed promising microsaltellite loci of which five revealed polymorphism and variation in microsatellite repeat number. Three of them were evaluated as appropriate to compare European and Asian populations. Population analysis with 20 microsatellite markers showed that Czech populations had lower population diversity than other European populations. Out of Czech populations one significantly differed from others in genetic analysis probably as a result of a founder effect. This findings indicated that there can be barriers that can complicate the dispersal of the pathogen, despite that otherwise genotype diversity in Europe is high and populations do not make a structure (Gross et al. 2014b; Orton et al. 2018). That there can be exceptional cases of genetic differenciation was also confirmed by the study of Nguyen et al. 2016a who found 4% genetic variation among habitats with varying levels of ash admixture.

Our first study (**Publication P1**) also reported presence of two different genotypes in one lesion. Multiple infections of shoots were already decribed (Bengtsson et al. 2014) and raised the question of the exact infection entrances and spread of the

pathogen within the host. This feature was specifically addressed in **Publication P4**.

In **Publication P4**, we were able to distinguish single pathogen genotypes within the petioles and also shoots and trace infection pathway of a particular *H. fraxineus* individuals from a petiole to a shoot, where it caused necrosis. This research was highly original in the experimental setup that consisted of labelling and tying the petioles of compound leaves to their shoots by threads to ensure that they are captured after fall and at the same time the process of colonization of the petioles by fungi is not disturbed and the method of collecting does not lead to premature detachment (Fig. 5).

Fig. 5: Leaves of *Fraxinus excelsior* after natural leaf fall still attached by threads to their shoot and labelled with their shoot position. Photo: Z. Haňáčková

Thanks to this pretreatment, we were able to collect all petioles belonging to one infected shoot after natural leaf fall and isolate *H. fraxineus* from all associated parts.

Based on the set of microsatellite markers from **Publication P1** and DNA directly extracted from the petioles, we distinguished multiple infections in particular petiole fragments. Genotyping of *H. fraxineus* isolates obtained by culturing these petiole fragments on the agar medium was then used to differentiate the particular microsatellite patterns in total DNA. Isolation and genotyping of *H. fraxineus* from the area of leaf scars of shoots then helped us to reveal the final genotypes causing necrosis. Known position of petioles allowed us to to reconstruct the infection pathway of each the pathogen genotype (Fig. 6).

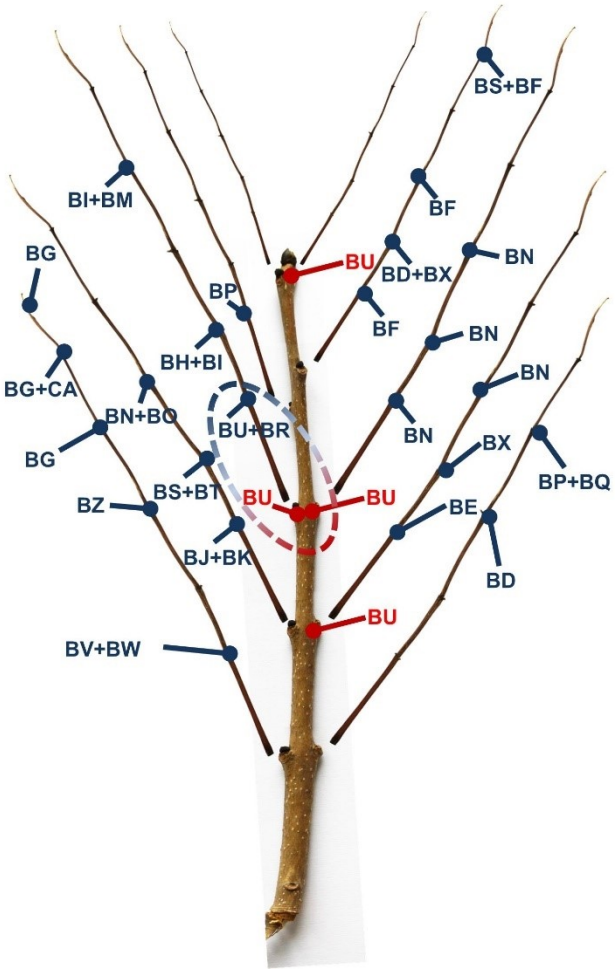

Fig. 6: Individual genotypes of *H. fraxineus* distinguished in a reconstructed shoot and respective petioles. Red color indicates the genotype causing shoot infection. Figure by O. Koukol.

One genotype was usually found in the shoot, which matched the genotype present in one of the petioles. This confirmed the presumed infection pathway through petiole-shoot junction. However, also three different genotypes in one shoot lesion were isolated from which two were paired with petiole infections and one probably originated from direct infection of the shoot showing the extent of the pathogen pressure on the host (Chandelier et al. 2014; Dvorak et al. 2016; Cross et al. 2017). We expected that length of the necrosis or either competition or faciliation among multiple strains of *H. fraxineus* affects the infection (Alizon et al. 2013). Number of invaded petiole segments by particular genotypes and number of other genotypes co-ocurring in the segments were analyzed to test effect of nutrient source and competition on successful infection, respectively. However the effect of none of these variables was confirmed. Since up to 27 genotypes of *H. fraxineus* were found in the petioles of one shoot, but usually only one was capable of infection, it seems that the infection route through the petiole and the crossing into the wood presents a strong bottleneck for the pathogen.

Nielsen et al. 2022 further extended our findings and showed that disease tolerant tree genotypes have also shorter petiole necrosis, which indicate plant defence mechanisms stopping growing strains. Authors supposed that these mechanisms will be probably different from *F. mandshurica* which can host high levels of the pathogen in leaves without shoot infection. One explanation could be also earlier switching to the necrotrophic phase after reaching a certain threshold of leaf inoculum level which can be high due to favourable condition in Europe compared to Asia (Hietala et al. 2022).

3.2 THE EFFECT OF *H. FRAXINEUS* ON SAPROTROPHS IN THE LITTER

The onset of the ash dieback was connected not only with the rapid spread of *H. fraxineus* across Europe, i. e. in large geographical scale, but also with its localized high abundances in ash tree stands. In ideal microclimatic conditions, virtually

every fallen petiole was blackened from the presence and saprotrophic growth of *H. fraxineus* (Fig. 7). Presumably, the colonisation pattern connected with production of black pseudosclerotical layer could be antagonistic towards other microfungi (Baral and Bemmann 2014). This was our working hypothesis that we transferred into the main question, whether the pathogen influences saprotrophs of ash petioles.

Fig. 7: Multiple apothecia of *H. fraxineus* on blackened petioles of *F. excelsior*, Jánské Lázně, August 2013. Photo: O. Koukol.

The main species that could be affected by *H. fraxineus* appeared to be *H. albidus*, which occupied the same niche on ash petioles and was probably present across the entire European ash distribution area in the past (Velenovský 1934; McKinney et al. 2012a; Baral and Bemmann 2014). Previous studies indicated that *H. albidus* could be locally extinct because this species was absent or only rarely found at localities after ash dieback invasion (McKinney et al. 2012a; Chandelier et al. 2014). However, other helotialean species fructifying on ash petioles especially in autumn, could also be affected.

In our study (**Publication P2**), we efficiently combined several approaches to evaluate the coexistence of *H. fraxineus* with other saprotrophs on a large scale (Czech Republic) and a smaller scale (single forest). To achieve the former, we collected (and also received from colleagues and students) multiple specimens of

petioles with apothecia resembling *H. fraxineus* or petioles only that were cultivated in damp chambers. Due to the limited morphological differences between *H. fraxineus* and *H. albidus*, we relied on molecular data in the identification, i.e. sequences of ITS rDNA. For the latter, we used spore traps installed at three forest sites coupled with detection of *H. albidus* using specific primers (Dvorak et al. 2016). In connection to the survey of recent impact of *H. fraxineus* on the distribution of *H. albidus*, we also attempted to reconstruct the historical distribution of the native saprotrophic species, that was supposed to be rather common and locally frequent by Velenovský (1934).

As expected, we did not record a single collection of *H. albidus*, which would allow a interpretation that this species is extinct in the Czech Republic as already speculated for Denmark (McKinney et al. 2012a). However, ascospores of *H. albidus* were detected on spore traps suggesting that this species is still present in the studied region. Interestingly, our recherche of later diversity surveys published in local mycological journals did not confirm abundant historical presence of *H. albidus* in ash stands. This has not been supported either by presence of specimens of this species in major Czech herbaria. We were able to study only eight collections from Herbarium of the Prague National Museum (PRM) labelled as *Helotium albidum*, *Hymenoscyphus* sp. and *Hymenoscyphus albidus*. Morphological survey and analysis of ITS rDNA confirmed that only four specimens collected between 1925 and 1942 belonged to *H. albidus*. Though we cannot exclude that this species was overlooked during previous field surveys, we concluded that it was rare in Czechia already prior to the onset of the ash dieback epidemy, similarly to the rest of the Europe (Baral and Bemmann 2014).

Based on the detection of *H. albidus* ascospores in the spore traps, we concluded that it is still present in nature, but its presence is masked by the dominance of *H. fraxineus*. It is difficult to predict the coexistence of these two species in the future, but rather promising results were obtained by Hietala et al. 2018a, who recorded a strong decline in sporulation of *H. fraxineus* suggesting that its invasion was followed by a population crash. Together with the confirmed coexistence of both species on multiple sites across southern Britain (King and Webber 2016), these results suggest that *H. albidus* may survive in Europe in the long term perspective. Our study also showed that the massive spread of *H. fraxineus* had limited effect on other fungal species present on the petioles in the litter. *Cyathicula fraxinophila*, another helotialean species specific for *F. excelsior* petioles in the litter was found on petioles from almost every fourth site and sometimes even on the same petiole

as *H. fraxineus*. These findings are consistent with Polish study of Kowalski and Bilański 2021, who observed *C. fraxinophila* on 14% of petioles. They also showed that most of the petioles colonized by *H. fraxineus* are simultaneously inhabited by at least one other fructifiing fungal species and spectrum of these fungi counted 26 taxa. Many of these species are generalists or use multiple substrates for sporulation and therefore they do not depend on the colonization of ash leaves, which probably explains their constant occurrence even under high infectious pressure of *H. fraxineus*. Some also use other parts of the petiole than *H. fraxineus*, and as antagonistic tests have showed (Kowalski and Bilański 2021), many will be reciprocally antagonistic to *H. fraxineus* that enable them to delimit own area in a petiole. A certain advantage could also be autumn fructification, which prevails in the occurring species and the spores thus germinate already on the senescent leaves (Kowalski and Bilański 2021). Recently, Hietala et al. 2022 reported that also *H. albidus* probably outlasts latent until the senescent phase of leaves and that its ecology is between saprotroph and weak pathogen, because it was recorded forming necrotic lesions on leaves in natural site.

3.3 ENDOPHYTIC COMMUNITY IN ASH SHOOTS AND ITS INTERACTIONS WITH *H. FRAXINEUS*

One of the natural barriers for the spread of the pathogen is potentially the endophytic community in ash leaves and wood. Obviously, this barrier is rather weak and does not prevent the successful spread of *H. fraxineus* in the invaded tissues because only trees with lesser susceptibility rather than resistant can be found in nature (McKinney et al. 2014a; Skovsgaard et al. 2017). However, the interaction of endophytic community with the susceptible and tolerant ash genotypes was not known. Moreover, leaves, that represent the part of a plant being affected by the pathogen with highest intensity, are rather studied in this respect (Cross et al. 2017; Schlegel et al. 2018; Becker et al. 2020; Griffiths et al. 2020; Agan et al. 2020; etc.). In view of that the spread of the pathogen within shoots into older woody parts is more devastating for the whole tree, we aimed at culturable endophytic microfungi from shoots of differently susceptible ash trees (**Publication P3**).

Shoots were sampled from pairs of trees growing in close distances. Sampling was carrying out for two years, from two locations, in summer and winter period. Pieces of shoots were surface sterilized and cultivated on wort agar. The obtained cultures were further identified morphologically and molecularly based on their ITS rDNA sequence. To screen the antagonistic potential of ash endophytes, we

subjected 49 isolated fungi to dual tests with *H. fraxineus* on agar with ash shoot extract and microcrystalline cellulose as a source of carbon.

Fungal communities of ash shoots were represented mainly by members of the classes Dothideomycetes and Sordariomycetes. Regarding the first question, it was found that trees more susceptible to ash dieback also hosted higher abundances of *Diaporthe* sp. – endophytes with pathogenic potential, even when the isolation was made from healthy tissues. More fungal species were found in trees relatively resistant to ash dieback, however no significant differences were found between the structure of the communities of susceptible and tolerant trees. Other studies also did not find an overall difference in the ash mycobiome of differently susceptible individuals (Schlegel et al. 2018; Kosawang et al. 2019; Griffiths et al. 2020; Ulrich et al. 2020; Agan et al. 2020). On the contrary, some confirmed the more frequent occurrence of pathogens in susceptible individuals and an increase in the overall diversity of fungi (Kosawang et al. 2018; Schlegel et al. 2018; Kosawang et al. 2019; Agostinelli et al. 2021). However, the number of species alone is unlikely to be authoritative, and the influence of species depends more on the functional composition of the community (Griffiths et al. 2020).

There were also seasonal changes between fungal communities. The season affected the fungal community in favor of pathogenic fungi in winter. It is known that the winter period contributes well to the development of fungi causing various cankers and wood necrosis, as the tree is in dormancy, does not activate plant defense and temperatures can be high enough for fungal activity (Lonsdale and Gibbs 1996; Agrios 2005). *H. fraxineus* probably uses the reduced plant defense during the winter too, even though its lesions are most growing during the summer (Bengtsson et al. 2014).

As shown by *in vitro* tests, the inhibition of *H. fraxineus* by endophytic fungi can be from 40 to 83%. The best ihnibition rates were achieved by fast growing fungi as *Botrytis cinerea* and *Phoma macrostoma var. incolorata*, which reduced the growth of *H. fraxineus* by at least 80%. The ecological role of both fungi can be both pathogenic and saprotrophic with an endophytic phase. While *B. cinerea* was detected only occasionally, *P. macrostoma* var. *incolorata* made up 10% of the summer community and could thus naturally form a barrier for *H. fraxineus*. The effect could be also synergistic with other fast growing fungi. However, the behavior of these fungi in natural substrates such as leaves or shoots is still unclear and extensive growth is not probably possible until senescence of tissues (Hietala et al. 2022). Moreover, in more than half of the tested fungi, an inhibition

comparable to the self-inhibition of *H. fraxineus* was found. Growth reduction of endophytes by *H. fraxineus* was also observed. We must also take into account that colonies of many potentially antagonistic species are reduced by abiotic factors at the time of *H. fraxineus* growth initiation in the shoot.

4 CONCLUSIONS

Knowledge of the precise functioning of the life cycle of pathogenic fungi is crucial for the precise timing of host protection. The main benefits of this work for phytopathology and related fields were thus the proof of the infection pathway of the pathogenic fungus *Hymenoscyphus fraxineus* from leaves to shoots of its host, *Fraxinus excelsior*. Even though the infection of shoots is the "dead end" of the life cycle for this fungus and the fungus does not need it for further spread, it is an important phase from the point of view of pathogenesis. The importance of this finding was demonstrated in following studies (Schlegel et al. 2018; Hietala et al. 2022; Nielsen et al. 2022) etc. During the methodological part of the study of the population structure of *H. fraxineus* on different scales, 5 new microsatellite markers were also developed, which were later used for studying colonization patterns of the pathogen in necrotic rootstocks and stem bases of ash (Meyn et al. 2019). Three of these markers were also found to be appropriate for comparing European and Asian populations. Presented results left open questions about the predictors determining successful shoot infection by a particular genotype.

The second part of the thesis contributed to the knowledge of several topics of fungal ecology with theoretical overlap into practical plant protection. It disapproved fears about the possible extinction of some native saprophytes of ash litter and at the same time pointed to the need for deeper knowledge about biology and ecology of the native fungi. The function of fungi in the defense capacity of trees is an often asked and still little known. This study found no significant differences in the diversity of the endophytic mycobiota of differently susceptible ash trees which was further confirmed in many studies (Schlegel et al. 2018; Kosawang et al. 2019; Griffiths et al. 2020; Ulrich et al. 2020; Agan et al. 2020). That support rather hypotheses about the influence of the tree itself on the composition of its microbial community. It is possible that we still do not have sufficient tools and approaches to assess the functioning of fungi in the microbiome of plants.

5 REFERENCES

Agan A, Drenkhan R, Adamson K, et al (2020) The relationship between fungal diversity and invasibility of a foliar niche—the case of ash dieback. J Fungi 6:1–27. doi: 10.3390/jof6030150

Agostinelli M, Cleary M, Martín JA, et al (2018) Pedunculate oaks (Quercus robur L.) differing in vitality as reservoirs for fungal biodiversity. Front Microbiol 9:1758. doi: 10.3389/fmicb.2018.01758

Agostinelli M, Nguyen D, Witzell J, Cleary M (2021) Mycobiome of Fraxinus excelsior with different phenotypic susceptibility to ash dieback. Front For Glob Chang 4:15. doi: 10.3389/ffgc.2021.580514

Agrios GN (2005) Plant Pathology, 5th edition. Academic Press, San Diego

Alizon S, de Roode JC, Michalakis Y (2013) Multiple infections and the evolution of virulence. Ecol Lett 16:556–567. doi: org/10.1111/ele.12076

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol Rev 21:51–66. doi: 10.1016/j.fbr.2007.05.003

Arnold AE, Mejía LC, Kyllo D, et al (2003) Fungal endophytes limit pathogen damage in a tropical tree. PNAS 100:15649–15654. doi: 10.1073/pnas.2533483100

Bakys R, Vasaitis R, Barklund P, et al (2009a) Investigations concerning the role of Chalara fraxinea in declining Fraxinus excelsior. Plant Pathol 58:284–292. doi: 10.1111/j.1365-3059.2008.01977.x

Bakys R, Vasaitis R, Barklund P, et al (2009b) Occurrence and pathogenicity of fungi in necrotic and non-symptomatic shoots of declining common ash (Fraxinus excelsior) in Sweden. Eur J For Res 128:51–60. doi: 10.1007/s10342-008-0238-2

Baldrian P (2017) Forest microbiome : diversity , complexity and dynamics. FEMS Microbiol Rev 41:109–130. doi: 10.1093/femsre/fuw040

Baral H-O, Bemmann M (2014) Hymenoscyphus fraxineus vs. Hymenoscyphus albidus – A comparative light microscopic study on the causal agent of European ash dieback and related foliicolous, stroma-forming species. Mycology 5:228–290. doi: 10.1080/21501203.2014.963720

Baral H-O, Queloz V, Hosoya T (2014) Hymenoscyphus fraxineus, the correct scientific name for the fungus causing ash dieback in Europe. IMA Fungus 5:79–80. doi: 10.5598/imafungus.2014.05.01.09

Becker R, Ulrich K, Behrendt U, et al (2020) Analyzing ash leaf-colonizing fungal communities for their bological control of Hymenoscyphus fraxineus. Front Microbiol 11:1–15. doi: 10.3389/fmicb.2020.590944

Bengtsson SBK, Barklund P, von Brömssen C, Stenlid J (2014) Seasonal pattern of lesion development in diseased Fraxinus excelsior infected by Hymenoscyphus pseudoalbidus. PLoS One 9:e76429. doi: 10.1371/journal.pone.0076429

Buck JW, Lachance M-A, Traquair JA (1998) Mycoflora of peach bark: population dynamics and composition. Can J Bot 76:345–354.

Buschbom J (2022) Genomic patterns and the evolutionary origin of an invasive fungal pathogen (Hymenoscyphus fraxineus) in Europe. Basic Appl Ecol 59:4–16. doi: 10.1016/j.baae.2021.12.006

Butin H, Kowalski T (1986) Die natürliche Astreinigung und ihre biologischen Voraussetzungen. III. Die Pilzflora von Ahorn, Erle, Birke, Hainbuche und Esche [The natural pruning of branches and their biological conditions. III. The fungal flora of common maple, gray alder, silver b. Eur J For Pathol 16:129–138.

Černý K, Havrdová L, Zlatník V, Hrabětová M (2016) Pěstování jasanu v prostředí s výskytem Hymenoscyphus fraxineus [certified methodology]. Průhonice, Czech Republic

Chandelier A, Gerarts F, San Martin G, et al (2016) Temporal evolution of collar lesions associated with ash dieback and the occurrence of Armillaria in Belgian forests. For Pathol 46:289–297.

doi: 10.1111/efp.12258

Chandelier A, Helson M, Dvorak M, Gischer F (2014) Detection and quantification of airborne inoculum of Hymenoscyphus pseudoalbidus using real-time PCR assays. Plant Pathol 63:1296–1305. doi: 10.1111/ppa.12218

Chen J (2012) Fungal Community Survey of Fraxinus excelsior in New Zealand [master thesis]. Swedish University of Agricultural Sciences

Chumanová E, Černý K, Brestovanská T, et al (2021) Atlas potenciální distribuce vybraných druhů invazních patogenů dřevin a jejich impaktu na lesní ekosystémy v ČR [specialized map]. VÚKOZ, v.v.i, Průhonice

Chumanová E, Romportl D, Havrdová L, et al (2019) Predicting ash dieback severity and environmental suitability for the disease in forest stands. Scand J For Res 34:254–266. doi: 10.1080/02827581.2019.1584638

Cleary MR, Arhipova N, Gaitnieks T, et al (2013a) Natural infection of Fraxinus excelsior seeds by Chalara fraxinea. For Pathol 43:83–85. doi: 10.1111/efp.12012

Cleary MR, Daniel G, Stenlid J (2013b) Light and scanning electron microscopy studies of the early infection stages of Hymenoscyphus pseudoalbidus on Fraxinus excelsior. Plant Pathol 62:1294–1301. doi: 10.1111/ppa.12048

Compant S, Samad A, Faist H, Sessitsch A (2019) A review on the plant microbiome : Ecology , functions , and emerging trends in microbial application. J Adv Res 19:29–37. doi: 10.1016/j.jare.2019.03.004

Cross H, Sønstebø JH, Nagy NE, et al (2017) Fungal diversity and seasonal succession in ash leaves infected by the invasive ascomycete Hymenoscyphus fraxineus. New Phytol 213.3:1405–1417. doi: 10.1111/nph.14204

Davydenko K, Vasaitis R, Stenlid J, Menkis A (2013) Fungi in foliage and shoots of Fraxinus excelsior in eastern Ukraine: a first report on Hymenoscyphus pseudoalbidus. For Pathol 43:462–467. doi: 10.1111/efp.12055

de Bary A (1879) Die Erscheinung der Symbiose. Verlag von Karl J. Trübner, Strassburg, Germany

De Gruyter J, Boerema GH, Van der Aa HA (2002) Contributions towards a monograph of Phoma (Coelomycetes) VI-2 Section Phyllostictoides: Outline of its taxa. Persoonia 18:1–53.

Desmazières JBHJ (1851) Plantes Cryptogames de France. Ann des Sci Nat Bot Série 3 16:323–324.

Drenkhan R, Sander H, Hanso M (2014) Introduction of Mandshurian ash (Fraxinus mandshurica Rupr.) to Estonia: Is it related to the current epidemic on European ash (F. excelsior L.)? Eur J For Res 133:769–781. doi: 10.1007/s10342-014-0811-9

Drenkhan R, Solheim H, Bogacheva A, et al (2017) Hymenoscyphus fraxineus is a leaf pathogen of local Fraxinus species in the Russian Far East. Plant Pathol 66:490–500. doi: 10.1111/ppa.12588

Dvorak M, Rotkova G, Botella L (2016) Detection of airborne inoculum of Hymenoscyphus fraxineus and H . albidus during seasonal fluctuations associated with absence of apothecia. Forests 7:1–14. doi: 10.3390/f7010001

Elfstrand M, Chen J, Cleary M, et al (2021) Comparative analyses of the Hymenoscyphus fraxineus and Hymenoscyphus albidus genomes reveals potentially adaptive differences in secondary metabolite and transposable element repertoires. BMC Genomics 22:1–15. doi: 10.1186/s12864-021-07837-2

Enderle R, Stenlid J, Vasaitis R (2019) An overview of ash (Fraxinus spp.) and the ash dieback disease in Europe. CAB Rev Perspect Agric Vet Sci Nutr Nat Resour. doi: 10.1079/PAVSNNR201914025

Fassatiová O (1986) Moulds and filamentous fungi in technical microbiology. Elsevier, Amsterdam

Franić I, Eschen R, Allan E, et al (2020) Drivers of richness and community composition of fungal endophytes of tree seeds. FEMS Microbiol Ecol 96:fiaa166. doi: 10.1093/femsec/fiaa166

Ganley RJ, Sniezko RA, Newcombe G (2008) Endophyte-mediated resistance against white pine blister rust in Pinus monticola. For Ecol Manage 255:2751–2760. doi: 10.1016/j.foreco.2008.01.052

Gennaro M, Gonthier P, Nicolotti G (2003) Fungal endophytic communities in healthy and declining Quercus robur L. and Q. cerris L. trees in northern Italy. J Phytopathol 151:529–534. doi: 10.1046/j.1439-0434.2003.00763.x

George JP, Sanders TGM, Timmermann V, et al (2022) European - wide forest monitoring substantiate the neccessity for a joint conservation strategy to rescue European ash species (Fraxinus spp .). Sci Rep 12:1–12. doi: 10.1038/s41598-022-08825-6

Giordano L, Gonthier P, Varese GC, et al (2009) Mycobiota inhabiting sapwood of healthy and declining Scots pine (Pinus sylvestris L.) trees in the Alps. Fungal Divers 38:69–83.

Granath G, Vicari M, Bazely DR, et al (2007) Variation in the abundance of fungal endophytes in fescue grasses along altitudinal and grazing gradients. Ecography (Cop) 30:422–430. doi: 10.1111/j.2007.0906-7590.05027.x

Gregorová B, Černý K, Holub V, et al (2006) Poškození dřevin a jeho příčiny. VÚKOZ, v.v.i, Praha

Griffith GS, Boddy L (1991) Fungal decomposition of twigs attached anglosperm. IV. Effect of water potential on interactions between fungi on agar and in wood. New Phytol 117:633–641.

Griffiths SM, Galambao M, Rowntree J, et al (2020) Complex associations between cross-kingdom microbial endophytes and host genotype in ash dieback disease dynamics. J Ecol 108:291–309. doi: 10.1111/1365-2745.13302

Gross A, Holdenrieder O (2013) On the longevity of Hymenoscyphus pseudoalbidus in petioles of Fraxinus excelsior. For Pathol 43:168–170. doi: 10.1111/efp.12022

Gross A, Holdenrieder O, Pautasso M, et al (2014a) Hymenoscyphus pseudoalbidus, the causal agent of European ash dieback. Mol Plant Pathol 15:5–21.

Gross A, Hosoya T, Queloz V (2014b) Population structure of the invasive forest pathogen Hymenoscyphus pseudoalbidus. Mol Ecol 23:2943–60. doi: 10.1111/mec.12792

Gross A, Sieber TN (2016) Virulence of Hymenoscyphus albidus and native and introduced Hymenoscyphus fraxineus on Fraxinus excelsior and Fraxinus pennsylvanica. Plant Pathol 65:655–663. doi: 10.1111/ppa.12450

Gross A, Zaffarano PL, Duo A, Grünig CR (2012) Reproductive mode and life cycle of the ash dieback pathogen Hymenoscyphus pseudoalbidus. Fungal Genet Biol 49:977–986. doi: 10.1016/j.fgb.2012.08.008

Guo L-D, Huang G-R, Wang Y (2008) Seasonal and tissue age influences on endophytic fungi of Pinus tabulaeformis (Pinaceae) in the Dongling Mountains, Beijing. J Integr Plant Biol 50:997–1003. doi: 10.1111/j.1744-7909.2008.00394.x

Halecker S, Wennrich JP, Rodrigo S, et al (2020) Fungal endophytes for biocontrol of ash dieback: The antagonistic potential of Hypoxylon rubiginosum. Fungal Ecol 45:100918. doi: 10.1016/j.funeco.2020.100918

Hammer Ø, Harper DAT, Ryan PD (2001) Paleontological statistics software package for education and data analysis. Palaeontol Electron 4:1–9. doi: 10.1016/j.bcp.2008.05.025

Haňáčková Z, Koukol O, Štursová M, et al (2015) Fungal succession in the needle litter of a montane Picea abies forest investigated through strain isolation and molecular fingerprinting. Fungal Ecol 13:157–166. doi: 10.1016/j.funeco.2014.09.007

Havrdová L, Zahradník D, Romportl D, et al (2017) Environmental and silvicultural characteristics influencing the extent of ash dieback in forest stands. Balt For 23:168–181.

Hayatgheibi H (2013) Studies on the microflora associated with the seeds of European ash (Fraxinus excelsior) and the infection biology of the pathogen Hymenoscyphus pseudoalbidus causing ash dieback [master thesis]. Swedish University of Agricultural Sciences

References

Hemery G (2008) Forest management and silvicultural responses to projected climate change impacts on European broadleaved trees and forests. Int For Rev 10:591–607. doi: 10.1505/ifor.10.4.591

Henning JA, Kinkel L, May G, et al (2021) Plant diversity and litter accumulation mediate the loss of foliar endophyte fungal richness following nutrient addition. Ecology 102:e03210. doi: 10.1002/ecy.3210

Hietala AM, Agan A, Nagy NE, et al (2022) The Native Hymenoscyphus albidus and the invasive Hymenoscyphus fraxineus are similar in their necrotrophic growth phase in ash leaves. Front Microbiol 13:1–14. doi: 10.3389/fmicb.2022.892051

Hietala AM, Børja I, Solheim H, et al (2018a) Propagule pressure build-up by the invasive Hymenoscyphus fraxineus following its introduction to an ash forest inhabited by the native Hymenoscyphus albidus. Front Plant Sci 9:1–11. doi: 10.3389/fpls.2018.01087

Hietala AM, Isabella B, Cross H, et al (2018b) Dieback of European ash: What can we learn from the microbial community and species-specific traits of endophytic fungi associated with ash? In: Pirttilä AM, Frank AC (eds) Endophytes of Forest Trees. Springer International PUblishing AG, pp 229–258

Hietala AM, Timmermann V, Børja I, Solheim H (2013) The invasive ash dieback pathogen Hymenoscyphus pseudoalbidus exerts maximal infection pressure prior to the onset of host leaf senescence. Fungal Ecol 6:302–308. doi: 10.1016/j.funeco.2013.03.008

Hosoya T, Otani Y, Furuya K (1993) Materials for the fungus flora of Japan (46). Trans Mycol Soc Japan 34:429–432.

Husson C, Caël O, Grandjean JP, et al (2012) Occurrence of Hymenoscyphus pseudoalbidus on infected ash logs. Plant Pathol 61:889–895. doi: 10.1111/j.1365-3059.2011.02578.x

Husson C, Scala B, Caël O, et al (2011) Chalara fraxinea is an invasive pathogen in France. Eur J Plant Pathol 130:311–324. doi: 10.1007/s10658-011-9755-9

Joshee S, Paulus BC, Park D, Johnston PR (2009) Diversity and distribution of fungal foliar endophytes in New Zealand Podocarpaceae. Mycol Res 113:1003–1015. doi: 10.1016/j.mycres.2009.06.004

Jumpponen A, Jones KL (2010) Seasonally dynamic fungal communities in the Quercus macrocarpa phyllosphere differ between urban and nonurban environments. New Phytol 186:496–513. doi: 10.1111/j.1469-8137.2010.03197.x

Juodvalkis A, Vasiliauskas A (2002) The extent and possible causes of dieback of ash stands in Lithuania. LZUU Moksl darbai, Biomed Moksl 56:17–22.

Kheireddine A, Essghaier B, Hedi A, et al (2018) New epiphytic yeasts able to reduce grey mold disease on apples. Plant Prot Sci 54:248–257. doi: 10.17221/103/2017-PPS

King KM, Webber JF (2016) Development of a multiplex PCR assay to discriminate native Hymenoscyphus albidus and introduced Hymenoscyphus fraxineus in Britain and assess their distribution. Fungal Ecol 23:79–85. doi: 10.1016/j.funeco.2016.06.002

Kosawang C, Buchvaldt Amby D, Bussaban B, et al (2018) Fungal communities associated with species of Fraxinus tolerant to ash dieback , and their potential for biological control. Fungal Biol 122:110–120. doi: 10.1016/j.funbio.2017.11.002

Kosawang C, Sørensen H, Kjær ED, et al (2019) Defining the twig fungal communities of Fraxinus species and Fraxinus excelsior genotypes with differences in susceptibility to ash dieback. Fungal Ecol 42:100859. doi: 10.1016/j.funeco.2019.08.003

Koukol O, Haňáčková Z, Dvořák M, Havrdová L (2016) Unseen , but still present in Czechia: Hymenoscyphus albidus detected by real-time PCR , but not by intensive sampling. Mycol Prog 15:1–9. doi: 10.1007/s11557-015-1149-5

Koukol O, Mrnka L, Kulhánková A, Vosátka M (2006) Competition of Scleroconidioma sphagnicola with fungi decomposing spruce litter needles. Can J Bot 84:469–476. doi:

10.1139/B06-021

Kowalski T (2006) Chalara fraxinea sp. nov. associated with dieback of ash (Fraxinus excelsior) in Poland. For Pathol 36:264–270.

Kowalski T (2001) O zamieraniu jesionów. Tryb Leśnika 4:6–7.

Kowalski T, Bartnik C (2010) Morphologial variation in colonies of Chalara fraxinea isolated from ash (Fraxinus excelsior L.) stems with symptoms of dieback and effects of temperature on colony growth and structure. Acta Agrobot 63:99–106. doi: 10.5586/aa.2010.012

Kowalski T, Bilański P (2021) Fungi detected in the previous year's leaf petioles of Fraxinus excelsior and their antagonistic potential against Hymenoscyphus fraxineus. Forests 12:1412. doi: 10.3390/f12101412

Kowalski T, Holdenrieder O (2009a) The teleomorph of Chalara fraxinea , the causal agent of ash dieback. For Pathol 39:304–308. doi: 10.1111/j.1439-0329.2008.00589.x

Kowalski T, Holdenrieder O (2009b) Pathogenicity of Chalara fraxinea. For Pathol 39:1–7. doi: 10.1111/j.1439-0329.2008.00565.x

Kowalski T, Kehr RD (1992) Endophytic fungal colonization of branch bases in several forest tree species. Sydowia 44:137–168.

Kowalski T, Łukomska A (2005) Badania nad zamieraniem jesionu (Fraxinus excelsior L.) w drzewostanach Nadleśnictwa Włoszczowa [The studies on ash dying (Fraxinus excelsior L.) in the Włoszczowa Forest Unit stands]. Acta Agrobot 58:429–440.

Kozlowski TT, Kramer PJ, Pallardy SG (1991) The Physiological Ecology of Woody Plants. Academic Press, Inc., SA, California

Kurtzman CP, Fell JW, Boekhout T (2011) The Yeasts, 5th ed. Elsevier, Amsterdam, The Netherlands

Kusari P, Kusari S, Spiteller M, Kayser O (2013) Endophytic fungi harbored in Cannabis sativa L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. Fungal Divers 60:137–151. doi: 10.1007/s13225-012-0216-3

Lacava PT, Azevedo JL (2014) Biological control of insect-pest and diseases by endophytes. In: Verma VC, Gange AC (eds) Advances in Endophytic Research. Springer India, New Delhi, pp 231–256

Lahiri A (2020) Endophytic diversity of Fraxinus excelsior L. (European ash) and its interaction with the dieback pathogen Hymenoscyphus fraxineus [doctoral thesis]. University of Dublin, Dublin, Ireland

Lahiri A, Murphy BR, Hodkinson TR (2021) Assessing genotypic and environmental effects on endophyte communities of Fraxinus (Ash) using culture dependent and independent DNA sequencing. J Fungi 7:565. doi: 10.3390/jof7070565

Lamit LJ, Lau MK, Sthultz CM, et al (2014) Tree genotype and genetically based growth traits structure twig endophyte communities. Am J Bot 101:467–478. doi: 10.3732/ajb.1400034

Landolt J, Gross A, Holdenrieder O, Pautasso M (2016) Ash dieback due to Hymenoscyphus fraxineus: what can we learn from evolutionary ecology? Plant Pathol 65:1056–1070. doi: 10.1111/ppa.12539

Latz MAC, Jensen B, Collinge DB, Jørgensen HJL (2018) Endophytic fungi as biocontrol agents: elucidating mechanisms in disease suppression. Plant Ecol Divers 11:555–567. doi: 10.1080/17550874.2018.1534146

Leslie JF, Summerell BA (2006) The Fusarium laboratory manual. Blackwell, Iowa

Levine JM, D'Antonio CM (1999) Elton revisited: a review of evidence linking diversity and invasibility. Oikos 87:15–26.

Lobo A, McKinney LV, Hansen JK, et al (2015) Genetic variation in dieback resistance in Fraxinus excelsior confirmed by progeny inoculation assay. For Pathol 45:379–387. doi:

10.1111/efp.12179

Lonsdale D, Gibbs JN (1996) Effects of climate change on fungal diseases of trees. In: Frankland JC, Magan N, Gadd GM (eds) Fungi and environmental change. Cambridge University Press, Cambridge, pp 1–19

Louda SM, Pemberton RW, Johnson MT, Follett PA (2003) Nontarget effects—The Achilles'heel of biological control? Retrospective analyses to reduce risk associated with biocontrol introductions. Annu Rev Entomol 48:365–396. doi: 10.1146/annurev.ento.48.060402.102800

Lygis V, Vasiliauskas R, Larsson KH, Stenlid J (2005) Wood-inhabiting fungi in stems of Fraxinus excelsior in declining ash stands of northern Lithuania, with particular reference to Armillaria cepistipes. Scand J For Res 20:337–346. doi: 10.1080/02827580510036238

Lyu D, Zajonc J, Pagé A, et al (2021) Plant holobiont theory: the phytomicrobiome plays a central role in evolution and success. MDPI 9:675. doi: 10.3390/ microorganisms9040675

Mansfield J, Brown I, Papp-Rupar M (2019) Life at the edge – the cytology and physiology of the biotroph to necrotroph transition in Hymenoscyphus fraxineus during lesion formation in ash. Plant Pathol 68:908–920. doi: 10.1111/ppa.13014

Martín JA, Witzell J, Blumenstein K, et al (2013) Resistance to Dutch elm disease reduces presence of xylem endophytic fungi in elms (Ulmus spp.). PLoS One 8:e56987. doi: 10.1371/journal.pone.0056987

Martinović T, Koukol O, Hirose D (2016) Distinct phylogeographic structure recognized within Desmazierella acicola. Mycologia 108:20–30. doi: 10.3852/14-291

McKinney LV, Thomsen IM, Kjær ED, et al (2012a) Rapid invasion by an aggressive pathogenic fungus (Hymenoscyphus pseudoalbidus) replaces a native decomposer (Hymenoscyphus albidus): a case of local cryptic extinction? Fungal Ecol 5:663–669. doi: 10.1016/j.funeco.2012.05.004

McKinney L V., Nielsen LR, Collinge DB, et al (2014a) The ash dieback crisis: genetic variation in resistance can prove a long-term solution. Plant Pathol 63:485–499. doi: 10.1111/ppa.12196

McKinney L V., Nielsen LR, Collinge DB, et al (2014b) The ash dieback crisis: genetic variation in resistance can prove a long-term solution. Plant Pathol 63:485–499. doi: 10.1111/ppa.12196

McKinney L V, Thomsen IM, Kjær ED, Nielsen LR (2012b) Genetic resistance to Hymenoscyphus pseudoalbidus limits fungal growth and symptom occurrence in Fraxinus excelsior. For Pathol 42:69–74. doi: 10.1111/j.1439-0329.2011.00725.x

McMullan M, Rafiqi M, Kaithakottil G, et al (2018) The ash dieback invasion of Europe was founded by two genetically divergent individuals. Nat Ecol Evol 2:1000–1008. doi: 10.1038/s41559-018-0548-9

Mejía LC, Rojas EI, Maynard Z, et al (2008) Endophytic fungi as biocontrol agents of Theobroma cacao pathogens. Biol Control 46:4–14. doi: 10.1016/j.biocontrol.2008.01.012

Meyn R, Langer GJ, Gross A, Langer EJ (2019) Fungal colonization patterns in necrotic rootstocks and stem bases of dieback-affected Fraxinus excelsior L. For Pathol. doi: 10.1111/efp.12520

Musolin DL, Selikhovkin A V., Shabunin DA, et al (2017) Between ash dieback and emerald ash borer: Two Asian invaders in Russia and the future of ash in Europe. Balt For 23:316–333.

Nemesio-Gorriz M, McGuinness B, Grant J, et al (2019) Lenticel infection in Fraxinus excelsior shoots in the context of ash dieback. iForest - Biogeosciences For 12:160–165. doi: 10.3832/ifor2897-012

Nemesio-Gorriz M, Menezes RC, Paetz C, et al (2020) Canditate metabolites for ash dieback tolerance in Fraxinus excelsior. J Exp Bot 71:6074–6083. doi: 10.1093/jxb/eraa306

Nguyen DT, Cleary MR, Enderle R, et al (2016a) Analyses of the ash dieback pathogen, Hymenoscyphus fraxineus , suggest role of tree species diversity on colonization and population structure differentiation. For Pathol 46:82–84. doi: 10.1111/efp.12236

Nguyen NH, Song Z, Bates ST, et al (2016b) FUNGuild: An open annotation tool for parsing fungal

community datasets by ecological guild. Fungal Ecol 20:241–248. doi: 10.1016/j.funeco.2015.06.006

Nielsen LR, Nagy NE, Piqueras S, et al (2022) Host – pathogen interactions in leaf petioles of common ash and Manchurian ash infected with Hymenoscyphus fraxineus. Microorganisms 10:375. doi: 10.3390/ microorganisms10020375

Oita S, Ibáñez A, Lutzoni F, et al (2021) Climate and seasonality drive the richness and composition of tropical fungal endophytes at a landscape scale. Commun Biol 4:313. doi: 10.1038/s42003-021-01826-7

Orton ES, Brasier CM, Bilham LJ, et al (2018) Population structure of the ash dieback pathogen , Hymenoscyphus fraxineus , in relation to its mode of arrival in the UK. Plant Pathol 67:255–264. doi: 10.1111/ppa.12762

Osono T (2008) Endophytic and epiphytic phyllosphere fungi of Camellia japonica: seasonal and leaf age-dependent variations. Mycologia 100:387–391. doi: 10.3852/07-110R1

Petrini O (1991) Fungal endophytes of tree leaves. In: Andrews JH, Hirano SS (eds) Microbial Ecology of Leaves. Springer New York, New York, NY, pp 179–197

Pliûra A, Heuertz M (2003) Technical guidelines for genetic conservation and use for common ash (Fraxinus excelsior). Rome

Pliūra A, Lygis V, Suchockas V, Bartkevičius E (2011) Performance of twenty four European Fraxinus excelsior populations in three Lithuanian progeny trials with a special emphasis on resistance to Chalara fraxinea. Balt For 17:17–34.

Pliūra A, Marčiulynienė D, Bakys R, Suchockas V (2014) Dynamics of genetic resistance to Hymenoscyphus pseudoalbidus in Juvenile Fraxinus excelsior clones. Balt For 20:10–27.

Poland TM, McCullough DG (2006) Emerald ash borer: Invasion of the urban forest and the threat to North America's ash resource. J For 104:118–124.

Prior R, Görges K, Yurkov A, Begerow D (2014) New isolation method for endophytes based on enzyme digestion. Mycol Prog 13:849–856. doi: 10.1007/s11557-014-0968-0

Przybył BK (2002) Fungi associated with necrotic apical parts of Fraxinus excelsior shoots. For Pathol 32:387–394.

Queloz V, Grünig CR, Berndt R, et al (2011) Cryptic speciation in Hymenoscyphus albidus. For Pathol 41:133–142. doi: 10.1111/j.1439-0329.2010.00645.x

Ragazzi A, Moricca S, Capretti P, et al (2003) Differences in composition of endophytic mycobiota in twigs and leaves of healthy and declining Quercus species in Italy. For Pathol 33:31–38. doi: 10.1046/j.1439-0329.2003.3062003.x

Rajala T, Velmala SM, Tuomivirta T, et al (2013) Endophyte communities vary in the needles of Norway spruce clones. Fungal Biol 117:182–190. doi: 10.1016/j.funbio.2013.01.006

Rajala T, Velmala SM, Vesala R, et al (2014) The community of needle endophytes reflects the current physiological state of Norway spruce. Fungal Biol 118:309–315. doi: 10.1016/j.funbio.2014.01.002

Rodriguez RJ, White JF, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–30. doi: 10.1111/j.1469-8137.2009.02773.x

Rong X, Rotondo F, Schisler DA, et al (2017) Tracking native and applied populations of Cryptococcus flavescens in the environment. Plant Gene 11:31–40. doi: 10.1016/j.plgene.2016.12.004

Rottstock T, Joshi J, Kummer V, Fischer M (2014) Higher plant diversity promotes higher diversity of fungal pathogens , while it decreases pathogen infection per plant. Ecology 95:1907–1917. doi: 10.1890/13-2317.1

Rozsypálek J, Dvořák M, Longauer R, et al (2017) Ash and ash dieback in the Czech Republic. In: Vasaitis R, Enderle R (eds) Dieback of European ash (Fraxinus spp.)–Consequences and Guidelines for Sustainable Management. Swedish University of Agricultural Sciences,

Uppsala,Sweden, pp 79–88

Saikkonen K, Faeth SH, Helander M, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 319–343.

Sansford CE (2013) Pest risk analysis for Hymenoscyphus pseudoalbidus (anamorph Chalara fraxinea) for the UK and the Republic of Ireland.

Schlegel M (2018) Diversity and ecology of fungal endophytes in face of an emerging tree disease [doctoral thesis]. ETH Zürich

Schlegel M, Dubach V, Buol L Von, Sieber TN (2016) Effects of endophytic fungi on the ash dieback pathogen. FEMS Microbiol Ecol 92:1–8. doi: 10.1093/femsec/fiw142

Schlegel M, Queloz V, Sieber TN (2018) The endophytic mycobiome of European ash and sycamore maple leaves – geographic patterns, host specificity and influence of ash dieback. Front Microbiol 9:2345. doi: 10.3389/fmicb.2018.02345

Schmidt CS, Lovecká P, Mrnka L, et al (2018) Distinct communities of poplar endophytes on an unpolluted and a risk element-polluted site and their plant growth-promoting potential in vitro. Microb Ecol 75:955–969. doi: 10.1007/s00248-017-1103-y

Scholtysik A, Unterseher M, Otto P, Wirth C (2013) Spatio-temporal dynamics of endophyte diversity in the canopy of European ash (Fraxinus excelsior). Mycol Prog 12:291–304. doi: 10.1007/s11557-012-0835-9

Schulz B, Boyle C, Draeger S, et al (2002) Endophytic fungi: a source of novel biologically active secondary metabolites*. 106:996–1004. doi: 10.1017?S0953756202006342

Schulz B, Haas S, Junker C, et al (2015) Fungal endophytes are involved in multiple balanced antagonisms. Curr Sci 109:39–45.

Siddique AB, Khokon AM, Unterseher M (2017) What do we learn from cultures in the omics age? High-throughput sequencing and cultivation of leaf- inhabiting endophytes from beech (Fagus sylvatica L.) revealed complementary community composition but similar correlations with local habitat conditions. MycoKeys 20:1–16. doi: 10.3897/mycokeys.20.11265

Sieber TN (2007) Endophytic fungi in forest trees: are they mutualists? Fungal Biol Rev 21:75–89. doi: 10.1016/j.fbr.2007.05.004

Sieber TN, Hugentobler C (1987) Endophytische Pilze in Blättern und Ästen gesunder und geschädigter Buchen (Fagus sylvatica L.)[Endophytic fungi in leaves and twigs of healthy and diseased beech trees (Fagus sylvatica L.) (in German with English abstract). Eur J For Pathol 17:411–425. doi: 10.1111/j.1439-0329.1987.tb01119.x

Skovsgaard JP, Thomsen IM, Skovgaard IM, Martinussen T (2010) Associations among symptoms of dieback in even-aged stands of ash (Fraxinus excelsior L.). For Pathol 40:7–18. doi: 10.1111/j.1439-0329.2009.00599.x

Skovsgaard JP, Wilhelm GJ, Thomsen IM, et al (2017) Silvicultural strategies for Fraxinus excelsior in response to dieback caused by Hymenoscyphus fraxineus. Forestry 90:455–472. doi: 10.1093/forestry/cpx012

Sønstebø JH, Vivian-Smith A, Adamson K, et al (2017) Genome-wide population diversity in Hymenoscyphus fraxineus points to an eastern Russian origin of European Ash dieback. bioRxiv 1–25.

Stener L-G (2013) Clonal differences in susceptibility to the dieback of Fraxinus excelsior in southern Sweden. Scand J For Res 28:205–216. doi: 10.1080/02827581.2012.735699

Stenlid J, Elfstrand M, Cleary M, et al (2017) Genomes of Hymenoscyphus fraxineus and Hymenoscyphus albidus encode surprisingly large cell wall degrading potential , balancing saprotrophic and necrotrophic signatures. Balt For 23:41–51.

Stodůlková E, Man P, Kuzma M, et al (2015) A highly diverse spectrum of naphthoquinone derivatives produced by the endophytic fungus Biatriospora sp. CCF 4378. Folia Microbiol (Praha) 60:259–267. doi: 10.1007/s12223-014-0366-7

Tan YP, Edwards J, Grice KRE, Shivas RG (2013) Molecular phylogenetic analysis reveals six new species of Diaporthe from Australia. Fungal Divers 61:251–260. doi: 10.1007/s13225-013-0242-9

Terhonen E, Blumenstein K, Kovalchuk A, Asiegbu FO (2019) Forest tree microbiomes and associated fungal endophytes: functional roles and impact on forest health. Forests 10:42. doi: 10.3390/f10010042

Tilman D (2004) Niche tradeoffs , neutrality , and community structure : A stochastic theory of resource competition , invasion , and community assembly. PNAS 101:10854–10861. doi: 10.1073/pnas.0403458101

Timmermann V, Børja I, Hietala AM, et al (2011) Ash dieback : pathogen spread and diurnal patterns of ascospore dispersal , with special emphasis on Norway *. EPPO Bull 41:14–20.

Tolasz R (2007) Climate atlas of Czechia. Czech Hydrometeorological Institute, Palacký University Olomouc, Prague, Olomouc

Ulrich K, Becker R, Behrendt U, et al (2020) A comparative analysis of ash leaf-colonizing bacterial communities identifies putative antagonists of Hymenoscyphus fraxineus. Front Microbiol 11:966. doi: 10.3389/fmicb.2020.00966

Unterseher M, Otto P, Morawetz W (2005) Species richness and substrate specificity of lignicolous fungi in the canopy of a temperate, mixed deciduous forest. Mycol Prog 4:117–132.

Unterseher M, Reiher A, Finstermeier K, et al (2007) Species richness and distribution patterns of leaf-inhabiting endophytic fungi in a temperate forest canopy. Mycol Prog 6:201–212. doi: 10.1007/s11557-007-0541-1

Unterseher M, Tal O (2006) Influence of small scale conditions on the diversity of wood decay fungi in a temperate, mixed deciduous forest canopy. Mycol Res 110:169–178. doi: 10.1016/j.mycres.2005.08.002

Vasaitis R, Enderle R (2017) Dieback of European Ash (Fraxinus spp.): Consequences and Guidelines for Sustainable Management. Uppsala,Sweden

Velenovský J (1934) Monographia Discomycetum Bohemiae, vol 1. Prague

Viterbo A, Inbar J, Hadar Y, Chet I (2007) Plant disease biocontrol and induced resistance via fungal mycoparasites. In: Kubicek CP, Druzhinina IS (eds) The mycota IV. Environmental and microbial relationships. Springer, Heidelberg, pp 127–146

Voglmayr H, Jaklitsch WM (2008) Prosthecium species with Stegonsporium anamorphs on Acer. Mycol Res 112:885–905. doi: 10.1016/j.mycres.2008.01.020

Volkovitsh MG, Bieńkowski AO, Orlova-Bienkowskaja MJ (2021) Emerald ash borer approaches the borders of the European union and kazakhstan and is confirmed to infest European ash. Forests 12:691. doi: 10.3390/f12060691

Vujanovic V (2021) Tremellomycetes yeasts in kernel ecological niche: Early indicators of enhanced competitiveness of endophytic and mycoparasitic symbionts against wheat pathobiota. Plants 10:905. doi: 10.3390/plants10050905

Webber JF, Gibbs JN (1984) Colonization of elm bark by Phomopsis oblonga. Trans Br Mycol Soc 82:348–352.

Wey T, Schlegel M, Stroheker S, Gross A (2016) MAT – gene structure and mating behavior of Hymenoscyphus fraxineus and Hymenoscyphus albidus. Fungal Genet Biol 87:54–63. doi: 10.1016/j.fgb.2015.12.013

Widler B, Müller E (1984) Untersuchungen über endophytische Pilze von Arclostaphylos uva-ursi (L .) Sprengel (Ericaceae)[Investigations on endophytic fungi of Arctostaphylos uva-ursi (L.) Sprengel (Ericaceae)] (in German with English abstract). Bot Helv 94:307–337.

Witzell J, Martín JA, Blumenstein K (2014) Ecological aspects of endophyte-based biocontrol of forest diseases. In: Verma VC, Gange AC (eds) Advances in endophytic research. Springer India, New Delhi, pp 321–333

Wylder B, Biddle M, King K, et al (2018) Evidence from mortality dating of Fraxinus excelsior

indicates ash dieback (Hymenoscyphus fraxineus) was active in England in 2004 – 2005. Forestry 91:434–443. doi: 10.1093/forestry/cpx059

Yadav AN, Verma P, Kour D, et al (2017) Plant microbiomes and its beneficial multifunctional plant growth promoting attributes. Int J Environ Sci Nat Resour 3:555601. doi: 10.19080/IJESNR.2017.03.555601

Yan JF, Broughton SJ, Yang SL, Gange a. C (2015) Do endophytic fungi grow through their hosts systemically? Fungal Ecol 13:53–59. doi: 10.1016/j.funeco.2014.07.005

Zhao Y, Hosoya T, Baral H, et al (2012) Hymenoscyphus pseudoalbidus, the correct name for Lambertella albida reported from Japan. Mycotaxon 122:25–41. doi: 10.5248/122.25

Zugmaier W, Bauer R, Oberwinkler F (1994) Mycoparasitism of some Tremella species. Mycologia 86:49–56.

PART B – ORIGINAL PAPERS

LOCAL POPULATION STRUCTURE OF HYMENOSCYPHUS FRAXINEUS SURVEYED BY AN ENLARGED SET OF MICROSATELLITE MARKERS

Zuzana Haňáčková[1,2,*], Ondřej Koukol[2], Ludmila Havrdová[1,3] & Andrin Gross[3]

[1]*Department of Biological Risks, Silva Tarouca Research Institute for Landscape and Ornamental Gardening Publ. Res. Inst., Květnové náměstí 391, 252 43 Průhonice, Czech Republic;* [2]*Department of Botany, Faculty of Science, Charles University in Prague, Benátská 2, 128, 01 Prague, Czech Republic;* [3]*Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Prague, Czech Republic;* [4]*Forest Pathology and Dendrology, Institute of Integrative Biology (IBZ), ETH Zurich, Zurich, Switzerland;* [*]*Corresponding author: E-mail: Zuzana.Hanackova@vukoz.cz*

Haňáčková et al. 2015. Forest Pathology, 45/5: 400–407, doi: 10.1111/efp.12185.

SUMMARY

The population structure of *Hymenoscyphus fraxineus*, the causal agent of ash dieback, was assessed at four closely located sites in the Czech Republic. To analyse the genetic variation, one Swiss and one Norwegian population with known population structures were selected as reference points. The analysis was performed using 16 previously published and five newly developed microsatellite markers. The quality of the new markers was assessed by sequencing the flanking region, identifying the type of the mutation and analysing the inheritance and linkage between all pairs of loci. In addition, markers were tested on 45 Japanese strains to confirm their usability on native *H. fraxineus* populations in Asia. One of the new markers was monomorphic in all European populations and one marker exhibited a high percent-age of null alleles in the Japanese samples. Twenty markers in the four Czech populations showed lower average gene diversity than in the other two European populations. One Czech population significantly differed from all the others, with a pairwise G_{ST} of approximately 0.2, the lowest allelic richness and very low average gene diversity, likely resulting from a founder effect. The amount of genetic differentiation between the four neighbouring Czech populations exceeds the expected value from previous Europe-wide population studies and suggests that local population dynamics can affect the population structure of *H. fraxineus*.

INTRODUCTION

Hymenoscyphus fraxineus is an invasive ascomycetous pathogen, which was likely introduced from Asia (Gross et al. 2014a; Zhao et al. 2012). Since the 1990s it has caused ash dieback in large areas of Europe (Timmermann et al. 2011). Preliminary population genetic investigations have revealed high genotypic diversity, indicating high rate of sexual reproduction (Kraj et al. 2012; Rytkönen et al. 2011). Subsequent studies concerning the population structure of *H. fraxineus* were in partial disagreement (Bengtsson et al. 2012; Gross et al. 2014a; Gross et al. 2012; Kraj and Kowalski 2014; Kraj et al. 2012; McKinney et al. 2012). For example, using microsatellite markers Gross et al. (2014a) and Bengtsson et al. (2012) demonstrated low genetic structure between populations from several European countries, and a study on populations in Denmark (McKinney et al. 2012) yielded corresponding results. By contrast, using RAMS markers, Kraj et al. (2012) have found a well-defined population structure, while differences between lowland and highland populations were shown. Because only isolates from necrotic lesions were used, this finding might be restricted to pathogen subpopulations able to induce stem necrosis. However, similar results were obtained with isolates from ascospores (Kraj and Kowalski 2014). Both studies characterised lowland populations by fewer number of markers, fewer polymorphic loci as well as a lesser degree of intra-population genetic variability.

Comparison of European and Japanese populations of *H. fraxineus* showed a much higher allelic richness in Japan (Gross et al. 2014a). There are, however, only eight microsatellite markers applicable for both groups thus far, whereas more loci are required to increase the resolution of genetic comparisons and to more accurately localise the source population.

In the Czech Republic, *H. fraxineus* was initially isolated in 2007 from *Fraxinus excelsior* cv. Pendula in the Křtiny Arboretum (South Moravia). Nevertheless, the first infections had likely occurred by the end of the 1990s (Jankovsky and Holdenrieder 2009), and at present, ash dieback can be found throughout the Czech Republic. The level of infection, however, differs among the vegetation types, where humidity seems to be the limiting factor for the pathogen (Havrdová and Černý 2013).

The aim of this study was to examine the population structure of *H. fraxineus* at four sites within the Czech Republic. Two other populations with known population structure from Switzerland and Norway were used to compare and

interpret our results. The analyses were carried out using 21 microsatellite markers, five of which were newly developed, and their suitability was tested on populations from the native range (Japan) and from the invaded area (Europe).

MATERIAL AND METHODS

Development of new microsatellite markers

Previously described sequencing data (Gross et al. 2012) were used to mine for microsatellite repeat loci. Twenty loci were selected, and primers that amplified a fragment of approximately 1 kb were designed with PRIMER3WEB (http://bioinfo.ut.ee/mprimer3/) (Table S1). Each locus was amplified in eight test strains of *H. fraxineus* (Table S2), and products were visualized on a 1 % (w/v) agarose gel. Subsequently, eight promising loci (without any null-lleles in test strains) were selected and all test strains were partially sequenced with the corresponding forward primer. Sequences were aligned using the multiple sequence alignment tool CLUSTALW, implemented in GENEIOUS v6.1.3 (Biomatters Ltd., Auckland, New Zealand). Two loci exhibited no polymorphisms, whereas six loci revealed size differences of which five were due to variation in the microsatellite repeat number. Sequenced alleles of the five polymorphic loci were submitted to GenBank (Table S3). For the five loci with mutations in the microsatellite repeat, new primers were designed and the forward primers were directly labelled with a fluorescent dye. A multiplex PCR (multiplex PCR no. 4) was developed, allowing to amplify all five loci in a single PCR run (Table S4).

Testing the new microsatellite markers

Mendelian inheritance of microsatellite markers was tested on 30–31 progeny isolates derived from three different apothecia collected in the field. A chi-squared-test was used to test for a 1 : 1 segregation of alleles (Table 1).

Linkage disequilibrium among all pairs of polymorphic loci, including 15 of the previously developed microsatellite markers, was calculated in ARLEQUIN v. 3.5.1.3 (Excoffier and Lischer 2010) using 10 000 steps in the Markov chain and 10 000 dememorization steps. The significance level $\alpha = 0.05$ was adjusted by the sequential Bonferroni correction procedure for each pair of loci (Rice 1989).

Table 1. Segregation patterns of four polymorphic microsatellite markers in progeny genotypes of three different apothecia.

Apothecia	Polymorphic locus	Allele_1 (bp)[1]	Allele_2(bp)[1]	Total progeny genotypes	p - χ^2 test
Fk02	mHp_076105	197 (16)	201 (14)	30	0.72
Fk02	mHp_076166	155 (15)	184 (15)	30	1.00
Fk08	mHp_068858	120 (16)	129 (14)	30	0.72
Fk08	mHp_076166	155 (14)	184 (16)	30	0.72
Fk08	mHp_086811	151 (17)	161 (13)	30	0.47
Fk19	mHp_068858	120 (14)	129 (17)	31	0.59
Fk19	mHp_076166	155 (12)	184 (19)	31	0.21

[1]Number of corresponding genotypes are given in parentheses

In addition, the microsatellite markers were tested on a set of 45 isolates from Japan (subsample of population JP_Sugadaira of Gross et al. 2014a) because previous studies revealed several microsatellite markers with a high percentage of amplification failures on strains from the native range of the fungus.

Sampling, isolation and microsatellite genotyping

Sampling of 1-year-old necrotic shoots was performed at four sites in the north of the Czech Republic, between February and April 2012 (Table 2, Fig. 1). The study sites were two nurseries (Ploužnice, Krásné Pole), a young ash plantation (Deštná) and a site with natural ash seedlings (Lovečkovice), located 21–37 km apart (Table 2). At each site, *H. fraxineus* was isolated from 20 necrotic lesions. Pieces of sapwood from the whole lower border of necrotic lesions were surface-sterilized as follows: 10 s
in ethanol (96%), 10 s in sodium hypochlorite (4.7% active chlorine), 10 s in ethanol (96%), a final wash in distilled water and drying on sterile paper tissue. Sapwood pieces were placed on malt extract agar with streptomycin (composition per 1 l: malt extract 30 g, peptone 5 g, agar 15 g, streptomycin 100 mg – added after autoclaving) and incubated at 18°C. Outgrowing mycelia with morphological features of *H. fraxineus* were kept on wort agar prepared from brewer's wort (final sucrose content 2% w/v). One to two strains from each lesion were analysed (however, it should be noted that two strains from a single lesion were isolated only in two cases in the Deštná population). The total number of strains (isolates) per population is shown in Table 2. Another two of the previously analysed populations (see Gross et al. 2014a) were selected for comparing genetic patterns: one from Switzerland and one from Norway, both collected in 2010 (Table 2). Together, 108 strains of European

H. fraxineus were analysed. Fungal DNA of the Czech populations was extracted by the ZR Fungal/Bacterial DNA kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions.

Table 2. Characteristics of study sites and *Hymenoscyphus fraxineus* populations.

Study site	Country	Coordinates				Sample Size	Altitude (m.a.s.l)	Stand character	Tree height (m)	Allelic richness	Number of alleles per locus	Gene diversity *He*
Lovečkovice	CZ	50.62083	N	14.26287	E	13	420-435	natural seeding	0.2	1.74	1.75	0.30
Deštná	CZ	50.52201	N	14.51732	E	19	240	planting	4	1.62	1.70	0.21
Ploužnice	CZ	50.63314	N	14.76381	E	19	291	nursery	1	1.78	1.85	0.31
Krásné Pole	CZ	50.85193	N	14.50256	E	16	440	nursery	0.5	1.90	1.95	0.35
Ebikon	CH	47.07412	N	8.30381	E	23	450	NA	NA	1.88	2.00	0.36
Oslo	N	59.92831	N	10.69423	E	18	43	NA	NA	1.86	1.90	0.38
Sugadaira	JP	36.52022	N	138.35101	E	45	1317	natural forest	> 20 m	–	–	–

NA, not available

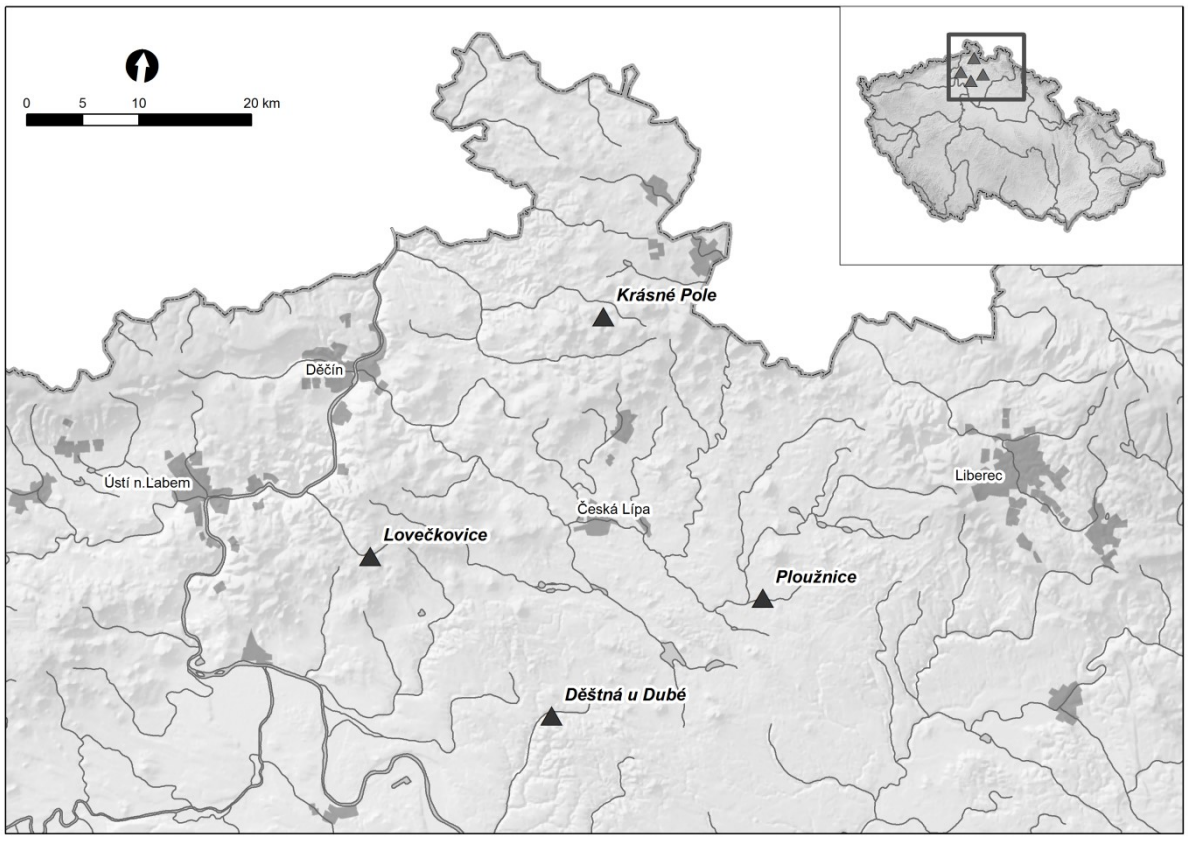

Fig. 1. Schematic map of northern Czech Republic (entire country in the upper right corner) showing the localities of the four Czech populations incorporated in the study.

All strains were genotyped with the new microsatellites described above and 16 microsatellites from Gross et al. (2012), using the adjusted multiplex PCRs specified in Table S4. Multiplex PCRs contained 2 µl 5× reaction buffer

(Promega,Dübendorf, Switzerland), 0.2 mM dNTP mix (Fermentas, St. Leon-Rot, Germany), primer concentrations as specified in Table S4 and 0.5 U GoTaq polymerase (Promega). PCRs and fragment analysis were carried out as described in Gross et al. (2012).

Population genetic analysis

Genetic analyses were conducted with 20 microsatellite markers. The mitochondrial locus mHp_mt_01 was excluded from the data because of frequent non-Mendelian inheritance, as identified in a previous study (Gross et al. 2014a). The R-package 'poppr v1.0.3' (R_Development_Core_Team 2009; Kamvar et al. 2014) was used to compute the number of multilocus genotypes and the index of association I_A (Brown et al. 1980; Smith et al. 1993). The I_A is expected to be zero if the alleles are distributed randomly between different genotypes, which would suggest random mating. The number of significant deviations from zero was tested by 1000 permutations using the allele permutation method of 'poppr'. The software SPAGeDi 1.4c (Hardy and Vekemans 2002) was used for the determination of allelic richness (normalised to the smallest populations size N=13) and Nei's average gene diversity *H*e (Nei 1987).

Pairwise population differentiation (G_{ST}) was calculated in Arlequin 3.5.1.2 (Excoffier and Lischer 2010) and tested for significance by 10 000 permutations. Population structure was explored by principal component analysis (PCA) using the R-package 'adegenet v1.3-9.2' (Jombart 2008; R_Development_Core_Team 2009). As a comparison, the Bayesian model-based clustering algorithm of the software STRUCTURE 2.3.2 (Pritchard et al. 2000) was used. The program minimises Hardy-Weinberg disequilibrium and the phase disequilibrium between loci within a given number of clusters *K*. After a burn-in period of 50 000 MCMC iterations, further 1 000 000 iterations were performed for ten independent runs for each *K* ranging from 1 to 8. No prior population information was supplemented, and the program was run under the admixture model with independent allele frequencies. The output of STRUCTURE was analysed with Structure Harvester Web v0.6.93 to determine the optimal number of clusters *K* (Earl and vonHoldt 2012).

RESULTS

New microsatellite markers for *Hymenoscyphus fraxineus*

Five new microsatellite markers were developed. The quality of the new markers was assessed by analysing (i) the type of the mutation between microsatellite alleles, (ii) their inheritance and (iii) the linkage among loci. In addition, to evaluate their usability on native *H. fraxineus* populations, the markers were tested on 45 *H. fraxineus* strains originating from Japan.

The sequencing of the microsatellite flanking region identified two sequence variants per marker. The mutation leading to sequence length variation always occurred in the microsatellite repeat region (Table 3). However, in marker mHp_076166, two single base indels (insertion or deletion) were also found in the flanking region, one of which was only found in the reference sequence. All loci revealed 1–6 additional single nucleotide polymorphisms (SNPs) in the flanking regions. In all but marker mHp_067560, the copy number of the microsatellite repeat motif differed by more than one repeat unit between the alleles. The onefold repeat shift at marker mHp_067560 was only detected once among all of the eight test strains (strain 2118_1, Table S3). In addition, a onefold repeat shift leading to a third allele at locus mHp_076166 was detected only once in a strain of the Czech population Ploužnice (Table 3). This allele, however, was not sequenced.

Table 3. New microsatellite loci and their characteristics in European populations (based on sequencing data and fragment analysis, see also Table S3): The periode size, number of alleles, microsatellite repeat motif, repeat copy number, sequence and fragment lengths and the type of the mutation (MS, microsatellite repeat; SNP, single nucleotide polymorphism; indels, insertion/deletion) is given.

Locus name	Period size (bp)	Repeat copy number	Repeat motif	Number of alleles	Allele size [bp]	Fragment lenghts	Mutation type
mHp_067560	3	7, 8	GAG	2	241, 245	242[1],245	MS, 3 SNPs
mHp_068858	3	6, 9	CAA	2	124, 133	120, 129	MS, 2 SNPs
mHp_076105	4	5, 6	ATGG	2	194, 198	197, 201	MS, 4 SNPs
mHp_076166	3	7, 17, 18	CTT	3	151, 180	155, 184, 187[2]	MS, 2 single bp indels, six SNPs
mHp_086811	3	5, 8	GAG	2	153, 162	151, 161	MS, 1 SNP

[1] Allele has been detected in the polymorphism screening only.
[2] Allele only found in a single strain in the Ploužnice population; allele not sequenced.

Microsatellite inheritance analysis revealed that of 30–31 progeny isolates within three different apothecia, only mHp_067560 was not polymorphic. The segregation patterns of the remaining markers did not significantly deviate from the 1 : 1 ratio expected under Mendelian inheritance (Table 1).

Significant pairwise linkage between old and new markers was only identified in individual cases. In addition, old markers mHp_080497 and mHp_073013 were in pairwise linkage in two Czech populations, that is Deštná and Lovečkovice.

Markers mHp_068858, mHp_076105 and mHp_086811 were polymorphic in Europe and also performed well on Japanese strains (Table 4). Locus mHp_076166 was polymorphic in Europe but failed to be amplified in 11 (24.4%) Japanese strains. In return, locus mHp_067560 was monomorphic for European populations (with the exception of the second allele found in the test population) but polymorphic in Japan (Table S3). Average gene diversity of the new microsatellites was 0.31 for European strains and 0.68 for Japanese strains (Table 4). The number of alleles varied from 1 to 3 in Europe, whereas the Japanese population showed 5–21 alleles per locus.

Table 4. Diversity of five new microsatellites in European and Japanese samples.

	Europe (N=108)			Japan (N=45)			
Locus	Number of alleles	Per cent of null alleles	ϕ H_e [1]	Number of alleles	Per cent of null alleles	ϕ H_e	Reccomended usage
mHp_067560	1 [2]	0.0	0.00	6	0.0	0.56	only Japanese populations, monomorph in Europe
mHp_068858	2	0.0	0.49	7	0.0	0.76	European and Japanese populations
mHp_076105	2	0.0	0.09	10	0.0	0.58	European and Japanese populations
mHp_076166	3	0.0	0.50	21	24.4	0.95	only European populations, null alleles in Japanese samples
mHp_086811	2	0.0	0.50	5	0.0	0.54	European and Japanese populations

[1] Average gene diversity
[2] A second allele was only found in the test strains (see Table S2)

Local genetic structure in the Czech Republic and comparison with two other European *Hymenoscyphus fraxineus* populations

All 108 European strains studied were recognized as individual multilocus genotypes. In addition, the two strains isolated from one lesion in the Deštná population yielded different genotypes. The index of association was not significantly different from zero in all populations (data not shown), indicating random recombination between loci. The number of alleles per locus varied from 1.70 to 1.95 in the Czech populations, whereas it was 2.00 for the Swiss population and 1.90 for the Norwegian population (Table 2). Likewise, gene diversity was

lower in the Czech populations, ranging from 0.21 to 0.35, while it was 0.36 and 0.38 in the Swiss and Norwegian populations, respectively (Table 2). The Deštná population showed the lowest gene diversity (H_e = 0.21) and significantly differed from all other tested populations, with the pairwise popula- tion G_{ST} value ranging from 0.10 to 0.28 (Table 5). G_{ST} values between the other Czech populations ranged from 0.01 to 0.02 and were not significant. However, comparisons to other European population were higher (G_{ST} = 0.02–0.11) and mostly significant. The PCA shows that the Deštná population is quite distinct. The other populations partially overlap and are not clearly separable from each other (Fig. 2).

Two clusters proved to be the best model, as determined by STRUCTURE analysis and subsequent analysis in Structure Harvester (Fig. 3). Log likelihood values for K = 2 were significantly better than for K = 1 (Wilcoxon rank sum test, $p < 0.001$) (Fig. 3b). In addition, delta (K) plots showed the highest value for K = 2 (Fig. 3c). However, the probability plots indicated that individuals of different populations were not allocated to one or the other cluster with a high confidence (Fig. 3a). Similarly, in all but the Deštná population, genotypes assigned to either cluster were present. Only in the Deštná population were individuals consistently assigned to the same cluster with moderate to high confidence.

Table 5. Matrix of pairwise population differentiation (G_{ST}) for Czech, Swiss and Norweigian populations of *Hymenoscyphus fraxineus.*

Population	Lovečkovice	Deštná	Ploužnice	Krásné Pole	Ebikon	Oslo
Lovečkovice	0.00000					
Deštná	0.20975*	0.00000				
Ploužnice	0.00799	0.09916*	0.00000			
Krásné Pole	0.00943	0.16511*	0.01850	0.00000		
Ebikon	0.03438	0.20506*	0.04983*	0.01607	0.00000	
Oslo	0.06955*	0.28307*	0.10886*	0.03879*	0.01730	0.00000

*Significant at P<0.05

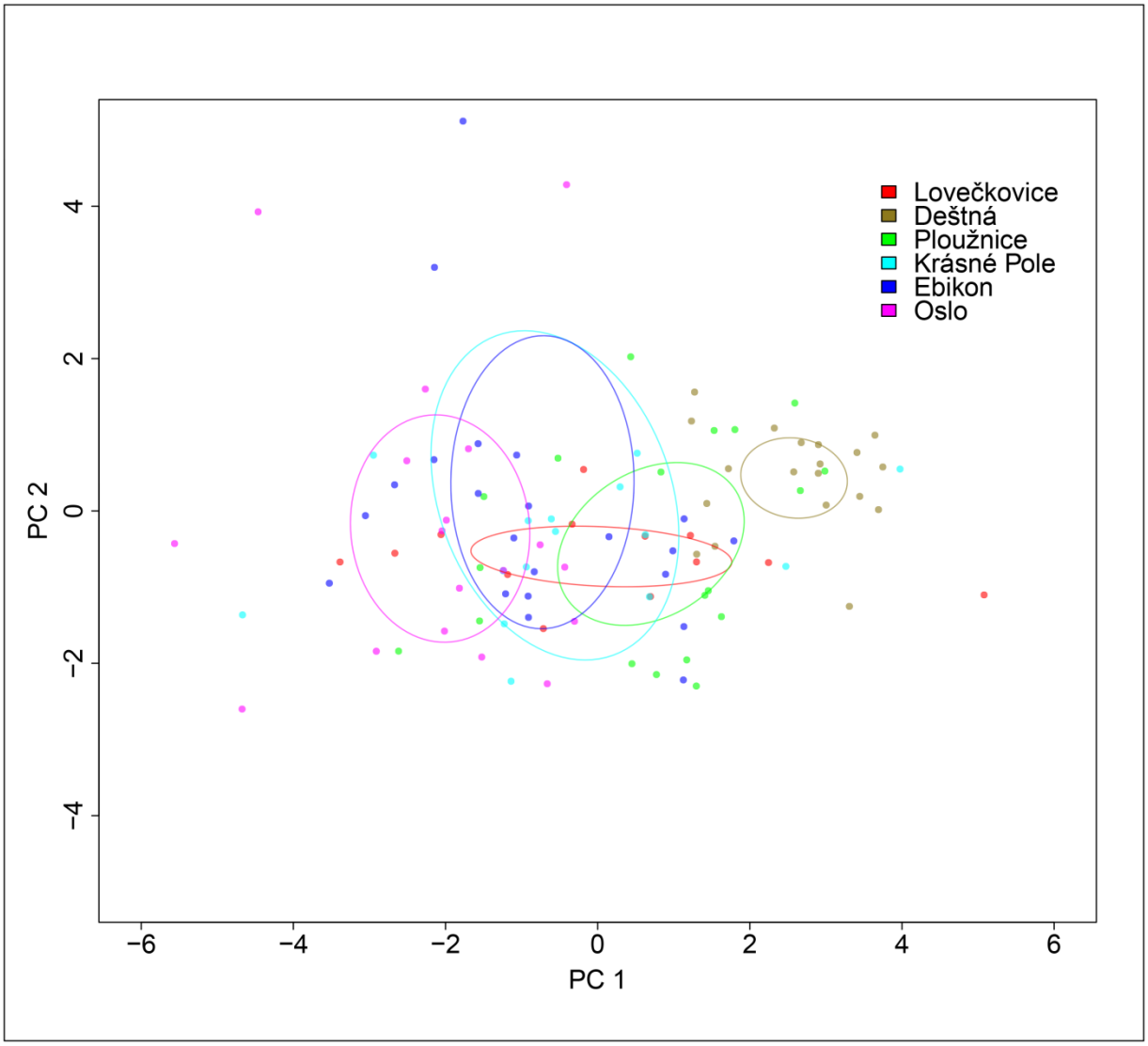

Fig. 2. Principal component analysis (PCA) of the Czech populations and the two reference populations from Norway and Switzerland. Individual populations are indicated by inertia ellipses and the different colours (see legend). Dots represent individual population members. PC1 explains 10.96% and PC2 8.1% of the total variance.

Discussion

During our study of the genetic diversity of *H. fraxineus* at four sites in the Czech Republic, 20 microsatellite markers were used in total. Five new markers were developed and their quality assessed carefully. Three markers were identified as

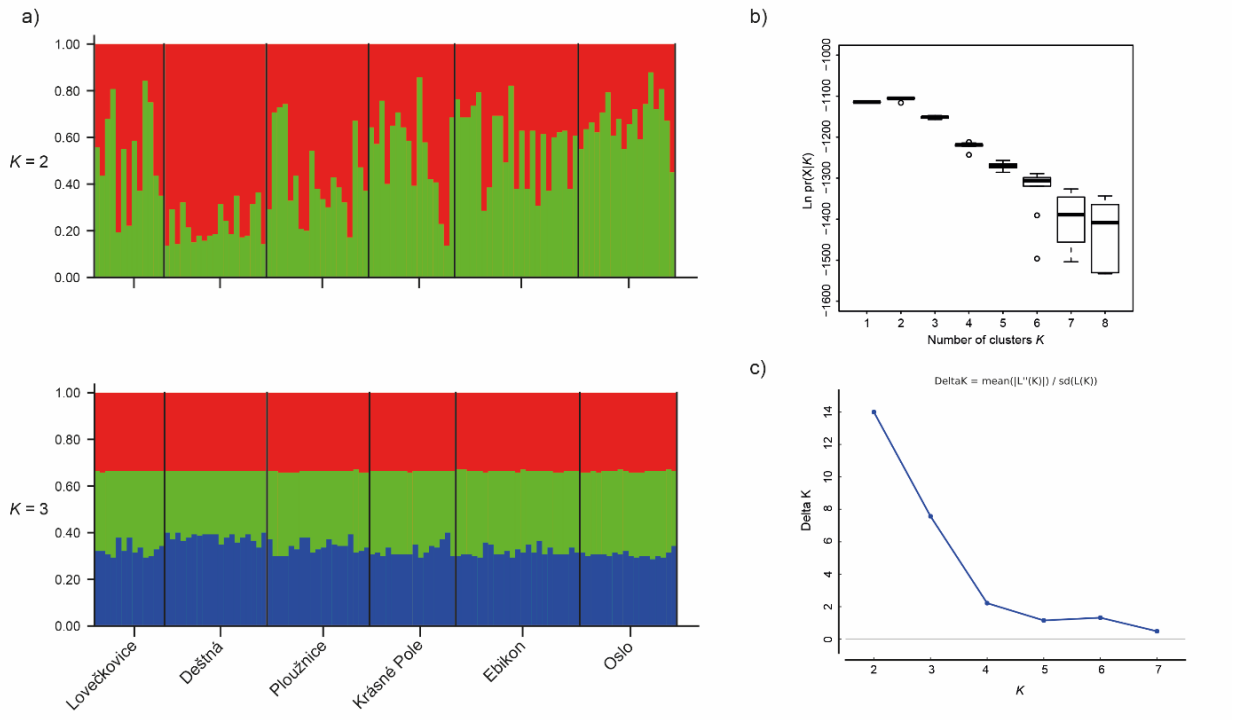

Fig. 3. Bayesian clustering analysis using the software STRUCTURE. (a) Assignment probability plots for K = 2 and K = 3 clusters of European Hymenoscyphus fraxineus genotypes. Individuals are represented by narrow bars divided into K segments showing the mean membership coefficient of an individual for a specific cluster. Vertical black lines delimit the populations that are indicated underneath the bar plots. (b) Boxplots of the log likelihood (LN pr(X|K)) of ten individual runs per cluster ranging from K = 1to K = 8, indicating that K = 2 is the model with the highest likelihood score. (c) Delta (K) plot to determine the optimal number of clusters in STRUCTURE analysis as suggested by Evanno et al. (2005). The highest delta (K) is observed at K = 2. However, the method is unable to differentiate between K = 1 and K = 2.

appropriate to compare the genetic diversity between populations in Europe and Japan. Only eight markers have proven valuable for this purpose so far (Gross et al. 2014a), and additional markers are necessary to elucidate the invasion history of *H. fraxineus*. Average gene diversity (H_e = 0.29) of Czech populations was considerably lower when compared to the two reference populations and to the European average H_e = 0.35 reported by Gross et al. (2014a). Only the Krásné Pole population reached a similar gene diversity (H_e = 0.35). The Deštná population represented an outlier in terms of low genetic diversity, which is likely the result of a founder event. A founder effect within the already bottlenecked European population has already been reported in the CH_Wilderswil population in a previous study (Gross et al. 2014a).

Interestingly, two strains isolated from the same lesion showed different genotypes. The possibility of multiple infections from one leaf scar has already

been indicated by Bengtsson et al. (2014). To confirm this, a more precise tracking of the infection event is necessary because the detection of multiple strains could also have been caused by the fusion of lesions from two nearby leaf scars or the combination of lesions from a leaf scar and direct entry (e.g. through a lenticel).

Concluding from the allelic diversity of the markers, Czech populations of *H. fraxineus* have a similar gene pool as that found in other European populations. Unsurprisingly, Czech *H. fraxineus* derived obviously from other European *H. fraxineus* populations. However, the considerable variation in genetic diversity and the two relatively undefined clusters found by STRUCTURE analysis among the geographically proximal populations was an unexpected result. *Hymenoscyphus fraxineus* was already present throughout the country (L. Havrdová, personal communication) and had been reported in all neighbouring countries (see fig. 1 in Timmermann et al. 2011) at the time of sampling. Ascospores of the pathogen are distributed by wind (Timmermann et al. 2011), and, as is established from disease monitoring programs, the disease front can move up to 75 km per year (Gross et al. 2014b), implicating a high migration potential. High migration rates lead to the prevention or dissolution of the population genetic structure (Wright 1949; Slatkin 1987), and this was shown for *H. fraxineus* in a Europe-wide study incorporating 32 populations and more than 1200 strains (Gross et al. 2014a). Therefore, factors acting against migration might have led to the observed pattern. Chandelier et al. (2014) showed a logarithmical decrease in the amount of *H. fraxineus* ascospores as height from the ground increased. Therefore, a few local genotypes may have generated a higher and earlier infection pressure (especially in the low and dense Czech stands) than *H. fraxineus* spores from surrounding areas, which may have hindered migration. Alternatively, other unknown epidemiological dynamics, for example low density of *F. excelsior* stands in surrounding areas or unfavourable conditions for infections, are also conceivable. Indeed, the ash stand in Deštná is surrounded by pine forests on sandy ground and agricultural fields with a very low density of ash. However, the result must not be overinterpreted because G_{ST} values between Czech populations other than Deštná were small and insignificant, and also the differences from the two foreign reference populations were not much higher. Our study is difficult to compare to those of Kraj et al. (2012) and Kraj and Kowalski (2014), which identified a clear difference between lowland and highland populations using RAMS markers. All our populations were sampled at a similar elevation and we used a single-locus, codominant marker type. There are several well-known technical and analytical drawbacks of multilocus markers

such as RAMS (see Sunnucks 2000), and therefore, the interesting findings of Kraj et al. should be challenged using more reliant genetic markers in the future.

Our results clearly point to our limited knowledge about epidemiology, disease dynamics and the ecological factors shaping them. Holdenrieder et al. (2004) called for transdisciplinary cooperation of forest pathologists and landscape ecologists, and such collaborative studies are needed to fully understand the forest pathogen *H. fraxineus.*

ACKNOWLEDGEMENTS

We would like to thank to the Genetic Diversity Centre (GDC) of ETH Zurich for providing their laboratory facilities. This study was supported by Ministry of Agriculture of the Czech Republic no. NAZV QJ1220218, by Institutional Support for Science and Research of the Ministry of Education, Youth and Sports of the Czech Republic, by a grant from COST Action FP1103 FRAXBACK for the short-term scientific mission of Z. H. in Switzerland.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Initial amplification of putatively polymorphic regions containing a microsatellite repeat.

Table S2. Strains used for the initial polymorphism screening.

Table S3. Sequenced alleles and their GenBank accession numbers.

Table S4. Four multiplex PCRs as used to genotype Czech populations.

REFERENCES

Bengtsson, S. B. K.; Vasaitis, R.; Kirisits, T.; Solheim, H.; Stenlid, J., 2012: Population structure of *Hymenoscyphus pseudoalbidus* and its genetic relationship to *Hymenoscyphus albidus*. Fungal Ecol. 5, 147–153.Bengtsson, S. B. K.; Barklund, P.; von Brömssen, C.; Stenlid, J., 2014: Seasonal pattern of lesion development in diseased *Fraxinus excelsior* infected by *Hymenoscyphus pseudoalbidus*. PLoS ONE 9, e76429.

Brown, A. H. D.; Feldman, M. W.; Nevo, E., 1980: Multilocus structure of natural populations of *Hordeum spontaneum*. Genetics 96, 523– 536.

Chandelier, A.; Helson, M.; Dvorak, M.; Gischer, F., 2014: Detection and quantification of airborne inoculum of *Hymenoscyphus pseudoalbidus* using real-time PCR assays. Plant. Pathol. 63, 1296–1305.

Earl, D. A.; vonHoldt, B. M., 2012: STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 4, 359–361.

Evanno, G.; Regnaut, S.; Goudet, J., 2005: Detecting the number of clusters of individuals using the software structure: a simulation study. Mol. Ecol. 14, 2611–2620.

Excoffier, L.; Lischer, H. E. L., 2010: Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Res. 10, 564–567.

Gross, A.; Grünig, C. R.; Queloz, V.; Holdenrieder, O., 2012: A molecular toolkit for population genetic investigations of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. Forest Pathol. 42, 252–264.

Gross, A.; Hosoya, T.; Queloz, V., 2014a: Population structure of the invasive forest pathogen *Hymenoscyphus pseudoalbidus*. Mol. Ecol. 23, 2943–2960.

Gross, A.; Holdenrieder, O.; Pautasso, M.; Queloz, V.; Sieber, T. N., 2014b: *Hymenoscyphus pseudoalbidus*, the causal agent of ash dieback. Mol. Plant Pathol. 15, 109–117.

Hardy, O. J.; Vekemans, X., 2002: spagedi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. Mol. Ecol. Notes 2, 618–620.

Havrdová, L.; Černý, K., 2013: The importance of air humidity in ash dieback epidemiology – preliminary results (in Czech with Engl. summary). Rep. Forest. Res. 58, 347–352.

Holdenrieder, O.; Pautasso, M.; Weisberg, P. J.; Lonsdale, D., 2004: Tree diseases and landscape processes: the challenge of landscape pathology. Trends Ecol. Evol. 19, 446–452.

Jankovsky, L.; Holdenrieder, O., 2009: *Chalara fraxinea* – ash dieback in the Czech Republic. Plant Prot. Sci. 45,74–78. Jombart, T., 2008: adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24, 1403–1405.

Kamvar, Z. N.; Tabima, J. F.; Grunwald, N., 2014: The poppr R package for genetic analysis of populations with mixed (clonal/sexual) reproduction. PeerJ 2, e281. doi: 10.7717/peerj.281

Kraj, W.; Kowalski, T., 2014: Genetic variability of *Hymenoscyphus pseudoalbidus* on ash leaf rachises in leaf litter of forest stands in Poland. J. Phytopathol. 162, 218–227.

Kraj, W.; Zarek, M.; Kowalski, T., 2012: Genetic variability of *Chalara fraxinea*, dieback cause of European ash (*Fraxinus excelsior* L.). Mycol. Prog. 11,37–45.

McKinney, L. V.; Thomsen, I. M.; Kjær, E. D.; Bengtsson, S. B. K.; Nielsen, L. R., 2012: Rapid invasion by an aggressive pathogenic fungus (*Hymenoscyphus pseudoalbidus*) replaces a native decomposer (*Hymenoscyphus albidus*): a case of local cryptic extinction? Fungal Ecol. 5, 663–669.

Nei, M., 1987: Molecular Evolutionary Genetics. New York, NY: Columbia University Press, 512 pp.

Pritchard, J. K.; Stephens, M.; Donnelly, P., 2000: Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

R_Development_Core_Team, 2009: R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing, 409 pp.

Rice, W. R., 1989: Analyzing tables of statistical tests. Evolution 43, 223–225. Rytkönen, A.; Lilja, A.; Drenkhan, R.; Gaitnieks, T.; Hantula, J., 2011: First record of *Chalara fraxinea* in Finland and genetic variation among isolates sampled from Äland, mainland Finland, Estonia and Latvia. Forest Pathol. 41, 169–174.

Slatkin, M., 1987: Gene flow and the geographic structure of natural populations. Science 236, 787–792.

Smith, J. M.; Smith, N. H.; Orourke, M.; Spratt, B. G., 1993: How clonal are bacteria? Proc. Natl. Acad. Sci. USA 90, 4384–4388.

Sunnucks, P., 2000: Efficient genetic markers for population biology. Trends Ecol. Evol. 15, 199–203.

Timmermann, V.; Børja, I.; Hietala, A. M.; Kirisits, T.; Solheim, H., 2011: Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. EPPO Bull. 41,14–20.

Wright, S., 1949: The genetical structure of populations. Ann. Eugen. 15, 323–354. Zhao, Y.-J.; Hosoya, T.; Baral, H.-O.; Hosaka, K.; Kakishima, M., 2012: *Hymenoscyphus pseudoalbidus*, the correct name for *Lambertella albida* reported from Japan. Mycotaxon 122,25–41.

UNSEEN, BUT STILL PRESENT IN CZECHIA: HYMENOSCYPHUS ALBIDUS DETECTED BY REAL-TIME PCR, BUT NOT BY INTENSIVE SAMPLING

Ondřej Koukol[1]*, Zuzana Haňáčková[1,2], Miloň Dvořák[3] & Ludmila Havrdová[2]

*[1]Department of Botany, Charles University in Prague, Faculty of Science, Benátská 2, CZ-128 01 Prague, Czech Republic, [2] Department of Biological Risks, Silva Tarouca Research Institute for Landscape and Ornamental Gardening Publ. Res. Inst., Květnové náměstí 391, CZ-252 43 Průhonice, Czech Republic, [3] Department of Forest Protection and Wildlife Management, Mendel University in Brno, Zemědělská 3, CZ-613 00 Brno, Czech Republic, *Corresponding author: Email: ondrej.koukol@natur.cuni.cz*

Koukol et al. 2016. Mycological Progress, 15/1: 1-9, doi: 10.1007/s11557-015-1149-5.

INTRODUCTION

Hymenoscyphus albidus (Gillet) W. Phillips and *H. fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (earlier referred to as *H. pseudoalbidus* Queloz et al. for the teleomorph and *Chalara fraxinea* T. Kowalski for the anamorph) (Helotiales, Leotiomycetes) represent two closely related species strikingly differing by their life histories. Whilst *H. albidus* is a saprotrophic ascomycete colonizing fallen petioles of common ash (*Fraxinus excelsior* L.) and narrow-leafed ash (*Fraxinus angustifolia* Vahl) leaves, *H. fraxineus* is a serious pathogen attacking living leaves and shoots of the same tree species (causing ash dieback disease) and later lives as a saprotroph on fallen petioles of leaves. Their overlapping niches result in presumed competition, with *H. fraxineus* being strongly favoured. Both species probably colonize living ash leaves already as endophytes (Baral and Bemmann 2014). *H. albidus* is disadvantaged in their colonization and outcompeted due to its later fructification and inactivity until leaf senescence (Hietala et al. 2013). In laboratory conditions, *H. fraxineus* showed faster growth than *H. albidus* on agar media (Kirisits et al. 2013), so it is probably a faster colonizer of petioles in nature. Both species have been shown to produce phytotoxic secondary metabolites and extracellular polyphenoloxidases that are involved in the infection process, but probably only the fact that *H. fraxineus* colonizes an exotic host triggers its necrotrophic behaviour (Gross et al. 2014).

H. albidus was once distributed in temperate and montane Europe (Baral and Bemmann 2014), but almost disappeared during the last two decades. Concerns of fungal decline are usually connected with change in climatic conditions, human

pollution, or loss of natural habitat (Heilmann-Clausen et al. 2015). Neither of these are, however, the cause for disappearance of *H. albidus*. The invasion of *H. fraxineus* is under suspicion for replacement of *H. albidus* in its natural habitat and threat of its extinction. *H. fraxineus* has rapidly spread across Europe within the last decade (Kowalski and Holdenrieder 2009; Queloz et al. 2011; Gross et al. 2014; Baral and Bemmann 2014) after its introduction from its native area in East Asia in the 1990s, and caused enormous losses to European ash populations. Until recently, *H. albidus* could be found at localities not affected by ash dieback, for example, in Scotland (McKinney et al. 2012), but *H. fraxineus* was recently introduced also to this area (Baral and Bemmann 2014). Both species together were recorded at several sites in central and northwestern France (Husson et al. 2011), but their coexistence might have only been temporary because *H. fraxineus* invaded these sites only very recently. Both species were also recorded using real-time PCR in Belgium. Low density of spores of *H. albidus* was recorded only in one of four stands (weakly infected by ash dieback) in September 2013 (Chandelier et al. 2014). The results of a survey carried out at three localities in Denmark, where apothecia of *H. albidus* were collected in 1989, 1993, and 1994, are alarming. All the localities were inhabited in 2005 only by *H. fraxineus* (McKinney et al. 2012), rendering *H. albidus* locally extinct in Denmark. This „local cryptic extinction" (McKinney et al. 2012) is not obvious in the field as both species form highly similar stipitate apothecia on petioles of previous year fallen ash leaves. Only the presence of croziers at the ascus base allows *H. fraxineus* to be morphologically distinguished from *H. albidus* (Baral and Bemmann 2014; Zhao et al. 2013).

In Czechia, the past distribution of *H. albidus* has never been thoroughly studied. Velenovský (1934) considered *H. albidus* (under the name "*Helotium albidum* (Rob.) Pat.", nom. illegit., Art. 53.1) as a rather common and locally frequent species. On the other hand, no other records dealing with fungal collections and notes on the distribution of fungi in Czechia between 1865 and 1948 exist in the literature (taking into account older synonyms of *H. albidus*). Similarly, only a few collections under the name *H. albidus* (also as *Helotium albidum*) were deposited in the Herbarium of the Prague National Museum (PRM, Prague, Czechia) from the 1920s onwards, compared to about 50 specimens of the morphologically similar *H. caudatus* (P. Karst.) Dennis. No specimens were present in other major herbaria in Czechia (Herbarium of Charles University in Prague and Herbarium of the Moravian Museum in Brno, V. Antonín pers. comm.). When Svrček (1984) revised specimens under the name *Helotium albidum* kept in PRM and studied by J. Velenovský, he described a new species *Conchatium fraxinophilum* Svrček, based on

three of these specimens that showed atypical characteristics. This species was later transferred to the genera *Cyathicula* by Baral (1993) and *Crocicreas* by Triebel and Baral (1996). Velenovský (1934), therefore, seems to have regarded *H. albidum* as common because he did not differentiate between these two species.

Since the first isolation of the anamorph of *H. fraxineus* within Czechia performed in September 2007 (Jankovský and Holdenrieder 2009), symptoms of ash dieback have been observed in numerous areas in Czechia with different severity (Havrdová and Černý 2012). Most recent research has targeted factors affecting the disease (Havrdová and Černý 2013) and a population study of *H. fraxineus* (Haňáčková et al. 2015). Morphological revision of more recent specimens of *H. albidus* and *H. fraxineus* from Czechia was performed with ambiguous results (M. Tomšovský and M. Šandová, pers. comm.) because the absence vs. presence of croziers is generally overlooked and requires considerable skill (Zhao et al. 2013). Apart from these peculiar morphological traits which yield unequivocal results, molecular data represent the only reliable method of distinguishing the two species. Recent progress in molecular techniques has enabled successful DNA extraction and further analyses (sequencing, fingerprinting) from fungal material in herbaria (Brock et al. 2009; Bruns et al. 1990; Inderbitzin et al. 2004). Owing to the lack of knowledge on the potential presence of *H. albidus* and *C. fraxinophila* in Czechia, we revised available herbarium specimens based on molecular data, carried out targeted sampling at historical localities within Czechia, and sampled apothecia on further localities to see whether *H. albidus* or *C. fraxinophila* may have been replaced by *H. fraxineus*.Sampling of fruiting structures was coupled with detection of airborne ascospores of *H. albidus* using species-specific real-time PCR assay. At four localities differing in level of *H. fraxineus* infection and without previous recordings of *H. albidus*, spore traps were installed to detect the presence of *H. albidus* that might have been overlooked during sampling of the apothecia.

MATERIAL AND METHODS

Herbarium specimens and recent collections

Herbarium specimens of *H. albidus* (also as *Helotium albidum*) were obtained from PRM. Each specimen was surveyed for the quantity of apothecia, and material for DNA extraction was harvested only upon permission by the curator on a case-by-case basis following guidelines aimed at safeguarding the collection for future users. Eight specimens sampled between 1925 and 2011 were approved for DNA

extraction. One to three apothecia were used for the extraction (not to exceed the 10 % of the total fungal material).

Recent collections of apothecia growing on ash petioles were made at localities with confirmed past presence of *H. albidus* and *C. fraxinophila*, and at numerous further localities in Czechia (Supplementary Table). Historical localities were relatively difficult to identify because of a scarcity of geographical information on labels. Usually, only nearby towns were mentioned without precise localization. Fortunately, Svrček (1984) mentioned in his revision more detailed localities probably based on personal knowledge of favourite areas of the authors. Several sampling sites were usually selected for each historical locality to avoid missing *H. albidus* in a given area. From each site, numerous peti- oles with apothecia were sampled to provide enough material for DNA extraction and new herbarium specimens. Further localities were selected with respect to the ash dieback disease. Sites with symptomless trees and thus supposed absence of *H. fraxineus* were preferred. Sites were visited between June and October 2013 and 2014 and in July 2015 to cover the season indicated on labels of historical specimens (July to September) with regard to the post poned period of fructification due to the relatively dry June and July 2013. In total, apothecia from 91 sampling sites were analysed; localities ranging from lowland to mountainous areas (170–1049 m a.s. l.), including solitary trees, urban areas, and natural forest stands.

Identification based on morphology

Ash petioles sampled in our study were first observed under the dissecting microscope to separate petioles with apothecia belonging to *H. fraxineus*/*H. albidus* and the remaining species. Apothecia of *H. fraxineus*/*H. albidus* that were recognized based on the surface blackening of petioles (pseudosclerotium) were not morphologically surveyed, but identified based on the DNA. Apothecia of the remaining species were examined mostly as rehydrated material after desiccation at 35 °C. Potassium hydroxide (3 % KOH) and lactic acid were used as mounting medium. The iodine reaction of the ascus apex was tested with Melzer'sreagent.

DNA extraction, fingerprinting, and sequence analysis

DNA analysis was targeted at reliable differentiation of the morphologically similar *H. albidus* and *H. fraxineus*, but DNAwas extracted also from apothecia of other species identified already based on morphology (*Cyathicula* spp. and other members of *Hymenoscyphus*). DNA extraction from herbarium specimens was performed using the ZR Fungal/ Bacterial DNA kit (Zymo Research, Orange, CA,

USA) according to the manufacturer's instructions. DNA from freshly collected specimens was extracted using the InstaGene matrix (Biorad, Hercules, CA, USA). Three subsamples consisting ofthree apothecia (each subsample from a different petiole) were used from each site for extraction. Apothecia from each subsample were immersed in 200 μL of the InstaGene Matrix suspension in a sterile Eppendorf phial with 2–5 glass beads (1.25–1.65 mm in diam., Roth GmbH, Karlsruhe, Germany) and crushed on a horizontal shaker for 10 min. Further incubation followed the manufacturer's instructions. PCR performed with crude supernatant yielded no product, so the supernatant was cleansed of enzymatic inhibitors using the OneStep PCR Inhibitor Removal Kit (ZymoResearch, Orange, CA, USA).

The M 13 core sequence primer (5 ′-GAGGGTGGCGGTTCT-3′) was used in arbitrary primed PCR (AP-PCR) for fingerprinting of apothecia from recent localities tentatively assigned to *H. fraxineus*. DNA extracted from a strain obtained from an ash dieback lesion was used as a positive control, and DNA from a strain of *H. albidus* (obtained from Institute of Forest Entomology, Forest Pathology and Forest Protection, University of Natural Resources and Life Sciences, Vienna, Austria, isolated by Thomas Kirisits, isolate CAR5) was used as a positive control of *H. albidus*. The PCR reaction and cycle followed Libkind et al. (2009). Band profiles were visualized on 1.5 % (w/v) TBE agarose gels stained with ethidium bromide. Because of intraspecific variability within *H. fraxineus*, several different fingerprints of this species were obtained, and representative DNA from each group was further amplified and sequenced to confirm the identity. The ITS1, 5.8S and ITS2 regions of rDNAwere amplified with the universal primer pair ITS1/ITS4 or the fungal specific primer pair ITS1F/ITS4 (Gardes and Bruns 1993; White et al. 1990). From two herbarium specimens, only the ITS1 region was amplified with the primer pair ITS1/ITS2 (White et al. 1990) in nested PCR.

The PCR products were viewed by electrophoresis on 1 % (w/v) TAE agarose gels stained with ethidium bromide. PCR products were purified with the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, Bade City, Taiwan). Both strands of the PCR fragments were sequenced with the primers ITS1/ITS1F and ITS4 (Faculty of Science, Charles University in Prague, Czechia).

Air sampling, DNA extraction, and real-time PCR conditions

Four localities differing in the level of infection, type of the stand (urban/forest area), and elevation were chosen: Hackerova školka (moderate infection, forest

nursery), Vranovice (extreme infection, floodplain forest), Boršov (high infection, suburban area), and Horní Benešov (low infection, suburban areas; for further details see Supplementary Table). At every locality a 7-day automatic volumetric spore trap (AMET Velké Bílovice, Czech Republic) based on the principle published by Hirst (1952) was installed to sample the air inoculum continuously. Melinex tape covered by petroleum jelly as a trapping medium was fixed to a revolving drum of the spore trap, facing an orifice through which the sampled air was sucked in. For this study the air of every locality was sampled three times through the season of expected *H. fraxineus* spore production. First sampling (June 20 – 21, 2014) was targeted to the beginning, second (August 1 – 2, 2014) to the peak, and the third (September 10 – 11, 2014) to the end of the spore production season. Duration of each of the three samplings was set to 48 h. After exposure, the tapes were cut into pieces simulating particular days and stored in 2 mL microtubes at -20 °C.

DNA was extracted separately according to the sampling days. Disruption of spores was processed similarly to Hospodsky et al. (2010): 0.3 g of 0.1 mm and 0.1 g of 0.5 mm balotina beads (Holland Mineraal, Deventer, Netherlands) were added to the microtube with the sample together with 250 μL of 0.1 % Nonidet P40 substitute (Sigma-Aldrich, St. Louis, MO, USA) and crushed for 10 min by 30 Hz in Mixer Mill MM400 (Retsch, Haan, Germany). For further processing the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) was used with extraction buffer described by Vainio et al. (1998) instead of lysis buffer supplied by the producer. Incubation of extraction buffer was prolonged to 60 min. In the last step, DNA of each sample was eluted only once with 100 μL of preheated elution buffer.

Real-time PCR with species-specific primers amplifying ITS1 region of rDNA and TaqMan probes was performed using a LightCycler 480 Instrument II (Roche Diagnostics, Basel, Switzerland) and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions (pre-incubation: 10 min, 95 °C followed by 45 cycles ofdenaturation: 10 s, 95 °C; annealing: 30 s, 60 °C; extension: 1 s, 72 °C - single acquisition mode). Primers were designed and their specificity was proved by Chandelier et al. (2010) for *H. fraxineus* and by Husson et al. (2011) for *H. albidus*. Their nucleotide sequences and names are mentioned in Table 1. Composition of the reaction mixture was following: 0.2 μL of each primer (final concentration 400 nM), 0.2 μL of TaqMan probe (200 nM), 5 μLof TaqMan Universal PCR Master Mix, 1.9 μL of sterile

deionized water, and 3 μL of template DNA. Every reaction was performed in three technical repetitions together with a negative control containing the master mix without template DNA.

Table 1 Specific primers and probes used for the real-time PCR

Nucleotide designation	Sequence	Source	Specific to
Halb-F	TATATTGTTGCTTTAGCAGGTCGC	Husson et al. (2011)	*H. albidus*
Halb-R	ATCCTCTAGCAGGCACGGTC	Husson et al. (2011)	*H. albidus*
Halb-P	YY-CCGGGGCGTTGGCCTCG-BHQ2 CCCTTGTGTATATTATATTGTTGCTTTA	Husson et al. (2011)	*H. albidus*
Cf-F	GC	Chandelier et al. (2010)	*H. fraxineus*
Cf-R	GGGTCCTCTAGCAGGCACAGT	Chandelier et al. (2010)	*H. fraxineus*
Cf-S	6FAM-TCTGGGCGTCGGCCTCGG-BHQ1	Chandelier et al. (2010)	*H. fraxineus*

The concentrations of *H. albidus* and *H. fraxineus* DNA in the samples were expressed as numbers ofcopies of the target sequence in 1 μL of template DNA (further only numbers of copies). These numbers of copies were quantified using a standard curve generated by reactions with different amounts of plasmid pCR 2.1-TOPO TA vector (Invitrogen, Carlsbad, CA, USA) by The LightCycler 480 Software (Roche Diag- nostics). Plasmids contained species-specific insert (PCR products amplified with primers described in Table 1). DNA was extracted from pure cultures of *H. fraxineus* (collection of Mendel University in Brno) and H. albidus (the same as for AP-PCR).

Numbers of copies in each sample and locality were tested for normality by Shapiro-Wilk test and compared by General linear model (GLM) approach. Parameters for models were calculated to determine the significance of the sampled locality and the ascospore concentrations of both fungi. All the statistical analyses were performed by STATISTICA version 12 (StatSoft, Tulsa, OK, USA).

RESULTS

The identification of *H. albidus* was confirmed based on ITS rDNA sequences in three out of eight herbarium specimens that were selected as having enough fungal structures for DNA extraction and analyses (PRM147373 from 1925, PRM683594 and PRM683595 from 1942, Table 2). PCR was repeatedly unsuccessful from specimen PRM148933 from 1928 (Fig. 1a,b), but it was identified as *H. albidus* based on the absence of croziers at the ascus base (Fig. 1c,d). Specimen PRM802801 from 1976 was labelled as *Hymenoscyphus* and turned out to belong to *C. fraxinophila* based on the lack of pseudosclerotial layers on petioles and morphology of apothecia. Three specimens belonged to *H. fraxineus* (PRM921903 from 2009, PRM899965 and PRM899715 from 2011), though they were originally identified as *H. albidus*. One specimen of *H. albidus* from Slovakia was also included in our study and turned out to belong to *H. fraxineus* (PRM857180 from 2005). One specimen from 1949 (PRM 683858, Žarošice (Moravia), leg. et det. V. Vacek) did not contain sufficient material for DNA extraction, but was already revised based on morphology (absence of croziers) by M. Tomšovský as *H. albidus*.

Our sampling in 2013 and 2014 aimed at finding *H. albidus* was performed on four historical sites, but all apothecia sam- pled herein turned out to belong to *H. fraxineus*. Similar results were obtained at remaining 87 localities in Czechia, where mostly *H. fraxineus* was found (Fig. 2, for collection details and identification of all species see Supplementary Table).

One herbarium specimen under the name *H. albidus* was identified as *C. fraxinophila* (PRM802801 from 1976), and *C. fraxinophila* was successfully found on the same locality also in 2013 (PRC2440), accompanied by *H. fraxineus* and *Cyathicula coronata* (Bull.) Rehm. *Cyathicula fraxinophila* was found at 21 and *C. coronata* at five sites (Fig. 2). These two species were identified based on their morphology. *Cyathicula fraxinophila* forms stipitate apothecia that are first closed and almost spherical and later open and expose the hymenium into the typical disc shape (Fig. 3a). The margin splits into numerous inconspicuous teeth in maturity. The outer surface of the excipulum is composed of hyphae, which carry crystals on their outside that are clearly visible in KOH and MLZ (Fig. 3c).

Table 2 Sequences obtained in this study from historical specimens specimen

Specimen code	Original name	Name after revision	Locality as appeared on the label	Collected by	Date of collection	GenBank Acession Nr.
PRM147373	*Helotium albidum*	*Hymenoscyphus albidus*	Bilichov	J. Velenovský	23.7.1925	HF937560
PRM148933	*Helotium albidum*	*Hymenoscyphus albidus*	Slané	M. Svrček	9. 1928	-
PRM683594	*Helotium albidum*	*Hymenoscyphus albidus*	Černošice (Bohemia)	V. Vacek	12.7.1942	HF937562
PRM683595	*Helotium albidum*	*Hymenoscyphus albidus*	Žarošice (Moravia)	V. Vacek	5.9.1942	HF937561
PRM802801	*Hymenoscyphus*	*Cyathicula fraxinophila*	Bohemia centralis: Praha-Jinonice, in valle rivi "Prokopský potok" prope Nová Ves	M. Svrček	26.9.1976	-
PRM857180	*Hymenoscyphus albidus*	*Hymenoscyphus fraxineus*	Slovakia, NP Nízké Tatry, Pavčina Lehota, PR Jelšie	O. Jindřich	12.9.2005	HF937557
PRM921903	*Hymenoscyphus albidus*	*Hymenoscyphus fraxineus*	Northern Bohemia, NP České Švýcarsko, NW of village of "Srbská Kamenice"	M. Chlebická	6.6.2009	HF937558
PRM899715	*Hymenoscyphus albidus*	*Hymenoscyphus fraxineus*	Central Bohemia, Bílý vrch hill between Mochov and Čelákovice	M. Chlebická	8.7.2011	HF937559
PRM899965	*Hymenoscyphus albidus*	*Hymenoscyphus fraxineus*	CHKO České středohoří, Bílé stráně u Litoměřic	M. Kříž	18.7.2011	HF937563

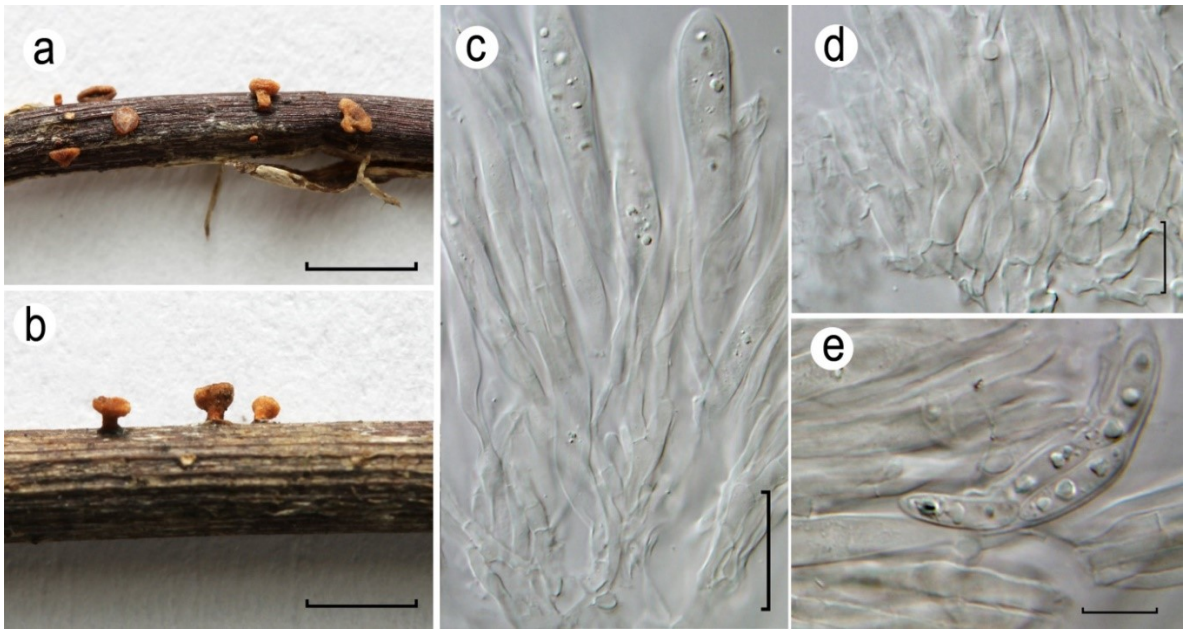

Fig. 1 *Hymenoscyphus albidus* (PRM148933). a, b.dried apothecia on the substrate (Scale bar = 2 mm). c, d. asci without croziers on their base (scale bar c=20 μm, d = 10 μm). e. ascospores (Scale bar = 10 μm)

The excipulum is formed of textura oblita with hyphae running parallel and immersed in a gelatinous substance (Fig. 3b). Asci emerging from croziers, pore blued in MLZ (Fig. 3d,f). Ascospores partly biseriate, straight to partly curved, with several small droplets close the tips, 13.5–16.5 μmlong and 2–3 μmwide (Fig. 3e). Sequences of ITS rDNA obtained from apothecia of *C. fraxinophila* were most closely related to sequences of *C. cyathoidea* (Bull.) Thüm. originating from the study of Pelaez et al. (2011). No exact match was found using the BLAST search in GenBank.

Real-time PCR detection of *H. albidus* and *H. fraxineus*

Concentrations of *H. albidus* ascospores detected at all four investigated localities ranged between 0.02 to 5.43 % of concentrations of *H. fraxineus* (Fig. 4). Concentrations of *H. albidus* ascospores showed normal distribution (W= 0.93; p= 0.15), unlike the concentrations of *H. fraxineus*, which tended to gamma distribution. The problem of normality was solved by log-transformation and deletion of two

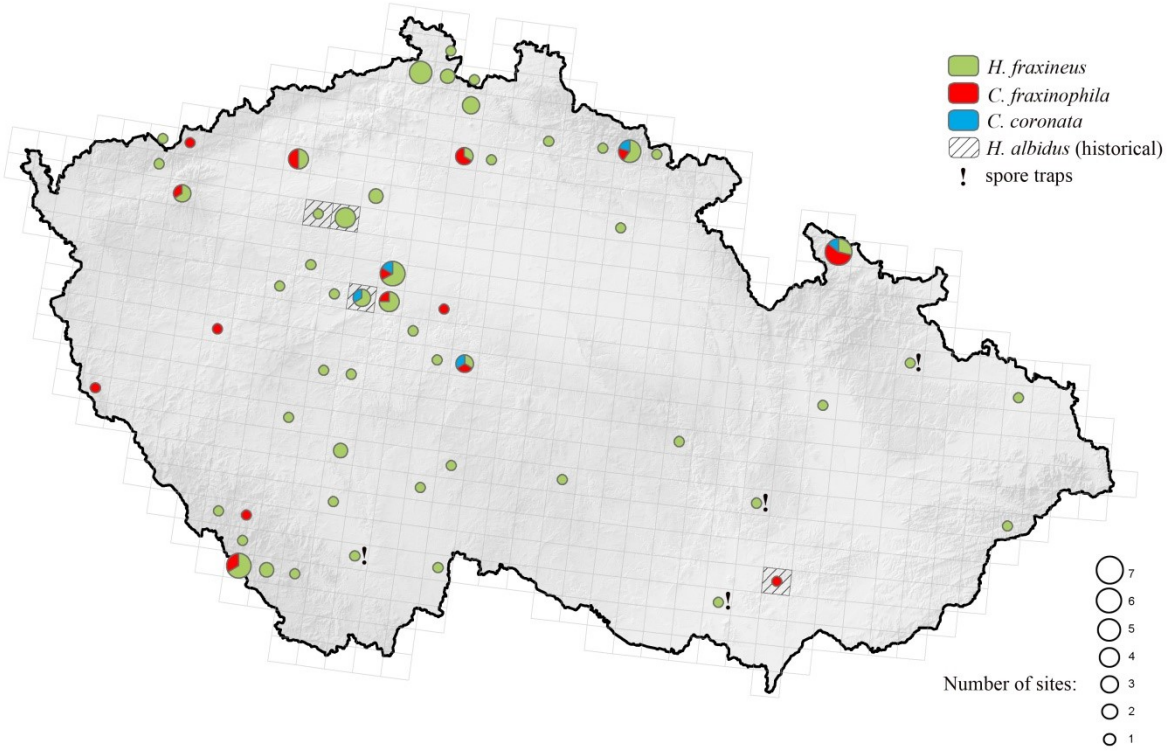

Fig. 2 Map ofhistorical records of *H. albidus*, collections of *H. fraxineus*, *C. fraxinophila*, and *C. coronata* in Czechia obtained during this study and location ofspore traps. Circles are proportional to number of sites present in a given square in the grid (Square = 11 km)

extreme values (W=0.95; p=0.36). The effect of the localities of air sampling was not significant, which shows limited influence of the different localities on the *H. albidus*/*H. fraxineus* ascospore concentration ratio. Therefore, the final model was recalculated only with the concentrations of ascospores as variables. Comparison of *H. fraxineus* x *H. albidus* ascospore concentration by GLM resulted in highly significant model (R2= 0.36; F=11.00; p=0.00):

$$Hfrax = 10^{3:607860 + 0.002670 \times Halb}$$

$$Halb = (\log Hfrax - 3.607860)/0.002670$$

where Hfrax = number of *H. fraxineus* copies and Halb = number of *H. albidus* copies of amplified fragment specific for *H. albidus* in 1 μL of the reaction mixture after real-time PCR.

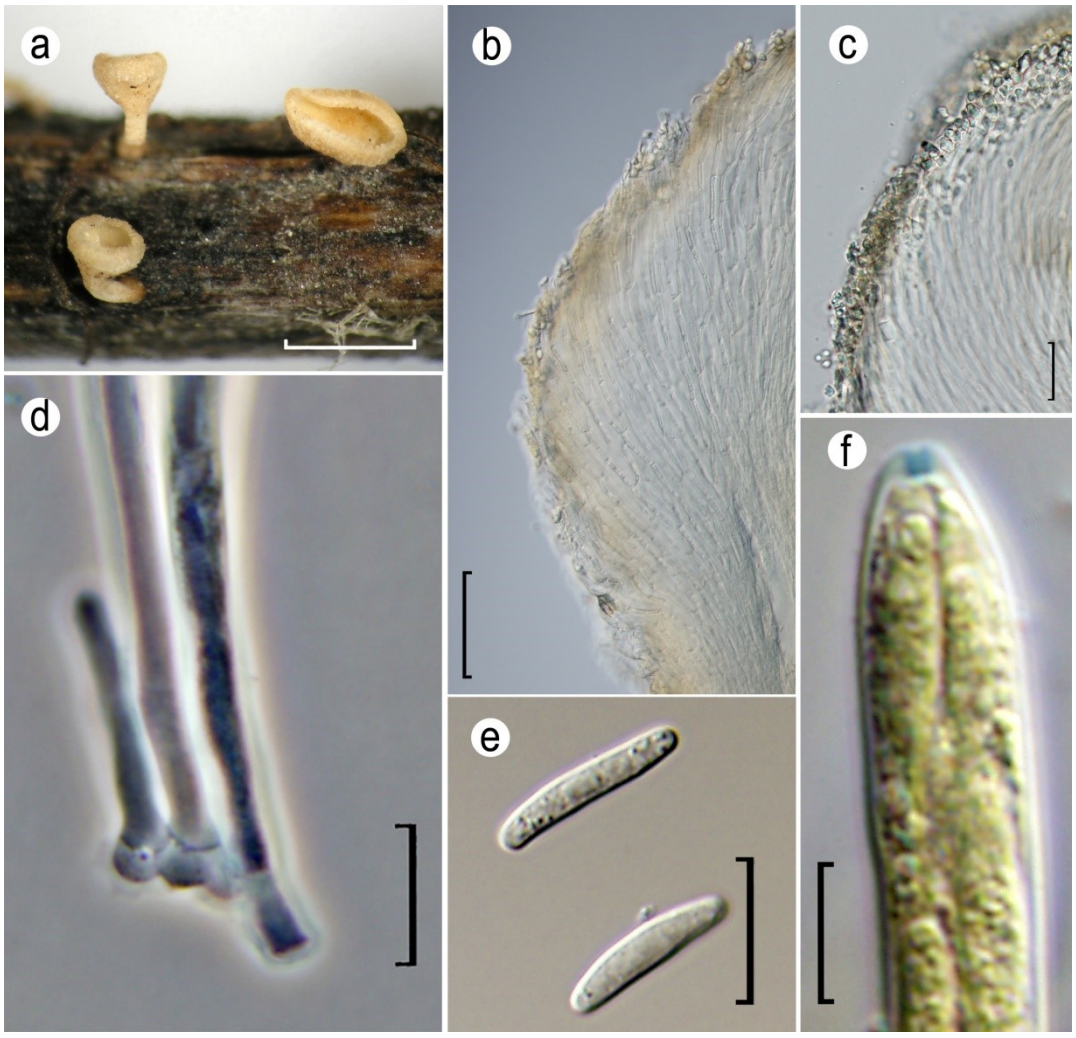

Fig. 3 *Cyathicula fraxinophila* (PRC1774). a. dried apothecia on the substrate (Scale bar = 1 mm). b. cross section showing textura oblita (Scale bar = 50 μm). c. detail of excipulum with crystalline substances (Scale bar = 20 μm). d, asci originating from croziers (in phase contrast, scale bar = 10 μm). e. ascospores (Scale bar = 10 μm). f. apical pore blued by iodine (in MLZ, scale bar = 10 μm

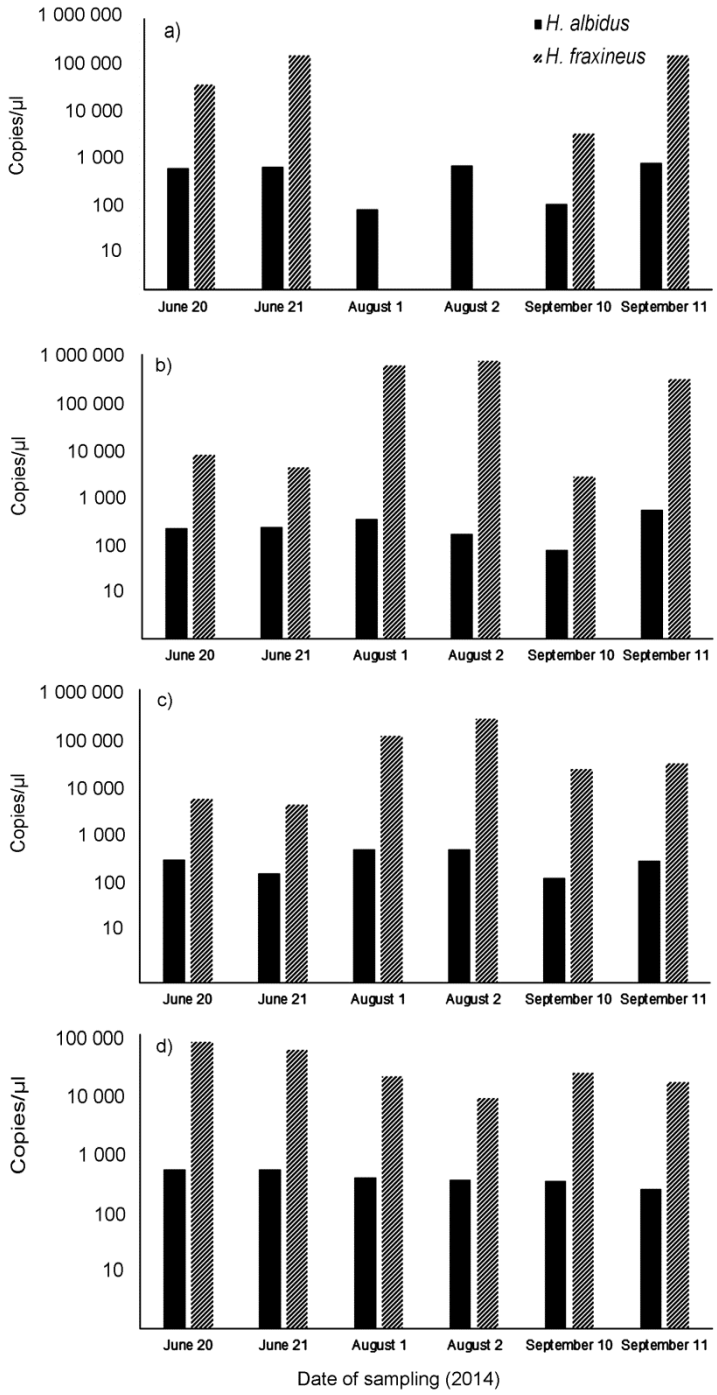

Fig. 4 Air inoculum of *H. albidus* and *H. fraxineus* in the four surveyed localities: a) Hackerova školka, b) Vranovice, c) Horní Benešov, and d) Boršov. Ascospore concentrations are expressed in numbers ofamplified species specific copies per 1 µL of template DNA (logarithmic scale)

DISCUSSION

Our revision of available herbarium specimens of *H. albidus* (or *Helotium albidum*) and literature survey indicate that this species was seemingly rare in Czechia prior to the onset of *H. fraxineus* expansion. This hypothesis is based on the absence of data and may be biased by potential neglecting of *H. albidus*, but rich records of other members of *Hymenoscyphus* (*Helotium*) in PRM indicate that this may not be the case.

The first revision ofsamples of *H. albidus* kept in PRM was done by Svrček (1984). Rather surprisingly, the specimen PRM148804 revised by Svrček as *H. albidus* contained petioles without the typical black pseudosclerotium. The fungus was morphologically reidentified by us as *C. fraxinophila*.Our three specimens sampled from the historical locality of PRM148804 (Zvánovice) in 2013 yielded *C. fraxinophila* together with numerous black ash petioles. Most probably, they belonged to *H. fraxineus*, but we could not confirm this in the absence of apothecia (DNA extracted from the pseudosclerotia yielded no PCR product).

Special attention has been paid to localities above 900 m. According to data of Queloz et al. (2011), *H. albidus* was still present in Switzerland at elevations above 850 m. Nevertheless, all of our 16 samplings from localities above this level (the highest from 1049 m a.s.l.) turned out to belong to *H. fraxineus*, which is in agreement with Baral and Bemmann (2014), who mentioned collections of *H. fraxineus* from the uppermost altitudinal limit of *F. excelsior* at 1600m above sea level.

As a result ofour sampling effort, *H. albidus* was not found in Czechia and potentially could be classified as extinct, or probably extinct (?EX, comprising missing plant and fungal species in Czechia since 1970) according to Holec and Beran (2006). McKinney et al. (2012) already assumed *H. albidus* to be extinct in Denmark based on the absence of its apothecia at revisited localities. However, our results of detection of airborne inoculum coupled with species-specific real-time PCR assay confirmed coexistence and co-fructification of both species. Hietala et al. (2013) already recorded *H. albidus* ascospores at 1 out of 5 days at one locality in a diseased pure ash stand in southern Norway. The estimated ascospore number of *H. albidus* was less than half percent of the estimated ascospore number of *H. fraxineus* (Hietala et al. 2013). Very low ascospore density in air was recorded also at a site with low disease incidence in southwestern Belgium (Chandelier et al. 2014). In our study, amounts of *H. albidus* spores were in all the tested cases

lower than *H. fraxineus*, but positive correlation supported by a logarithmic model shows common coexistence of these two species. Furthermore, the lack of any influence of the sampled locality (differing by disease level) on the *H. albidus*/*H. fraxineus* ratio shows on insensibility of this relationship to the severity of the ash dieback disease. In the absence of long-term studies it is not clear whether *H. albidus* is being suppressed by *H. fraxineus* (McKinney et al. 2012; Hietala et al. 2013), because the ratio of the *H. albidus* inoculum is only a few percent of *H. fraxineus*. On the other hand, *H. albidus* might have been rare also in the absence of *H. fraxineus*, and the latter species only occupied free niches. The fact that apothecia of *H. albidus* were not collected at the localities where the spore traps proved their presence may be caused by the huge amount of *H. fraxineus* apothecia; they may reach counts of several thousand per square meter (Hietala et al. 2013).

Compared to *H. albidus*, apothecia of *C. fraxinophila* were recently found at one historical locality (Zvánovice; the other five localities listed by Svrček (1986) were not visited) and 20 other sites (Supplementary Table). We assume that this species is not declining. *Cyathicula fraxinophila* was usually found later in the season, mostly in September and October. Mature apothecia of *C. fraxinophila* were either growing out of non- blackened petioles or regions, or they rarely even penetrated the pseudosclerotial layer of *H. fraxineus* with senescent apothecia. A comparison of data from historical samplings showed the same pattern. Collections mentioned by Svrček (1986) were sampled between September 26th and November 20th, so no shift in the fructification period due to pressure from *H. fraxineus* can be expected. Our results are in a strong contrast to Baral and Bemmann (2014), who considered *C. fraxinophila* threatened by invasion of *H. fraxineus*. They based this assumption on drop of records of *C. fraxinophila* in the last decade compared to its rich occurrence before 1995. The life cycle of *C. fraxinophila* is not known, but two scenarios are possible. In the first, ascospores infect fallen leaves, and new apothecia are formed on the petioles after thorough colonization. In the second, ascospores geminate on living leaves, and the fungus develops as an endophyte. Apothecia are thus formed after leaf abscission on already precolonized petioles. The former hypothesis seems more probable because ash leaves are shed during the time of *C. fraxinophila* fructification, thus making their endophytic colonization inefficient. A BLASTsearch of a sequence obtained from apothecia of *C. fraxinophila* did not match records obtained in previous studies of endophytic mycobiota of ash leaves (Unterseher et al. 2007; Bakys et al. 2009), nor was this species isolated by Gherghel et al. (2013), which also points to the former scenario.

Cyathicula coronata was found on ash petioles at five sites. At each site, only 1–2 apothecia were present, suggesting that ash petioles represent merely an alternative niche to dead herbaceous stems occupied by this species. Svrček (1986) also mentioned *C. coronata* accompanying *C. fraxinophila* on ash petioles.

Finally, two further species of *Hymenoscyphus* were found at six sites, but their identity remains unclear. The micromorphology of their dried apothecia was close to *H. caudatus* and *H. virgultorum* (Vahl) W. Phillips, but guttulation of living ascospores would have been needed to confirm the identification (H.-O. Baral, pers. comm.)

Our study indicates that the effect of *H. fraxineus* on helotialean fungi colonizing ash petioles is not detrimental. *Cyathicula fraxinophila* was found at approximately 24 % of the sites, sometimes accompanied by *H. fraxineus* on the same petiole. *Cyathicula fraxinophila* is genetically related to *Glarea lozoyensis* Bills & F. Peláez, a producer of the antifungal lipopeptide pneumocandin (Pelaez et al. 2011). Provided that *C. fraxinophila* also possesses this ability, it may be a promising agent able to compete with *H. fraxineus* on ash petioles in litter

Acknowledgments We thank Markéta Šandová for her eager help in finding herbarium specimens in PRM, Dušan Romportl for visualization of the collections and numerous collectors (in alphabetical order): H. Deckerová, K. Černý, M. Hrabětová, C. Korittová, K. Novotná, K. Pešicová, V. Pešková, V. Pouska, P. Seifertová, and P. Štochlová. We also thank to T. Kirisits and K. Kräutler for providing strain of *H. albidus*. Special thanks are due to Hans-Otto Baral for serving as a presubmission reviewer. This study was supported by Ministry of Agriculture of the Czech Republic no. NAZV QJ1220218 and by Institutional Support for Science and Research ofthe Ministry ofEducation, Youth, and Sports of the Czech Republic.

Electronic supplementary material The online version of this article (doi:10.1007/s11557-015-1149-5) contains supplementary material, which is available to authorized users.

REFERENCES

Bakys R, Vasaitis R, Barklund P, Ihrmark K, Stenlid J (2009) Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. Plant Pathol 58(2):284–292. doi:10.1111/j. 1365-3059.2008.01977.x

Baral H-O (1993) Beiträge zur Taxonomie der Discomyceten III. Zeitschrift für Mykologie 59(1):3–22

Baral H-O, Bemmann M (2014) *Hymenoscyphus fraxineus* vs. *Hymenoscyphus albidus* – A comparative light microscopic study on the causal agent of European ash dieback and related foliicolous, stroma-forming species. Mycology 5(4):228–290. doi:10.1080/ 21501203.2014.963720

Brock PM, Doring H, Bidartondo MI (2009) How to know unknown fungi: the role of a herbarium. New Phytologist 181(3):719–724. doi:10.1111/j.1469-8137.2008.02703.x

Bruns TD, Fogel R, Taylor JW (1990) Amplification and sequencing of DNA from fungal herbarium specimens. Mycologia 82(2):175–184. doi:10.2307/3759846

Chandelier A, André F, Laurent F (2010) Detection of *Chalara fraxinea* in common ash (*Fraxinus excelsior*) using real time PCR. For Pathol 40(2):87–95. doi:10.1111/j.1439-0329.2009.00610.x

Chandelier A, Helson M, Dvorak M, Gischer F (2014) Detection and quantification of airborne inoculum of *Hymenoscyphus pseudoalbidus* using real-time PCR assays. Plant Pathol 63(6): 1296–1305. doi:10.1111/ppa.12218

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gherghel F, Fussi B, Donges K, HausteinM, JakobKM, Müller K, Piškur B, Hauptman T, Lenz HD, Konnert M, Kost G, Rexer KH, Stenlid J (2013) The development of a species-specific test to detect *Hymenoscyphus pseudoalbidus* in ash tissues. For Pathol 44(2): 137–144. doi:10.1111/efp.12078

Gross A, Holdenrieder O, Pautasso M, Queloz V, Sieber TN (2014) *Hymenoscyphus pseudoalbidus*, the causal agent of European ash dieback. Mol Plant Pathol 15(1):5–21. doi:10.1111/mpp.12073

Haňáčková Z, Koukol O, Havrdová L, Gross A, Cleary M (2015) Local population structure of *Hymenoscyphus fraxineus* surveyed by an enlarged set of microsatellite markers. For Pathol 45(5):400–407. doi:10.1111/efp.12185

Havrdová L, Černý K (2012) Invaze *Chalara fraxinea* v CHKO Lužické hory – předběžné výsledky výzkumu [The research on *Chalara fraxinea* invasion in Lužické hory Landscape Protected Area – pre- liminary results] (The research on *Chalara fraxinea* invasion in Lužické hory Landscape Protected Area – preliminary results). Acta Pruhoniciana 100:137–145

Havrdová L, Černý K (2013) Význam vlhkosti vzduchu v epidemiologii nekrózy jasanu – předběžné výsledky výzkumu [The importance of air humidity in Ash Dieback epidemiology - preliminary results] (The importance of air humidity in Ash Dieback epidemiology - preliminary results). Zprávy Lesnického Výzkumu 58(4):347–352

Heilmann-Clausen J, Barron ES, Boddy L, Dahlberg A, Griffith GW, Norden J, Ovaskainen O, Perini C, Senn-Irlet B, Halme P (2015) A fungal perspective on conservation biology. Conserv Biol 29(1): 61–68. doi:10.1111/cobi.12388

Hietala AM, Timmermann V, Børja I, SolheimH (2013) The invasive ash dieback pathogen *Hymenoscyphus pseudoalbidus* exerts maximal infection pressure prior to the onset of host leaf senescence. Fungal Ecol 6(4):302–308. doi:10.1016/j.funeco.2013.03.008

Hirst JM (1952) An automatic volumetric spore-trap. Ann Appl Biol 39(2):257–265. doi:10.1111/j.1744-7348.1952.tb00904.x

Holec J, Beran M (eds) (2006) Červený seznam hub (makromycetů) České republiky [Red list of fungi (macromycetes) of the Czech Republic], vol 24. Příroda, Praha

Hospodsky D, Yamamoto N, Peccia J (2010) Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. Appl Environ Microbiol 76(21):7004–7012. doi:10. 1128/AEM.01240-10

Husson C, Scala B, Caël O, Frey P, Feau N, Ioos R, Marçais B (2011) *Chalara fraxinea* is an invasive pathogen in France. Eur J Plant Pathol 130(3):311–324. doi:10.1007/s10658-011-9755-9

Inderbitzin P, Lim SR, Volkmann-Kohlmeyer B, Kohlmeyer J, Berbee ML (2004) The phylogenetic position of *Spathulospora* based on DNA sequences from dried herbarium material. Mycol Res 108(7): 737–748

Jankovský L, Holdenrieder O (2009) *Chalara fraxinea* – Ash Dieback in the Czech Republic. Plant Prot Sci 45(2):74–78

Kirisits T, Dämpfle L, Kräutler K, Woodward S (2013) *Hymenoscyphus albidus* is not associated with an anamorphic stage and displays slower growth than *Hymenoscyphus pseudoalbidus* on agar media. For Pathol 43(5):386–389. doi:10.1111/efp.12042

Kowalski T, Holdenrieder O (2009) Pathogenicity of *Chalara fraxinea*. For Pathol 39(1):1–7. doi:10.1111/j.1439-0329.2008.00565.x

Libkind D, Moliné M, Sampaio JP, Van Broock M (2009) Yeasts from high-altitude lakes: influence of UV radiation. FEMS Microbiol Ecol 69(3):353–362. doi:10.1111/j.1574-6941.2009.00728.x

McKinney LV, Thomsen IM, Kjær ED, Bengtsson SBK, Nielsen LR (2012) Rapid invasion by an aggressive pathogenic fungus (*Hymenoscyphus pseudoalbidus*) replaces a native decomposer (*Hymenoscyphus albidus*): a case of local cryptic extinction? Fungal Ecol 5(6):663–669. doi:10.1016/j.funeco.2012.05.004

Pelaez F, Collado J, Platas G,Overy DP, Martin J, Vicente F, Gonzalez del Val A, Basilio A, De la Cruz M, Tormo JR, Fillola A, Arenal F, Villareal M, Rubio V, Baral HO, Galan R, Bills GF (2011) Phylogeny and intercontinental distribution of the pneumocandin- producing anamorphic fungus *Glarea lozoyensis*. Mycology 2(1):1– 17. doi:10.1080/21501203.2010.544334

Queloz V, Grünig CR, Berndt R, Kowalski T, Sieber TN, Holdenrieder O (2011) Cryptic speciation in *Hymenoscyphus albidus*. For Pathol 41(2):133–142. doi:10.1111/j.1439-0329.2010.00645.x

Svrček M (1984) A taxonomic revision of inoperculate discomycetes described by J. Velenovský in the genus *Helotium*, preserved in National Museum, Prague. Acta Musei Nationalis Pragae, Series B 40(3-4):129–215

Svrček M (1986) New or less known Discomycetes. XIV. Česká Mykologie 40(4):203–217

Triebel D, Baral H-O (1996) Notes on the ascus types in *Crocicreas* (Leotiales, Ascomycetes) with a characterization of selected taxa. Sendtnera 3:199–218

Unterseher M, Reiher A, Finstermeier K, Otto P, Morawetz W (2007) Species richness and distribution patterns of leaf-inhabiting endophytic fungi in a temperate forest canopy. Mycol Prog 6(3):201– 212. doi:10.1007/s11557-007-0541-1

Vainio EJ, KorhonenK, Hantula J (1998) Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. Mycol Res 102(2):187–192. doi:10.1017/s0953756297004577

Velenovský J (1934) Monographia Discomycetum Bohemiae, vol 1., Prague

White TJ, Bruns TD, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: InnisMA,GelfandDH, Sninsky JJ,White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, California, USA, pp 315–322

Zhao Y-J, Hosoya T, Baral H-O, Hosaka K, Kakishima M (2013) *Hymenoscyphus pseudoalbidus*, the correct name for *Lambertella albida* reported from Japan. Mycotaxon 122(1):25–41. doi:10.5248/122.2

FUNGAL ENDOPHYTES IN ASH SHOOTS – DIVERSITY AND INHIBITION OF *HYMENOSCYPHUS FRAXINEUS*

Zuzana Haňáčková[1,2*], Ludmila Havrdová[1], Karel Černý[1], Daniel Zahradník[1] & Ondřej Koukol[2]

[1]*Department of Biological Risks, Silva Tarouca Research Institute for Landscape and Ornamental Gardening Publ. Res. Inst., Květnové náměstí 391, Průhonice 252 43, Czech* Republic, [2]*Department of Botany, Faculty of Science, Charles University, Benátská 2, Prague 128 01, Czech Republic, *Corresponding author: E-mail: zuzana.kolarova@seznam.cz*

Haňáčková et al. 2017. Baltic Forestry, 23/1: 89-106.

ABSTRACT

The population of European ash (*Fraxinus excelsior*) in Europe is severely affected by ash dieback disease caused by *Hymenoscyphus fraxineus*. Endophytic fungi are known to influence tree fitness and there are efforts to use them directly or indirectly in the biological control of tree pathogens. To assess possible variation in the fungal community depending on the health status of the tree, three pairs of ash dieback relatively resistant and ash dieback susceptible adult trees were selected from two locations. The diversity of fungal endophytes in healthy ash shoots was investigated in the summer and winter season by agar culture isolations. To screen for the antagonistic potential of ash endophytes to *H. fraxineus*, 48 isolated taxa were tested in dual cultures with the pathogen. Distinctive seasonal changes were observed in the identified fungal communities. Endophytes with a presumptive saprotrophic functional role increased in the summer, whereas presumptive pathogenic taxa increased in winter. Furthermore, species diversity was significantly higher in the winter. Higher frequencies of *Diaporthe* sp. 1 and *Diaporthe* sp. 2 were recorded in susceptible trees than in resistant trees. However, no significant differences were found between community structures. The growth of *H. fraxineus* was significantly reduced by 36 endophytes, with inhibition rates ranging from 42 to 83%. The best inhibition results were obtained for fast growing fungi such as *Botrytis cinerea* and *Phoma macrostoma* var. *incolorata.*

Key words: tree genotype, endophyte community, dual cultures, antagonism, *Hymenoscyphus fraxineus*

INTRODUCTION

The recent spread of *Hymenoscyphus fraxineus*, the causal agent of ash dieback, resulted in a substantial threat to native ash stands and forestry (McKinney et al. 2014, Landolt et al. 2016). Several European countries with a high ratio of ash in forestry production reported high losses of adult trees (Pliūra et al. 2011, McKinney et al. 2014). Artificial re-establishment of ash stands is no longer recommended due to the high probability of disease return (Pliūra et al. 2014). Therefore, interest among practitioners to plant the species has been limited (McKinney et al. 2014b). Moreover, the production of ash seedlings in nurseries has also been affected due to the effective spread of the pathogen and the lack of customer interest (Havrdová et al. this publication).

Some ash trees show less susceptibility to ash dieback than others; a heavily damaged tree may be observed in proximity to a tree having almost no signs of dieback. These differences have been connected with the genetic variability of individual trees (Pliūra et al. 2011, McKinney et al. 2012, Stener 2013, Lobo et al. 2015). However, tree genotype, along with tree physiology, can also influence the endophytic fungal community (Rajala et al. 2013, Rajala et al. 2014). Tree pathogen resistance can even lead to thorough reduction of endophytic colonization in target tissue (Martín et al. 2013). Endophytes are known for their defensive power against various tree pathogens. Although a supportive role has been attributed to the fungal communities of trees (Arnold et al. 2003, Ganley et al. 2008, Mejía et al. 2008, Witzell et al. 2014), there are also concerns about isolates of fungal species having the opposite function; some of them may contribute to the development of tree diseases (Bengtsson et al. 2014).

Fungal endophytes and saprotrophs of European ash (*Fraxinus excelsior*) have previously been investigated in leaves (Unterseher et al. 2007, Scholtysik et al. 2013, Cross et al. 2016), living branches (Kowalski and Kehr 1992) and attached dead branches (Butin and Kowalski 1986, Griffith and Boddy 1991, Unterseher et al. 2005, Unterseher and Tal 2006). An intensive search for the causal agent of ash dieback led to surveys in the past decade of fungi in ash shoots, branches, roots, buds, litter petioles and seeds (Przybył 2002, Kowalski and Łukomska 2005, Bakys et al. 2009a, Chen 2012, Cleary et al. 2013, Davydenko et al. 2013). In addition to *H. fraxineus*, the results indicated the presence of other potential pathogens, but pathogenicity to ash was confirmed only for a few of the tested species (Przybył 2002, Bakys et al. 2009a).

Mechanisms by which endophytes can influence the presence of pathogens in a tree consist of direct effects including antibiosis by metabolites, competition for nutrients or space and mycoparasitism, and indirect effects including induced systemic resistance of the tree (Viterbo et al. 2007, Lacava and Azevedo 2014). Preliminary tests of antagonism *in vitro* are beneficial in searching for potential biological control agents with direct effects on unwanted fungi (Mejía et al. 2008). Such screening may also reveal a possible inverse impact of the pathogen on native fungal species. The first studies with *H. fraxineus* showed that it created an inhibition zone in the presence of other fungi *in vitro* (Kowalski and Holdenrieder 2009, Kowalski and Bartnik 2010). Schulz et al. (2015) reported mutual antibiosis between *H. fraxineus* and ash endophytes resulting in reduced concentrations of phytotoxins produced by *H. fraxineus* in co-cultures. Recently, Schlegel et al. (2016) showed that ash leaf endophytes inhibited germination of *H. fraxineus* ascospores.

Although there have been many studies of tree endophytes, their diversity and spatio-temporal dynamics are still mostly unknown, which impedes the ability to utilize endophytes in forest protection (Louda et al. 2003; Jumpponen and Jones 2010; Witzell et al. 2014). Seasonal changes have been observed in the fungal community of the phyllosphere of deciduous trees (e. g. Sieber and Hugentobler 1987, Unterseher et al. 2007, Jumpponen and Jones 2010). However, less is known about the winter fungal community of coniferous and evergreen plants, as well as of bark of deciduous trees in temperate zones (Widler and Müller 1984, Buck et al. 1998, Osono 2008, Guo et al. 2008, Joshee et al. 2009). Although high-throughput sequencing methods may reveal total fungal communities, the origin of fungi (endophytic vs. epiphytic) may be difficult to assess (Jumpponen and Jones 2010). An advantage of cultivation methods is the yield of fungal isolates that can be further used for polyphasic identification and possible other applications such as antagonistic assays (Prior et al. 2014).

The aim of our study was to investigate the differences between fungal communities of healthy shoots of trees severely damaged by ash dieback and of those of relatively resistant trees. The comparison was made in two different seasons (summer and winter) which differ not only in climatic conditions but also in activity of the pathogen. A further objective was to assess the competitive ability of fungal endophytes naturally occurring in *F. excelsior* against *H. fraxineus*. For this purpose, isolated endophytes were tested in dual cultures *in vitro* on a nutrient-poor medium.

MATERIALS AND METHODS

Locations and collection of material

Field sampling was performed in July 2012 and February 2013 at two locations in the Czech Republic: Luž (50.8391469N, 14.6502289E, Lusatian mountains, 650 m a.s.l., mean annual temperature 6-7°C, mean annual precipitation 800-1000 mm) and Heřmanice (50.6555558N, 14.3852781E, Central Bohemian Uplands, 422 m a.s.l., mean annual temperature 7-8°C, mean precipitation 600-650 mm). Approximate meteorological data were obtained from the Climate Atlas of Czechia (Tolasz et al. 2007). Both localities had been exposed to infection pressure of *H. fraxineus* during previous years and trees showed various levels of ash dieback. The two locations consisted of windbreaks of adult ash trees along meadows and pastures, respectively. Three pairs of *F. excelsior* trees were chosen from each location. The distance between trees in a pair was 4 to 12 m to ensure similar conditions in the pair (growth conditions, sources of fungal inoculum, etc.). One tree from each pair visibly suffered from ash dieback (crown defoliation 30-50%) and the other was relatively resistant (crown defoliation up to 10%). Eight healthy looking one-year shoots were cut off from each tree at a height of approximately six metres and processed the next day.

Isolation and identification of fungal endophytes

In the laboratory, shoots were washed in tap water, divided into bark and wood and cut into fragments of 5×3-4 mm. Bark fragments were surface sterilized in 96% ethanol for 30 s, 0.47% sodium hypochlorite for 60 s, 96% ethanol for 30 s and finally rinsed in distilled water. The time of sterilization was shortened to 10 s in each disinfection agent for wood fragments. Five pieces of wood and five pieces of bark from one shoot were placed in 9 cm Petri dishes with 2% wort agar (2WA) prepared from brewery wort (Staropramen Brewery, Prague, Czech Republic). The final sucrose content was adjusted to 2% w/v, and the suspension was supplied with 18 g L-1 agar (Fassatiová 1986). Petri dishes were incubated in the dark at 20°C and checked weekly for the growth of fungi for four weeks for the summer sampling and six weeks for the winter sampling (due to almost no growth of fungi in the first two weeks). Emerging colonies were grouped into morphospecies based on phenotype characteristics and one isolate of each morphospecies was subcultured on 2WA for subsequent morphological identification and extraction of DNA. Molecular data were obtained using standard procedures for DNA extraction, PCR amplification with primers amplifying the ITS1-5.8S-ITS2 rDNA

region and sequencing (Haňáčková et al. 2015). The morphological and molecular data were taken into consideration in the final identification of taxa (Suppl. Table 2) and a currently accepted name was assigned to a taxon according to the Index Fungorum (http://www.indexfungorum.org/names/names.asp) or another relevant taxonomic source (e.g. recent monographies).

Antagonistic assays in dual cultures

Forty-nine isolates of fungal endophytes were tested against two isolates of *H. fraxineus*; these isolates of *H. fraxineus* were obtained from infected shoots of trees in the same locations (LUZ from Luž and HER from Heřmanice). All endophytes originated from *F. excelsior* except for *Biatriospora* sp. (strain CCF4378, obtained from the Culture Collection of Fungi, Charles University in Prague, Faculty of Science, Czech Republic), which was isolated from *Ulmus glabra* and was also recently detected in *Acer pseudoplatanus* (Kelnarová I., unpublished results). This isolate was considered to be a promising agent due to its broad spectrum of toxic secondary metabolites (Stodůlková et al. 2015). To approach natural conditions in our assays, agar with the addition of ash shoot extract and microcrystalline cellulose as a source of carbon was used in dual cultures. The extract was prepared as follows: *F. excelsior* shoots were cut into small fragments, boiled in water (30 g of fragments per 1 l of deionized water) for 20 min and filtered through a fine cloth. Agar medium was prepared from 1 l of the filtered suspension amended with 18 g of agar and 20 g of microcrystalline cellulose (Serva, Germany). Because of the slow growth of *H. fraxineus*, the pathogen was inoculated onto the Petri dish one week before an endophyte. Agar plugs with mycelium (5 mm diameter, taken from the actively growing margin of the colony) were placed 4 cm apart from each other on a 9 cm Petri dish. For the self-inhibition test, the identical *H. fraxineus* isolate was used instead of an endophyte. The negative control consisted of pairing *H. fraxineus* with a plug of agar. Each treatment was replicated three times and the growth of the pathogen was measured as a colony radius on a connective line between both colonies and on a line 45° up and down from this line after 14 and 28 days of co-cultivation at 18°C. At the end of cultivation, five plugs from the interaction zone or from the margin of the *H. fraxineus* colony (in the case of no contact) were transferred to 2WA to reisolate the pathogen to confirm viability of *H. fraxineus* in the contact zone and distinguish between replacement and overgrowing only (Koukol et al. 2006).

Data analysis

Isolated endophytes were classified into three presumptive groups: pathogens, saprotrophs, or endophytes with unknown ecology, according to available records about given species or higher taxa in the literature or GenBank (http://www.ncbi.nlm.nih.gov). The colonization frequency of each taxon was calculated as the proportion of colonies from the total number of colonies per sample and the Shannon index was defined for fungal communities. Shannon indices were compared using paired *t*-tests. The dependency of the occurrence of particular fungal species on season and the health status of the tree was assessed using contingency table chi-squared tests.

To compare the inhibition ability of endophytes, data for both isolates of *H. fraxineus* were assessed together due to similar variances. The sum of *H. fraxineus* growth in three directions after 28 days was converted to a relative scale (1 = maximal growth of *H. fraxineus* without other fungus, 0 = no growth of *H. fraxineus*) and was further explained as an inhibition effect of an endophyte. Differences in the inhibition effects of endophytes were assessed using two-way ANOVA. Homogeneous groups of endophytes (inhibition effect of endophytes in the same group is not significantly different) were defined using Tukey multiple comparisons of means. The statistical analyses were performed in PAST: Paleontological Statistics Software Package for Education and Data Analysis (Hammer et al. 2001) and Statistica 7.0 (StatSoft, Inc., Tulsa, OK).

RESULTS

Endophytic community

In total, the isolation yielded 884 colonies representing 58 fungal species; of these species, 35 were obtained in the summer and 38 in the winter (Table 1). Twenty species were isolated only in the summer, 23 only in winter and 15 were present in both seasons. Shannon diversity was significantly higher in the winter ($P < 0.001$). The number of fungal colonies also increased from 382 in summer to 502 in winter.

Irrespective of the health status of the tree, the species richness of saprotrophs decreased and the species richness of pathogens increased in the winter (Table 2). The number of species was higher in the shoots of resistant trees than in susceptible trees (32 and 26, respectively), but the number of colonies was identical (442 in each).

Table 1. Frequencies of isolation of fungi (%) in summer and winter sampling for all samples and for susceptible/resistant trees. When known, the ecological role is provided for each taxon: p = pathogen, s = saprotroph, ps = both strategies, ? = unknown ecology

Order	Fungal taxa	Ecology	Summer (all)	Summer (susceptible|resistant)	Winter (all)	Winter (susceptible/resistant)
Pleosporales	*Alternaria alternata*	ps	1.83	(1.52|2.16)	1.99	(2.04|1.95)
	Coniothyrium sp.	ps	–	(-|-)	0.8	(1.63|-)
	Dendrothyrium sp.	p	–	(-|-)	1.39	(-|2.72)
	*Lophiostoma corticola**	s	1.31	(2.03|0.54)	6.18	(6.12|6.23)
	Lophiostoma sp.*	s	–	(-|-)	12.35	(15.5|9.34)
	Phoma macrostoma	ps	4.97	(5.08|4.86)	–	(-|-)
	Phoma macrostoma var. *incolorata*	ps	10.21	(8.12|12.43)	2.79	(2.86|2.72)
	Phoma sp.	s	0.79	(0.51|1.08)	1.59	(-|3.11)
	Pleospora herbarum	ps	0.26	(-|0.54)	–	(-|-)
	Pleosporales sp. 1*	?	4.45	(2.54|6.49)	0.4	(-|0.78)
	Pleosporales sp. 2	s	0.79	(0.51|1.08)	–	(-|-)
	Pleosporales sp. 3*	?	–	(-|-)	8.76	(6.94|10.5)
	Pyrenochaeta corni	ps	1.83	(1.02|2.7)	1.39	(0.82|1.95)
Dothideales	*Aureobasidium pullulans**	s	19.11	(20.81|17.3)	5.98	(4.9|7)
Capnodiales	*Cladosporium* sp.*	?	2.88	(2.54|3.24)	–	(-|-)
incertae sedis	Dothideomycetes sp. 1*	?	0.79	(1.02|0.54)	20.32	(22.0|18.6)
	Dothideomycetes sp. 2	?	0.52	(1.02|-)	–	(-|-)
	Dothideomycetes sp. 3	s	–	(-|-)	0.2	(0.41|-)
	Dothideomycetes sp. 4	?	–	(-|-)	0.2	(-|0.39)
	All Dothideomycetes		49.74	(46.72|52.96)	64.34	(63.27|65.38)
Xylariales	*Annulohypoxylon cohaerens*	s	0.26	(0.51|-)	0.2	(-|0.39)
	Annulohypoxylon	s	0.26	(0.51|-)	1	(0.41|1.56)
	Anthostomella pinea	ps	2.88	(1.02|4.86)	1.99	(1.63|2.33)
	Lopadostoma turgidum	s	–	(-|-)	0.2	(0.41|-)
	Nemania serpens	s	0.26	(0.51|-)	–	(-|-)
	Xylaria longipes	s	0.26	(0.51|-)	–	(-|-)
	Xylariales sp. 1*	?	–	(-|-)	0.8	(0.82|0.78)
	Xylariales sp. 2	?	–	(-|-)	0.2	(0.41|-)
Diaporthales	*Apiognomonia errabunda*	p	–	(-|-)	0.2	(-|0.39)
	Cryptodiaporthe hystrix	ps	–	(-|-)	0.6	(0.82|0.39)
	Diaporthe sp. 1*	?	4.97	(6.6|3.24)	–	(-|-)
	Diaporthe sp. 2*	?	–	(-|-)	13.35	(17.5|9.34)
	*Prosthecium platanoidis**	p	–	(-|-)	2.79	(2.04|3.5)
	Prosthecium pyriforme	p	0.26	(0.51|-)	–	(-|-)
	Valsa sp.*	p	1.5	(0.51|1.62)	–	(-|-)
Hypocreales	*Gibberella avenacea*	ps	1.83	(2.54|1.08)	1	(-|1.95)
	*Gibberella baccata**	ps	3.14	(3.05|3.24)	0.4	(-|0.78)
Sordariales	*Chaetomium globosum**	s	1.5	(1.52|0.54)	–	(-|-)
	Sordaria fimicola	s	0.79	(1.02|0.54)	–	(-|-)
Coniochaeta	*Coniochaeta* sp.*	ps	0.26	(-|0.54)	–	(-|-)
incertae sedis	Sordariomycetes sp. 1	?	0.26	(-|0.54)	–	(-|-)
	Sordariomycetes sp. 2	?	–	(-|-)	1	(0.41|1.56)
	Sordariomycetes sp. 3	?	–	(-|-)	0.4	(0.82|-)
	All Sordariomycetes		17.54	(18.81|16.2)	24.1	(25.32|22.97)

Table 1. (Continued)

Order	Fungal taxa	Ecology	Summer (all)	Summer (susceptible|resistant)	Winter (all)	Winter (susceptible/resistant)
Helotiales	*Botrytis cinerea*	ps	0.52	(0.51|0.54)	–	(-|-)
	Bulgaria inquinans	s	0.79	(1.02|0.54)	–	(-|-)
	Helotiales sp.*	s	–	(-|-)	4.58	(4.49|4.67)
	Pezicula sporulosa	p	–	(-|-)	1	(2.04|-)
	Phialocephala sp.	s	–	(-|-)	0.4	(0.41|0.39)
incertae sedis	Leotiomycetes sp. 1*	?	–	(-|-)	1	(1.22|0.78)
	Leotiomycetes sp. 2	?	–	(-|-)	0.2	(-|0.39)
	All Leotiomycetes		1.31	(1.53|1.08)	7.17	(8.16|6.23)
Eurotiales	*Aspergillus pseudoglaucus*	s	1.57	(2.03|1.08)	–	(-|-)
	Aspergillus versicolor	s	0.52	(-|1.08)	0.2	(-|0.39)
	*Penicillium citrinum**	s	2.88	(4.06|1.62)	–	(-|-)
Chaetothyriales	*Phaeomoniella* sp.*	p	–	(-|-)	2.79	(2.04|3.5)
	All Eurotiomycetes		4.97	(6.09|3.78)	2.99	(2.04|3.89)
Polyporales	*Bjerkandera adusta*	s	–	(-|-)	0.2	(-|0.39)
Agaricales	*Coprinellus disseminatus*	s	–	(-|-)	0.2	(-|0.39)
	All Agaricomycetes		–	(-|-)	0.4	(-|0.78)
Sporidiobolales	*Rhodotorula mucilaginosa**	s	4.97	(7.11|2.7)	–	(-|-)
	All Microbotryomycetes		4.97	(7.11|2.7)	–	(-|-)
Pezizales	*Desmazierella acicola*	s	0.26	(-|0.54)	–	(-|-)
	All Pezizomycetes		0.26	(-|0.54)	–	(-|-)
incertae sedis	unidentified yeast*	?	21.2	(19.8|22.7)	1	(1.22|0.78)
	All *incertae sedis*		21.2	(19.8|22.7)	1	(1.22|0.78)

* indicates a significant difference in the frequency of the taxa between the seasons ($P < 0.05$)

The most frequent taxa in summer were unidentified yeast (21%), *Aureobasidium pullulans* (19%) and *Phoma macrostoma var. incolorata* (10%), whereas Dothideomycetes sp. 1 (20%), *Diaporthe* sp. 2 (13%) and *Lophiostoma* sp. (12%) dominated in the winter. Seasonal changes in frequencies were significant for 23 species (Table 1). Seasonality was apparent for several frequent species, e. g. *A. pullulans*, Dothideomycetes sp. 1, *Diaporthe* sp. 2, both varieties of *P. macrostoma*, both species of *Lophiostoma* or Pleosporales sp. 3. In the winter, *Diaporthe* sp. 2 was significantly associated with susceptible trees ($P = 0.02$). Similarly, *Diaporthe* sp. 1 reached higher numbers in susceptible trees in the summer, but this finding was not statistically significant ($P = 0.095$). Nevertheless, total communities did not significantly differ between susceptible and resistant trees.

Table 2. Species richness and number of fungal colonies isolated from healthy ash shoots as a function of season and with attributed ecological group. Isolates with unknown ecology were not included

	Summer		Winter	
Ecological group of fungi	No.of colonies	Species richness	No. of colonies	Species richness
Saprotrophs	149	16	167	13
Pathogens	5	2	41	7
Both strategies	105	10	55	8

The growth of *H. fraxineus* in dual cultures

The inhibition of *H. fraxineus* was significantly affected by the endophyte, by the used isolate of *H. fraxineus* and by their interaction in dual cultures after 28 days (Table 3). When the results from both isolates were combined, 36 endophytes had a significantly inhibiting effect on the growth of *H. fraxineus*. The inhibitions varied from 42-83%. The best inhibition rates, over 80%, were achieved by *Botrytis cinerea* and *Phoma macrostoma* var. *incolorata* (Figure 1). Another 26 species caused growth inhibition of *H. fraxineus* by at least 50%. In contrast, 11 species had lower antagonistic effects than *H. fraxineus* itself, i.e. less than 39 ± 0.05% inhibition, but these species belonged to one homogeneous group with self-inhibition of *H. fraxineus*. When inhibition rates were compared with self-inhibition of *H. fraxineus*, only 17 endophytes had significantly higher effect than self-inhibition. No reisolation of *H. fraxineus* was yielded after interaction with three endophytes: *Gibberella baccata*, *Lopadostoma turgidum* and *Nemania serpens* suggesting replacement of *H. fraxineus* by these endophytes. However, morphological changes on mycelium were not observed. Dual cultures with some species with good inhibition rates, e.g., *B. cinerea* and *Alternaria alternata*, also resulted in poor reisolation (1 positive out of 5) of the pathogen. However, the results of many other endophytes differed for the two *H. fraxineus* isolates. The LUZ isolate was not reisolated after interaction with 14 endophytes, whereas the HER isolate was not reisolated after interaction with three endophytes. *Biatriospora* sp. affected *H. fraxineus* with 40 ± 0.05% inhibition and reisolation of the pathogen was successful (3 positive out of 5). The inhibition rates of endophytes for both *H. fraxineus* isolates together and for each isolate individually and the results of reisolation are available in the Supplementary Data (Suppl. Table 1).

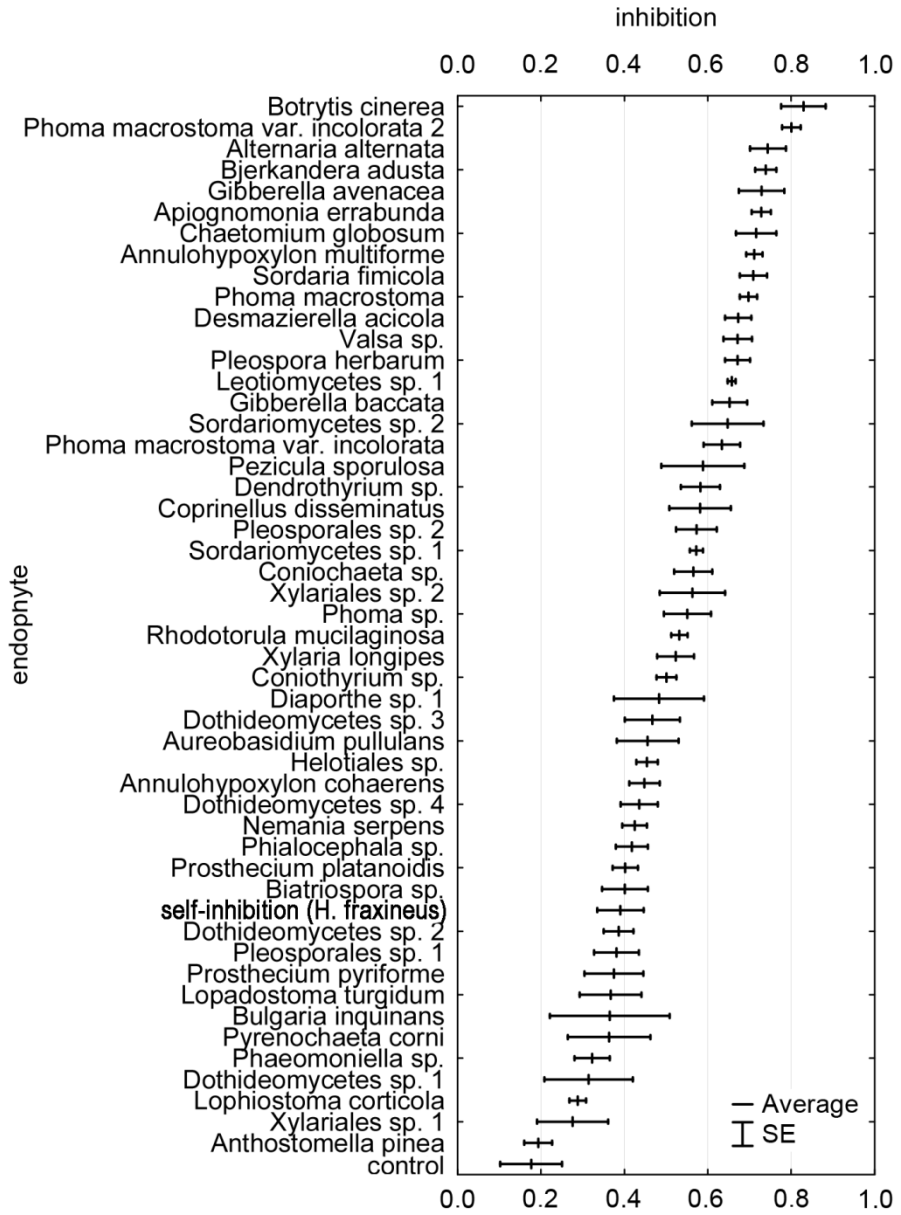

Figure 1. Average growth inhibition of *H. fraxineus* caused by endophytes isolated from healthy ash shoots (including *Biatriospora* sp. from CCF). Horizontal bars indicate standard errors. Identical isolates of *H. fraxineus* were used to test the self-inhibition and a pairing with an agar plug was used in the control treatment

Table 3. Results of the two-way ANOVA of *H. fraxineus* growth inhibition by endophytes in dual culture. (Df = degrees of freedom, Sum Sq = sum of squares, Mean Sq = mean squares, E = endophyte, I = isolate of *H. fraxineus*)

Source of variation	Df	Sum Sq	Mean Sq	F value	Pr(> F)
Endophyte (E)	50	7.668	0.15336	15.421	<2e-16 ***
Isolate *H.f.* (I)	1	0.061	0.06104	6.138	0.0141 *
E*I	50	2.694	0.05389	5.419	<2e-16 ***
Residuals	199	1.979	0.00994		

H.f., Hymenoscyphus fraxineus

DISCUSSION

To be complementary to previous studies aimed at (micro)fungi colonizing ash twigs and shoots, we combined data characterising the composition of the fungal community from two different seasons and two types of trees differing in health condition with the antagonistic potential of isolated fungal species against *H. fraxineus*. Using this approach, we were able to obtain a complex view of the function of endophytic mycobiota against the spread of *H. fraxineus* in shoot tissue.

The fungal community and seasonal changes

We recorded similar magnitudes of the overall species number as in previous studies using the same substrate, considering some variation in the sampling design and isolation methods. However, the dominant species differed substantially. *Aureobasidium pullulans*, one of the most frequently isolated species in our study and in the study by Davydenko et al. (2013), was recorded with medium frequency by Bakys et al. (2009b) and only rarely by Kowalski and Kehr (1992) (Table 4). Some taxa were congruent with endophytes from other ash tissues. An unknown member of Pleosporales (Pleosporales sp. 1) was isolated from ash shoots with advanced necrosis and also from living leaves (Bakys et al. 2009b)and to Fungal sp. MT0843 in Scholtysik et al. 2013, respectively). *Coniothyrium* sp. was recorded in necrotic ash leaves and shoot bark (Bakys et al. 2009a). *Dendrothyrium* sp., *Bjerkandera adusta* and *Coprinellus disseminatus* were recorded in ash shoots with dead tops (Bakys et al. 2009b). *Pyrenochaeta corni* was noted as an endophyte of living ash leaves (Scholtysik et al. 2013) and current year seeds (Cleary et al. 2013a). Our approach most likely excluded some yeasts that are typical for the bark and wood of *F. excelsior* (Bakys et al. 2009b, Chen 2012). We isolated just two yeasts: *Rhodotorula mucilaginosa* and an unidentified yeast.

Table 4. A comparison of endophytic fungi detected in healthy ash (*F. excelsior*) shoots from different countries

	Bakys et al. 2009a	Chen 2012*	Davydenko et al. 2013	Kowalski and Kehr 1992*	present study	
Country of origin	Sweden	New Zealand	Ukraine	Germany, Poland	Czech Republic	
Sampling season (month)	summer (June)	winter (August)	spring (May)	thourough summer and autumn	summer (July)	winter (February)
Number of samples	58 (wood with bark)	34 bark + 35 wood	68 (wood with bark)	70 (wood with bark)	92 (wood with bark)	92 (wood with bark)
Isolation of endophytes	cultivation (Hagem agar)	cultivation (1%MEA) and direct DNA extraction with cloning	direct DNA extraction and sequencing of fungal ITS rRNA	cultivation (2% MEA)	cultivation (2% wort agar)	
Number of species	20	16 and 53	7	47	35	38
Dominant species	*Alternaria alternata*	Ascomycota sp.	*Alternaria arborescens*	*Alternaria alternata*	*Aureobasidium pullulans*	*Diaporthe* sp. 2
	Epicocum nigrum	*Fusarium* sp.	*Aureobasidium pullulans*	*Pezicula cinnamomea*	*Phoma macrostoma* var. *incolorata*	Dothideomycetes sp. 1
	Giberella avenacea	unidentified sp. 119	*Cladosporium cladosporioides*	*Phomopsis* spp.	unidentified yeast	*Lophiostoma* sp.
			Cladosporium tenuissimum			
Species congruently recorded in present study	*Alternaria alternata*	*Gibberella baccata*	*Aureobasidium pullulans*	*Aureobasidium pullulans*		
	Aureobasidium pullulans	*Lophiostoma corticola*		*Alternaria alternata*		
	Diaporthe sp. 1			*Prosthecium pyriforme*		
	Gibberella avenacea			*Nemania serpens*		
	Valsa sp.					

*only endophytes identified to the species level were compared due to a lack of accession numbers

Several species that we recorded as singletons were never before recorded in any type of ash tissue. For example, we recorded *Desmazierella acicola*, a saprotroph of pine needles (Martinović et al. 2016) and *Prosthecium platanoides*, which was previously only known to be restricted to dead branches of *Acer pseudoplatanus* (Voglmayr and Jaklitsch 2008). Similar findings were noted by Hayatgheibi (2013), who isolated *Lophodermium pinastri*, an endophyte and later saprotroph of pine needles, from ash seeds. These findings may represent accidental infections from surrounding vegetation (Kowalski and Kehr 1992, Joshee et al. 2009), and suggest interesting host flexibility, but probably without any significance for a given fungal species and impact on the host.

We revealed a clear seasonal change in the fungal community of ash shoots with a higher species richness of pathogenic fungi in the winter (Table 2). The dormant season of trees probably facilitates the growth of pathogens, especially when winters are mild (Lonsdale and Gibbs 1996). Moreover, the decrease in carbohydrate content in twigs during the winter and the translocation of carbohydrates to storage tissues – frequently roots (Kozlowski et al. 1991) – can reduce the survival or activity of saprotrophs in shoot tissues. Winter shoots were older than summer shoots, which could also increase the number of species, which is known to be somewhat positively correlated with the age of tissue (Widler and Müller 1984, Guo et al. 2008).

The fungal community of trees differing in health status

The influence of tree health status on fungal endophytes was studied in healthy shoots of six pairs of *F. excelsior*. Susceptible ash trees hosted more colonies of *Diaporthe* sp. 1 and *Diaporthe* sp. 2. *Diaporthe* species (with anamorphs assigned to *Phomopsis*) have often been isolated as endophytes (Sieber 2007) and frequently reported as plant pathogens (Tan et al. 2013). Trees suffering from ash dieback thus probably enable wider expansion of other parasites in plant tissue as observed on *Phomopsis oblonga* in trees affected by *Ophiostoma* spp. (Webber and Gibbs 1984). Additionally, reduced defensive capacity of a tree can trigger a shift of an endophyte to a pathogenic phase (Sieber 2007). However, it is difficult to differentiate whether there are beneficial effects of *H. fraxineus* on the development of other pathogens or if tissues previously colonized by other pathogens accelerate the spread of *H. fraxineus* and progress of the ash dieback.

Sieber and Hugentobler (1987) focused on endophytic assemblages in healthy leaves and twigs of healthy and diseased beech trees (*Fagus sylvatica*). Similar to our results, they did not find differences between endophytic communities from those substrates. In contrast to our results, *Diaporthe eres* had higher frequencies in the leaves of healthy trees, which was attributed to supposedly higher water capacity compared to diseased trees. Similarly, Gennaro et al. (2003) did not demonstrate a distinction between whole endophytic communities of healthy and declining *Quercus* species. However, these authors noted a significantly lower Shannon-Wiener index for leaves, twigs and buds of declining *Q. robur* and shoots of declining *Q. cerris*. Although trees in our study did not differ significantly in species diversity, we isolated more species from resistant trees. Ragazzi et al. (2003) found significantly higher colonization frequencies of given fungal species in twigs and symptomless leaves of declining *Quercus* species in comparison to

healthy ones. These authors emphasized that this pattern was especially evident for species that can switch to a pathogenic lifestyle. It seems that trees in a better state of health can host more diversified fungal communities, or higher species diversity of fungi balances the self-assertion of opportunistic pathogens. A tree probably chooses from several methods of protecting itself against fungal pathogens. A high production of phenolic compounds can also limit fungal spread, and in that case, low species diversity of endophytes is a sign of resistance. This feature of a tree can even be restricted to a particular type of tissue (Martín et al. 2013). The end of active restriction of the endophytic fungal community may be recognized after tissue dieback, when a shoot becomes accessible for various pathogens and saprotrophs. At a certain moment of infection, species richness of fungal taxa in necrotic shoots significantly increases (Bakys et al. 2009a, Davydenko et al. 2013).

The influence of ash endophytes on *H. fraxineus* growth *in vitro*

Here, we examined the inhibition effect of native endophytes colonizing European *F. excelsior* in interactions with non-native pathogenic *H. fraxineus*. Medium with ash extract was used to simulate natural conditions for interactions because this antagonistic screening will be followed by *in planta* tests in ash seedlings. Different media and cultivation conditions can influence the results of antagonism substantially (Kusari et al. 2013). The use of media rich in nutrients such as MEA or PDA could support isolates in faster growth or production of secondary metabolites that cannot be synthesized in plant tissue as endophytes. There are also obvious limits of the pairwise testing, which ignores the collective effect of endophytes. This study tried to overcome this bias by using a simultaneous view of the mosaic of endophytes present in ash shoots. The majority of the endophytes used in our study inhibited the growth of both tested *H. fraxinus* isolates. Nevertheless, inhibition rates of more than half endophytes were comparable with self-inhibition of *H. fraxineus*. Similarly, Schulz et al. (2015) reported that 57 of 59 tested endophytes inhibited *H. fraxineus*. However, only 19 of those 59 reached inhibition rates greater than 30%.

The endophytes with the best inhibition rates were mostly fast growing species and some of them, such as *P. macrostoma* var. *incolorata, G. baccata* or *A. pullulans*, reached high frequencies in shoots in summer. The synergistic effect of these endophytes could reduce the number of *H. fraxineus* strains infecting shoots in the late summer or autumn. The lower frequencies of these endophytes in the winter could contribute to the spread of *H. fraxineus*. However, *P. macrostoma* (both

varieties) (De Gruyter et al. 2002) and *G. baccata* are weak parasites (Leslie and Summerell 2006). These species were previously isolated from ash shoots with necrosis and might not cause disease, but might profit from *H. fraxineus* infection (Przybył 2002, Bakys et al. 2009a). Not all genotypes of a particular species are able to be virulent to a particular host. Long co-evolution of the endophyte with the host tree is assumed to result in lower susceptibility of the tree. High colonization frequencies of an endophyte are even considered to be a signal of low virulence (Sieber 2007). Surprisingly, *Biatriospora* sp., known to produce a mixture of antagonistic compounds (Stodůlková et al. 2015), had only intermediate effect on the pathogen.

Although we did not establish controls for endophytes, reduction in their growth was often apparent, likely as a result of reciprocal antagonism, which is assumed for most of the endophytic microbes. Antagonism among different isolates of the same species is no exception (Schulz et al. 2015, Yan et al. 2015). The impact of *H. fraxineus* on the native community of endophytes was recently outlined by Schulz et al. (2015), who reported that 13 of 55 tested ash endophytes were inhibited by more than 30% by *H. fraxineus*.

CONCLUSION

The endophytic fungal community of selected ash shoots did not differ between trees susceptible to ash dieback and resistant trees. This finding could indicate that the endophyte community has a lower impact on the inhibition of *H. fraxineus,* and that trees with better health status defend themselves by other mechanisms. Although dual tests with ash endophytes demonstrated their significant influence on *H. fraxineus* growth and suggested which should be tested in future *in vivo* trials, reciprocal antagonism was observed as well; thus, many species do not represent an obstacle for *H. fraxineus,* suggesting that a neutral outcome of the interaction can occur *in vivo*. In contrast, a decrease in some fast-growing species in the winter could facilitate the growth of *H. fraxineus* in colder months. Reduced numbers of tree saprotrophs in the winter also might be relevant for the success of other pathogenic fungi.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Agriculture of the Czech Republic no. NAZV QJ1220218 and by Institutional Support for Science and Research of the Ministry of Education, Youth and Sports of the Czech Republic.

SUPPORTING INFORMATION

The online version of this article available at: https://www.balticforestry.mi.lt/bf/PDF_Articles/2017-23[1]/BalticForestry2017.1_089-106.pdf contains supplementary material.

REFERENCES

Agan A, Drenkhan R, Adamson K, et al (2020) The relationship between fungal diversity and invasibility of a foliar niche—the case of ash dieback. J Fungi 6:1–27. doi: 10.3390/jof6030150

Agostinelli M, Cleary M, Martín JA, et al (2018) Pedunculate oaks (Quercus robur L.) differing in vitality as reservoirs for fungal biodiversity. Front Microbiol 9:1758. doi: 10.3389/fmicb.2018.01758

Agostinelli M, Nguyen D, Witzell J, Cleary M (2021) Mycobiome of Fraxinus excelsior with different phenotypic susceptibility to ash dieback. Front For Glob Chang 4:15. doi: 10.3389/ffgc.2021.580514

Agrios GN (2005) Plant Pathology, 5th edition. Academic Press, San Diego

Alizon S, de Roode JC, Michalakis Y (2013) Multiple infections and the evolution of virulence. Ecol Lett 16:556–567. doi: org/10.1111/ele.12076

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol Rev 21:51–66. doi: 10.1016/j.fbr.2007.05.003

Arnold AE, Mejía LC, Kyllo D, et al (2003) Fungal endophytes limit pathogen damage in a tropical tree. PNAS 100:15649–15654. doi: 10.1073/pnas.2533483100

Bakys R, Vasaitis R, Barklund P, et al (2009a) Investigations concerning the role of Chalara fraxinea in declining Fraxinus excelsior. Plant Pathol 58:284–292. doi: 10.1111/j.1365-3059.2008.01977.x

Bakys R, Vasaitis R, Barklund P, et al (2009b) Occurrence and pathogenicity of fungi in necrotic and non-symptomatic shoots of declining common ash (Fraxinus excelsior) in Sweden. Eur J For Res 128:51–60. doi: 10.1007/s10342-008-0238-2

Baldrian P (2017) Forest microbiome : diversity , complexity and dynamics. FEMS Microbiol Rev 41:109–130. doi: 10.1093/femsre/fuw040

Baral H-O, Bemmann M (2014) Hymenoscyphus fraxineus vs. Hymenoscyphus albidus – A comparative light microscopic study on the causal agent of European ash dieback and related foliicolous, stroma-forming species. Mycology 5:228–290. doi: 10.1080/21501203.2014.963720

Baral H-O, Queloz V, Hosoya T (2014) Hymenoscyphus fraxineus, the correct scientific name for the fungus causing ash dieback in Europe. IMA Fungus 5:79–80. doi: 10.5598/imafungus.2014.05.01.09

Becker R, Ulrich K, Behrendt U, et al (2020) Analyzing ash leaf-colonizing fungal communities for their bological control of Hymenoscyphus fraxineus. Front Microbiol 11:1–15. doi: 10.3389/fmicb.2020.590944

Bengtsson SBK, Barklund P, von Brömssen C, Stenlid J (2014) Seasonal pattern of lesion development in diseased Fraxinus excelsior infected by Hymenoscyphus pseudoalbidus. PLoS One 9:e76429. doi: 10.1371/journal.pone.0076429

Buck JW, Lachance M-A, Traquair JA (1998) Mycoflora of peach bark: population dynamics and composition. Can J Bot 76:345–354.

Buschbom J (2022) Genomic patterns and the evolutionary origin of an invasive fungal pathogen (Hymenoscyphus fraxineus) in Europe. Basic Appl Ecol 59:4–16. doi: 10.1016/j.baae.2021.12.006

Butin H, Kowalski T (1986) Die natürliche Astreinigung und ihre biologischen Voraussetzungen.

III. Die Pilzflora von Ahorn, Erle, Birke, Hainbuche und Esche [The natural pruning of branches and their biological conditions. III. The fungal flora of common maple, gray alder, silver b. Eur J For Pathol 16:129–138.

Černý K, Havrdová L, Zlatník V, Hrabětová M (2016) Pěstování jasanu v prostředí s výskytem Hymenoscyphus fraxineus [certified methodology]. Průhonice, Czech Republic

Chandelier A, Gerarts F, San Martin G, et al (2016) Temporal evolution of collar lesions associated with ash dieback and the occurrence of Armillaria in Belgian forests. For Pathol 46:289–297. doi: 10.1111/efp.12258

Chandelier A, Helson M, Dvorak M, Gischer F (2014) Detection and quantification of airborne inoculum of Hymenoscyphus pseudoalbidus using real-time PCR assays. Plant Pathol 63:1296–1305. doi: 10.1111/ppa.12218

Chen J (2012) Fungal Community Survey of Fraxinus excelsior in New Zealand [master thesis]. Swedish University of Agricultural Sciences

Chumanová E, Černý K, Brestovanská T, et al (2021) Atlas potenciální distribuce vybraných druhů invazních patogenů dřevin a jejich impaktu na lesní ekosystémy v ČR [specialized map]. VÚKOZ, v.v.i, Průhonice

Chumanová E, Romportl D, Havrdová L, et al (2019) Predicting ash dieback severity and environmental suitability for the disease in forest stands. Scand J For Res 34:254–266. doi: 10.1080/02827581.2019.1584638

Cleary MR, Arhipova N, Gaitnieks T, et al (2013a) Natural infection of Fraxinus excelsior seeds by Chalara fraxinea. For Pathol 43:83–85. doi: 10.1111/efp.12012

Cleary MR, Daniel G, Stenlid J (2013b) Light and scanning electron microscopy studies of the early infection stages of Hymenoscyphus pseudoalbidus on Fraxinus excelsior. Plant Pathol 62:1294–1301. doi: 10.1111/ppa.12048

Compant S, Samad A, Faist H, Sessitsch A (2019) A review on the plant microbiome : Ecology , functions , and emerging trends in microbial application. J Adv Res 19:29–37. doi: 10.1016/j.jare.2019.03.004

Cross H, Sønstebø JH, Nagy NE, et al (2017) Fungal diversity and seasonal succession in ash leaves infected by the invasive ascomycete Hymenoscyphus fraxineus. New Phytol 213.3:1405–1417. doi: 10.1111/nph.14204

Davydenko K, Vasaitis R, Stenlid J, Menkis A (2013) Fungi in foliage and shoots of Fraxinus excelsior in eastern Ukraine: a first report on Hymenoscyphus pseudoalbidus. For Pathol 43:462–467. doi: 10.1111/efp.12055

de Bary A (1879) Die Erscheinung der Symbiose. Verlag von Karl J. Trübner, Strassburg, Germany

De Gruyter J, Boerema GH, Van der Aa HA (2002) Contributions towards a monograph of Phoma (Coelomycetes) VI-2 Section Phyllostictoides: Outline of its taxa. Persoonia 18:1–53.

Desmazières JBHJ (1851) Plantes Cryptogames de France. Ann des Sci Nat Bot Série 3 16:323–324.

Drenkhan R, Sander H, Hanso M (2014) Introduction of Mandshurian ash (Fraxinus mandshurica Rupr.) to Estonia: Is it related to the current epidemic on European ash (F. excelsior L.)? Eur J For Res 133:769–781. doi: 10.1007/s10342-014-0811-9

Drenkhan R, Solheim H, Bogacheva A, et al (2017) Hymenoscyphus fraxineus is a leaf pathogen of local Fraxinus species in the Russian Far East. Plant Pathol 66:490–500. doi: 10.1111/ppa.12588

Dvorak M, Rotkova G, Botella L (2016) Detection of airborne inoculum of Hymenoscyphus fraxineus and H . albidus during seasonal fluctuations associated with absence of apothecia. Forests 7:1–14. doi: 10.3390/f7010001

Elfstrand M, Chen J, Cleary M, et al (2021) Comparative analyses of the Hymenoscyphus fraxineus and Hymenoscyphus albidus genomes reveals potentially adaptive differences in secondary metabolite and transposable element repertoires. BMC Genomics 22:1–15. doi: 10.1186/s12864-021-07837-2

Enderle R, Stenlid J, Vasaitis R (2019) An overview of ash (Fraxinus spp.) and the ash dieback disease in Europe. CAB Rev Perspect Agric Vet Sci Nutr Nat Resour. doi: 10.1079/PAVSNNR201914025

Fassatiová O (1986) Moulds and filamentous fungi in technical microbiology. Elsevier, Amsterdam

Franić I, Eschen R, Allan E, et al (2020) Drivers of richness and community composition of fungal endophytes of tree seeds. FEMS Microbiol Ecol 96:fiaa166. doi: 10.1093/femsec/fiaa166

Ganley RJ, Sniezko RA, Newcombe G (2008) Endophyte-mediated resistance against white pine blister rust in Pinus monticola. For Ecol Manage 255:2751–2760. doi: 10.1016/j.foreco.2008.01.052

Gennaro M, Gonthier P, Nicolotti G (2003) Fungal endophytic communities in healthy and declining Quercus robur L. and Q. cerris L. trees in northern Italy. J Phytopathol 151:529–534. doi: 10.1046/j.1439-0434.2003.00763.x

George JP, Sanders TGM, Timmermann V, et al (2022) European - wide forest monitoring substantiate the neccessity for a joint conservation strategy to rescue European ash species (Fraxinus spp .). Sci Rep 12:1–12. doi: 10.1038/s41598-022-08825-6

Giordano L, Gonthier P, Varese GC, et al (2009) Mycobiota inhabiting sapwood of healthy and declining Scots pine (Pinus sylvestris L.) trees in the Alps. Fungal Divers 38:69–83.

Granath G, Vicari M, Bazely DR, et al (2007) Variation in the abundance of fungal endophytes in fescue grasses along altitudinal and grazing gradients. Ecography (Cop) 30:422–430. doi: 10.1111/j.2007.0906-7590.05027.x

Gregorová B, Černý K, Holub V, et al (2006) Poškození dřevin a jeho příčiny. VÚKOZ, v.v.i, Praha

Griffith GS, Boddy L (1991) Fungal decomposition of twigs attached anglosperm. IV. Effect of water potential on interactions between fungi on agar and in wood. New Phytol 117:633–641.

Griffiths SM, Galambao M, Rowntree J, et al (2020) Complex associations between cross-kingdom microbial endophytes and host genotype in ash dieback disease dynamics. J Ecol 108:291–309. doi: 10.1111/1365-2745.13302

Gross A, Holdenrieder O (2013) On the longevity of Hymenoscyphus pseudoalbidus in petioles of Fraxinus excelsior. For Pathol 43:168–170. doi: 10.1111/efp.12022

Gross A, Holdenrieder O, Pautasso M, et al (2014a) Hymenoscyphus pseudoalbidus, the causal agent of European ash dieback. Mol Plant Pathol 15:5–21.

Gross A, Hosoya T, Queloz V (2014b) Population structure of the invasive forest pathogen Hymenoscyphus pseudoalbidus. Mol Ecol 23:2943–60. doi: 10.1111/mec.12792

Gross A, Sieber TN (2016) Virulence of Hymenoscyphus albidus and native and introduced Hymenoscyphus fraxineus on Fraxinus excelsior and Fraxinus pennsylvanica. Plant Pathol 65:655–663. doi: 10.1111/ppa.12450

Gross A, Zaffarano PL, Duo A, Grünig CR (2012) Reproductive mode and life cycle of the ash dieback pathogen Hymenoscyphus pseudoalbidus. Fungal Genet Biol 49:977–986. doi: 10.1016/j.fgb.2012.08.008

Guo L-D, Huang G-R, Wang Y (2008) Seasonal and tissue age influences on endophytic fungi of Pinus tabulaeformis (Pinaceae) in the Dongling Mountains, Beijing. J Integr Plant Biol 50:997–1003. doi: 10.1111/j.1744-7909.2008.00394.x

Halecker S, Wennrich JP, Rodrigo S, et al (2020) Fungal endophytes for biocontrol of ash dieback: The antagonistic potential of Hypoxylon rubiginosum. Fungal Ecol 45:100918. doi: 10.1016/j.funeco.2020.100918

Hammer Ø, Harper DAT, Ryan PD (2001) Paleontological statistics software package for education and data analysis. Palaeontol Electron 4:1–9. doi: 10.1016/j.bcp.2008.05.025

Haňáčková Z, Koukol O, Štursová M, et al (2015) Fungal succession in the needle litter of a montane Picea abies forest investigated through strain isolation and molecular fingerprinting. Fungal Ecol 13:157–166. doi: 10.1016/j.funeco.2014.09.007

Havrdová L, Zahradník D, Romportl D, et al (2017) Environmental and silvicultural characteristics influencing the extent of ash dieback in forest stands. Balt For 23:168–181.

Hayatgheibi H (2013) Studies on the microflora associated with the seeds of European ash (Fraxinus excelsior) and the infection biology of the pathogen Hymenoscyphus pseudoalbidus causing ash dieback [master thesis]. Swedish University of Agricultural Sciences

Hemery G (2008) Forest management and silvicultural responses to projected climate change impacts on European broadleaved trees and forests. Int For Rev 10:591–607. doi: 10.1505/ifor.10.4.591

Henning JA, Kinkel L, May G, et al (2021) Plant diversity and litter accumulation mediate the loss of foliar endophyte fungal richness following nutrient addition. Ecology 102:e03210. doi: 10.1002/ecy.3210

Hietala AM, Agan A, Nagy NE, et al (2022) The Native Hymenoscyphus albidus and the invasive Hymenoscyphus fraxineus are similar in their necrotrophic growth phase in ash leaves. Front Microbiol 13:1–14. doi: 10.3389/fmicb.2022.892051

Hietala AM, Børja I, Solheim H, et al (2018a) Propagule pressure build-up by the invasive Hymenoscyphus fraxineus following its introduction to an ash forest inhabited by the native Hymenoscyphus albidus. Front Plant Sci 9:1–11. doi: 10.3389/fpls.2018.01087

Hietala AM, Isabella B, Cross H, et al (2018b) Dieback of European ash: What can we learn from the microbial community and species-specific traits of endophytic fungi associated with ash? In: Pirttilä AM, Frank AC (eds) Endophytes of Forest Trees. Springer International PUblishing AG, pp 229–258

Hietala AM, Timmermann V, Børja I, Solheim H (2013) The invasive ash dieback pathogen Hymenoscyphus pseudoalbidus exerts maximal infection pressure prior to the onset of host leaf senescence. Fungal Ecol 6:302–308. doi: 10.1016/j.funeco.2013.03.008

Hosoya T, Otani Y, Furuya K (1993) Materials for the fungus flora of Japan (46). Trans Mycol Soc Japan 34:429–432.

Husson C, Caël O, Grandjean JP, et al (2012) Occurrence of Hymenoscyphus pseudoalbidus on infected ash logs. Plant Pathol 61:889–895. doi: 10.1111/j.1365-3059.2011.02578.x

Husson C, Scala B, Caël O, et al (2011) Chalara fraxinea is an invasive pathogen in France. Eur J Plant Pathol 130:311–324. doi: 10.1007/s10658-011-9755-9

Joshee S, Paulus BC, Park D, Johnston PR (2009) Diversity and distribution of fungal foliar endophytes in New Zealand Podocarpaceae. Mycol Res 113:1003–1015. doi: 10.1016/j.mycres.2009.06.004

Jumpponen A, Jones KL (2010) Seasonally dynamic fungal communities in the Quercus macrocarpa phyllosphere differ between urban and nonurban environments. New Phytol 186:496–513. doi: 10.1111/j.1469-8137.2010.03197.x

Juodvalkis A, Vasiliauskas A (2002) The extent and possible causes of dieback of ash stands in Lithuania. LZUU Moksl darbai, Biomed Moksl 56:17–22.

Kheireddine A, Essghaier B, Hedi A, et al (2018) New epiphytic yeasts able to reduce grey mold disease on apples. Plant Prot Sci 54:248–257. doi: 10.17221/103/2017-PPS

King KM, Webber JF (2016) Development of a multiplex PCR assay to discriminate native Hymenoscyphus albidus and introduced Hymenoscyphus fraxineus in Britain and assess their distribution. Fungal Ecol 23:79–85. doi: 10.1016/j.funeco.2016.06.002

Kosawang C, Buchvaldt Amby D, Bussaban B, et al (2018) Fungal communities associated with species of Fraxinus tolerant to ash dieback , and their potential for biological control. Fungal Biol 122:110–120. doi: 10.1016/j.funbio.2017.11.002

Kosawang C, Sørensen H, Kjær ED, et al (2019) Defining the twig fungal communities of Fraxinus species and Fraxinus excelsior genotypes with differences in susceptibility to ash dieback.

Fungal Ecol 42:100859. doi: 10.1016/j.funeco.2019.08.003

Koukol O, Haňáčková Z, Dvořák M, Havrdová L (2016) Unseen , but still present in Czechia: Hymenoscyphus albidus detected by real-time PCR , but not by intensive sampling. Mycol Prog 15:1–9. doi: 10.1007/s11557-015-1149-5

Koukol O, Mrnka L, Kulhánková A, Vosátka M (2006) Competition of Scleroconidioma sphagnicola with fungi decomposing spruce litter needles. Can J Bot 84:469–476. doi: 10.1139/B06-021

Kowalski T (2006) Chalara fraxinea sp. nov. associated with dieback of ash (Fraxinus excelsior) in Poland. For Pathol 36:264–270.

Kowalski T (2001) O zamieraniu jesionów. Tryb Leśnika 4:6–7.

Kowalski T, Bartnik C (2010) Morphologial variation in colonies of Chalara fraxinea isolated from ash (Fraxinus excelsior L.) stems with symptoms of dieback and effects of temperature on colony growth and structure. Acta Agrobot 63:99–106. doi: 10.5586/aa.2010.012

Kowalski T, Bilański P (2021) Fungi detected in the previous year's leaf petioles of Fraxinus excelsior and their antagonistic potential against Hymenoscyphus fraxineus. Forests 12:1412. doi: 10.3390/f12101412

Kowalski T, Holdenrieder O (2009a) The teleomorph of Chalara fraxinea , the causal agent of ash dieback. For Pathol 39:304–308. doi: 10.1111/j.1439-0329.2008.00589.x

Kowalski T, Holdenrieder O (2009b) Pathogenicity of Chalara fraxinea. For Pathol 39:1–7. doi: 10.1111/j.1439-0329.2008.00565.x

Kowalski T, Kehr RD (1992) Endophytic fungal colonization of branch bases in several forest tree species. Sydowia 44:137–168.

Kowalski T, Łukomska A (2005) Badania nad zamieraniem jesionu (Fraxinus excelsior L.) w drzewostanach Nadleśnictwa Włoszczowa [The studies on ash dying (Fraxinus excelsior L.) in the Włoszczowa Forest Unit stands]. Acta Agrobot 58:429–440.

Kozlowski TT, Kramer PJ, Pallardy SG (1991) The Physiological Ecology of Woody Plants. Academic Press, Inc., SA, California

Kurtzman CP, Fell JW, Boekhout T (2011) The Yeasts, 5th ed. Elsevier, Amsterdam, The Netherlands

Kusari P, Kusari S, Spiteller M, Kayser O (2013) Endophytic fungi harbored in Cannabis sativa L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. Fungal Divers 60:137–151. doi: 10.1007/s13225-012-0216-3

Lacava PT, Azevedo JL (2014) Biological control of insect-pest and diseases by endophytes. In: Verma VC, Gange AC (eds) Advances in Endophytic Research. Springer India, New Delhi, pp 231–256

Lahiri A (2020) Endophytic diversity of Fraxinus excelsior L. (European ash) and its interaction with the dieback pathogen Hymenoscyphus fraxineus [doctoral thesis]. University of Dublin, Dublin, Ireland

Lahiri A, Murphy BR, Hodkinson TR (2021) Assessing genotypic and environmental effects on endophyte communities of Fraxinus (Ash) using culture dependent and independent DNA sequencing. J Fungi 7:565. doi: 10.3390/jof7070565

Lamit LJ, Lau MK, Sthultz CM, et al (2014) Tree genotype and genetically based growth traits structure twig endophyte communities. Am J Bot 101:467–478. doi: 10.3732/ajb.1400034

Landolt J, Gross A, Holdenrieder O, Pautasso M (2016) Ash dieback due to Hymenoscyphus fraxineus: what can we learn from evolutionary ecology? Plant Pathol 65:1056–1070. doi: 10.1111/ppa.12539

Latz MAC, Jensen B, Collinge DB, Jørgensen HJL (2018) Endophytic fungi as biocontrol agents: elucidating mechanisms in disease suppression. Plant Ecol Divers 11:555–567. doi: 10.1080/17550874.2018.1534146

Leslie JF, Summerell BA (2006) The Fusarium laboratory manual. Blackwell, Iowa

Levine JM, D'Antonio CM (1999) Elton revisited: a review of evidence linking diversity and invasibility. Oikos 87:15–26.

Lobo A, McKinney LV, Hansen JK, et al (2015) Genetic variation in dieback resistance in Fraxinus excelsior confirmed by progeny inoculation assay. For Pathol 45:379–387. doi: 10.1111/efp.12179

Lonsdale D, Gibbs JN (1996) Effects of climate change on fungal diseases of trees. In: Frankland JC, Magan N, Gadd GM (eds) Fungi and environmental change. Cambridge University Press, Cambridge, pp 1–19

Louda SM, Pemberton RW, Johnson MT, Follett PA (2003) Nontarget effects—The Achilles'heel of biological control? Retrospective analyses to reduce risk associated with biocontrol introductions. Annu Rev Entomol 48:365–396. doi: 10.1146/annurev.ento.48.060402.102800

Lygis V, Vasiliauskas R, Larsson KH, Stenlid J (2005) Wood-inhabiting fungi in stems of Fraxinus excelsior in declining ash stands of northern Lithuania, with particular reference to Armillaria cepistipes. Scand J For Res 20:337–346. doi: 10.1080/02827580510036238

Lyu D, Zajonc J, Pagé A, et al (2021) Plant holobiont theory: the phytomicrobiome plays a central role in evolution and success. MDPI 9:675. doi: 10.3390/ microorganisms9040675

Mansfield J, Brown I, Papp-Rupar M (2019) Life at the edge – the cytology and physiology of the biotroph to necrotroph transition in Hymenoscyphus fraxineus during lesion formation in ash. Plant Pathol 68:908–920. doi: 10.1111/ppa.13014

Martín JA, Witzell J, Blumenstein K, et al (2013) Resistance to Dutch elm disease reduces presence of xylem endophytic fungi in elms (Ulmus spp.). PLoS One 8:e56987. doi: 10.1371/journal.pone.0056987

Martinović T, Koukol O, Hirose D (2016) Distinct phylogeographic structure recognized within Desmazierella acicola. Mycologia 108:20–30. doi: 10.3852/14-291

McKinney LV, Thomsen IM, Kjær ED, et al (2012a) Rapid invasion by an aggressive pathogenic fungus (Hymenoscyphus pseudoalbidus) replaces a native decomposer (Hymenoscyphus albidus): a case of local cryptic extinction? Fungal Ecol 5:663–669. doi: 10.1016/j.funeco.2012.05.004

McKinney L V., Nielsen LR, Collinge DB, et al (2014a) The ash dieback crisis: genetic variation in resistance can prove a long-term solution. Plant Pathol 63:485–499. doi: 10.1111/ppa.12196

McKinney L V., Nielsen LR, Collinge DB, et al (2014b) The ash dieback crisis: genetic variation in resistance can prove a long-term solution. Plant Pathol 63:485–499. doi: 10.1111/ppa.12196

McKinney L V, Thomsen IM, Kjær ED, Nielsen LR (2012b) Genetic resistance to Hymenoscyphus pseudoalbidus limits fungal growth and symptom occurrence in Fraxinus excelsior. For Pathol 42:69–74. doi: 10.1111/j.1439-0329.2011.00725.x

McMullan M, Rafiqi M, Kaithakottil G, et al (2018) The ash dieback invasion of Europe was founded by two genetically divergent individuals. Nat Ecol Evol 2:1000–1008. doi: 10.1038/s41559-018-0548-9

Mejía LC, Rojas EI, Maynard Z, et al (2008) Endophytic fungi as biocontrol agents of Theobroma cacao pathogens. Biol Control 46:4–14. doi: 10.1016/j.biocontrol.2008.01.012

Meyn R, Langer GJ, Gross A, Langer EJ (2019) Fungal colonization patterns in necrotic rootstocks and stem bases of dieback-affected Fraxinus excelsior L. For Pathol. doi: 10.1111/efp.12520

Musolin DL, Selikhovkin A V., Shabunin DA, et al (2017) Between ash dieback and emerald ash borer: Two Asian invaders in Russia and the future of ash in Europe. Balt For 23:316–333.

Nemesio-Gorriz M, McGuinness B, Grant J, et al (2019) Lenticel infection in Fraxinus excelsior shoots in the context of ash dieback. iForest - Biogeosciences For 12:160–165. doi: 10.3832/ifor2897-012

Nemesio-Gorriz M, Menezes RC, Paetz C, et al (2020) Canditate metabolites for ash dieback

tolerance in Fraxinus excelsior. J Exp Bot 71:6074–6083. doi: 10.1093/jxb/eraa306

Nguyen DT, Cleary MR, Enderle R, et al (2016a) Analyses of the ash dieback pathogen, Hymenoscyphus fraxineus , suggest role of tree species diversity on colonization and population structure differentiation. For Pathol 46:82–84. doi: 10.1111/efp.12236

Nguyen NH, Song Z, Bates ST, et al (2016b) FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecol 20:241–248. doi: 10.1016/j.funeco.2015.06.006

Nielsen LR, Nagy NE, Piqueras S, et al (2022) Host – pathogen interactions in leaf petioles of common ash and Manchurian ash infected with Hymenoscyphus fraxineus. Microorganisms 10:375. doi: 10.3390/ microorganisms10020375

Oita S, Ibáñez A, Lutzoni F, et al (2021) Climate and seasonality drive the richness and composition of tropical fungal endophytes at a landscape scale. Commun Biol 4:313. doi: 10.1038/s42003-021-01826-7

Orton ES, Brasier CM, Bilham LJ, et al (2018) Population structure of the ash dieback pathogen , Hymenoscyphus fraxineus , in relation to its mode of arrival in the UK. Plant Pathol 67:255–264. doi: 10.1111/ppa.12762

Osono T (2008) Endophytic and epiphytic phyllosphere fungi of Camellia japonica: seasonal and leaf age-dependent variations. Mycologia 100:387–391. doi: 10.3852/07-110R1

Petrini O (1991) Fungal endophytes of tree leaves. In: Andrews JH, Hirano SS (eds) Microbial Ecology of Leaves. Springer New York, New York, NY, pp 179–197

Pliûra A, Heuertz M (2003) Technical guidelines for genetic conservation and use for common ash (Fraxinus excelsior). Rome

Pliūra A, Lygis V, Suchockas V, Bartkevičius E (2011) Performance of twenty four European Fraxinus excelsior populations in three Lithuanian progeny trials with a special emphasis on resistance to Chalara fraxinea. Balt For 17:17–34.

Pliūra A, Marčiulynienė D, Bakys R, Suchockas V (2014) Dynamics of genetic resistance to Hymenoscyphus pseudoalbidus in Juvenile Fraxinus excelsior clones. Balt For 20:10–27.

Poland TM, McCullough DG (2006) Emerald ash borer: Invasion of the urban forest and the threat to North America's ash resource. J For 104:118–124.

Prior R, Görges K, Yurkov A, Begerow D (2014) New isolation method for endophytes based on enzyme digestion. Mycol Prog 13:849–856. doi: 10.1007/s11557-014-0968-0

Przybył BK (2002) Fungi associated with necrotic apical parts of Fraxinus excelsior shoots. For Pathol 32:387–394.

Queloz V, Grünig CR, Berndt R, et al (2011) Cryptic speciation in Hymenoscyphus albidus. For Pathol 41:133–142. doi: 10.1111/j.1439-0329.2010.00645.x

Ragazzi A, Moricca S, Capretti P, et al (2003) Differences in composition of endophytic mycobiota in twigs and leaves of healthy and declining Quercus species in Italy. For Pathol 33:31–38. doi: 10.1046/j.1439-0329.2003.3062003.x

Rajala T, Velmala SM, Tuomivirta T, et al (2013) Endophyte communities vary in the needles of Norway spruce clones. Fungal Biol 117:182–190. doi: 10.1016/j.funbio.2013.01.006

Rajala T, Velmala SM, Vesala R, et al (2014) The community of needle endophytes reflects the current physiological state of Norway spruce. Fungal Biol 118:309–315. doi: 10.1016/j.funbio.2014.01.002

Rodriguez RJ, White JF, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–30. doi: 10.1111/j.1469-8137.2009.02773.x

Rong X, Rotondo F, Schisler DA, et al (2017) Tracking native and applied populations of Cryptococcus flavescens in the environment. Plant Gene 11:31–40. doi: 10.1016/j.plgene.2016.12.004

Rottstock T, Joshi J, Kummer V, Fischer M (2014) Higher plant diversity promotes higher diversity of fungal pathogens , while it decreases pathogen infection per plant. Ecology 95:1907–1917. doi: 10.1890/13-2317.1

Rozsypálek J, Dvořák M, Longauer R, et al (2017) Ash and ash dieback in the Czech Republic. In: Vasaitis R, Enderle R (eds) Dieback of European ash (Fraxinus spp.)–Consequences and Guidelines for Sustainable Management. Swedish University of Agricultural Sciences, Uppsala,Sweden, pp 79–88

Saikkonen K, Faeth SH, Helander M, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 319–343.

Sansford CE (2013) Pest risk analysis for Hymenoscyphus pseudoalbidus (anamorph Chalara fraxinea) for the UK and the Republic of Ireland.

Schlegel M (2018) Diversity and ecology of fungal endophytes in face of an emerging tree disease [doctoral thesis]. ETH Zürich

Schlegel M, Dubach V, Buol L Von, Sieber TN (2016) Effects of endophytic fungi on the ash dieback pathogen. FEMS Microbiol Ecol 92:1–8. doi: 10.1093/femsec/fiw142

Schlegel M, Queloz V, Sieber TN (2018) The endophytic mycobiome of European ash and sycamore maple leaves – geographic patterns, host specificity and influence of ash dieback. Front Microbiol 9:2345. doi: 10.3389/fmicb.2018.02345

Schmidt CS, Lovecká P, Mrnka L, et al (2018) Distinct communities of poplar endophytes on an unpolluted and a risk element-polluted site and their plant growth-promoting potential in vitro. Microb Ecol 75:955–969. doi: 10.1007/s00248-017-1103-y

Scholtysik A, Unterseher M, Otto P, Wirth C (2013) Spatio-temporal dynamics of endophyte diversity in the canopy of European ash (Fraxinus excelsior). Mycol Prog 12:291–304. doi: 10.1007/s11557-012-0835-9

Schulz B, Boyle C, Draeger S, et al (2002) Endophytic fungi: a source of novel biologically active secondary metabolites*. 106:996–1004. doi: 10.1017?S0953756202006342

Schulz B, Haas S, Junker C, et al (2015) Fungal endophytes are involved in multiple balanced antagonisms. Curr Sci 109:39–45.

Siddique AB, Khokon AM, Unterseher M (2017) What do we learn from cultures in the omics age? High-throughput sequencing and cultivation of leaf- inhabiting endophytes from beech (Fagus sylvatica L.) revealed complementary community composition but similar correlations with local habitat conditions. MycoKeys 20:1–16. doi: 10.3897/mycokeys.20.11265

Sieber TN (2007) Endophytic fungi in forest trees: are they mutualists? Fungal Biol Rev 21:75–89. doi: 10.1016/j.fbr.2007.05.004

Sieber TN, Hugentobler C (1987) Endophytische Pilze in Blättern und Ästen gesunder und geschädigter Buchen (Fagus sylvatica L.)[Endophytic fungi in leaves and twigs of healthy and diseased beech trees (Fagus sylvatica L.) (in German with English abstract). Eur J For Pathol 17:411–425. doi: 10.1111/j.1439-0329.1987.tb01119.x

Skovsgaard JP, Thomsen IM, Skovgaard IM, Martinussen T (2010) Associations among symptoms of dieback in even-aged stands of ash (Fraxinus excelsior L.). For Pathol 40:7–18. doi: 10.1111/j.1439-0329.2009.00599.x

Skovsgaard JP, Wilhelm GJ, Thomsen IM, et al (2017) Silvicultural strategies for Fraxinus excelsior in response to dieback caused by Hymenoscyphus fraxineus. Forestry 90:455–472. doi: 10.1093/forestry/cpx012

Sønstebø JH, Vivian-Smith A, Adamson K, et al (2017) Genome-wide population diversity in Hymenoscyphus fraxineus points to an eastern Russian origin of European Ash dieback. bioRxiv 1–25.

Stener L-G (2013) Clonal differences in susceptibility to the dieback of Fraxinus excelsior in southern Sweden. Scand J For Res 28:205–216. doi: 10.1080/02827581.2012.735699

Stenlid J, Elfstrand M, Cleary M, et al (2017) Genomes of Hymenoscyphus fraxineus and Hymenoscyphus albidus encode surprisingly large cell wall degrading potential , balancing saprotrophic and necrotrophic signatures. Balt For 23:41–51.

Stodůlková E, Man P, Kuzma M, et al (2015) A highly diverse spectrum of naphthoquinone derivatives produced by the endophytic fungus Biatriospora sp. CCF 4378. Folia Microbiol (Praha) 60:259–267. doi: 10.1007/s12223-014-0366-7

Tan YP, Edwards J, Grice KRE, Shivas RG (2013) Molecular phylogenetic analysis reveals six new species of Diaporthe from Australia. Fungal Divers 61:251–260. doi: 10.1007/s13225-013-0242-9

Terhonen E, Blumenstein K, Kovalchuk A, Asiegbu FO (2019) Forest tree microbiomes and associated fungal endophytes: functional roles and impact on forest health. Forests 10:42. doi: 10.3390/f10010042

Tilman D (2004) Niche tradeoffs , neutrality , and community structure : A stochastic theory of resource competition , invasion , and community assembly. PNAS 101:10854–10861. doi: 10.1073/pnas.0403458101

Timmermann V, Børja I, Hietala AM, et al (2011) Ash dieback : pathogen spread and diurnal patterns of ascospore dispersal , with special emphasis on Norway *. EPPO Bull 41:14–20.

Tolasz R (2007) Climate atlas of Czechia. Czech Hydrometeorological Institute, Palacký University Olomouc, Prague, Olomouc

Ulrich K, Becker R, Behrendt U, et al (2020) A comparative analysis of ash leaf-colonizing bacterial communities identifies putative antagonists of Hymenoscyphus fraxineus. Front Microbiol 11:966. doi: 10.3389/fmicb.2020.00966

Unterseher M, Otto P, Morawetz W (2005) Species richness and substrate specificity of lignicolous fungi in the canopy of a temperate, mixed deciduous forest. Mycol Prog 4:117–132.

Unterseher M, Reiher A, Finstermeier K, et al (2007) Species richness and distribution patterns of leaf-inhabiting endophytic fungi in a temperate forest canopy. Mycol Prog 6:201–212. doi: 10.1007/s11557-007-0541-1

Unterseher M, Tal O (2006) Influence of small scale conditions on the diversity of wood decay fungi in a temperate, mixed deciduous forest canopy. Mycol Res 110:169–178. doi: 10.1016/j.mycres.2005.08.002

Vasaitis R, Enderle R (2017) Dieback of European Ash (Fraxinus spp.): Consequences and Guidelines for Sustainable Management. Uppsala,Sweden

Velenovský J (1934) Monographia Discomycetum Bohemiae, vol 1. Prague

Viterbo A, Inbar J, Hadar Y, Chet I (2007) Plant disease biocontrol and induced resistance via fungal mycoparasites. In: Kubicek CP, Druzhinina IS (eds) The mycota IV. Environmental and microbial relationships. Springer, Heidelberg, pp 127–146

Voglmayr H, Jaklitsch WM (2008) Prosthecium species with Stegonsporium anamorphs on Acer. Mycol Res 112:885–905. doi: 10.1016/j.mycres.2008.01.020

Volkovitsh MG, Bieńkowski AO, Orlova-Bienkowskaja MJ (2021) Emerald ash borer approaches the borders of the European union and kazakhstan and is confirmed to infest European ash. Forests 12:691. doi: 10.3390/f12060691

Vujanovic V (2021) Tremellomycetes yeasts in kernel ecological niche: Early indicators of enhanced competitiveness of endophytic and mycoparasitic symbionts against wheat pathobiota. Plants 10:905. doi: 10.3390/plants10050905

Webber JF, Gibbs JN (1984) Colonization of elm bark by Phomopsis oblonga. Trans Br Mycol Soc 82:348–352.

Wey T, Schlegel M, Stroheker S, Gross A (2016) MAT – gene structure and mating behavior of Hymenoscyphus fraxineus and Hymenoscyphus albidus. Fungal Genet Biol 87:54–63. doi: 10.1016/j.fgb.2015.12.013

Widler B, Müller E (1984) Untersuchungen über endophytische Pilze von Arclostaphylos uva-ursi (

L .) Sprengel (Ericaceae)[Investigations on endophytic fungi of Arctostaphylos uva-ursi (L.) Sprengel (Ericaceae)] (in German with English abstract). Bot Helv 94:307–337.

Witzell J, Martín JA, Blumenstein K (2014) Ecological aspects of endophyte-based biocontrol of forest diseases. In: Verma VC, Gange AC (eds) Advances in endophytic research. Springer India, New Delhi, pp 321–333

Wylder B, Biddle M, King K, et al (2018) Evidence from mortality dating of Fraxinus excelsior indicates ash dieback (Hymenoscyphus fraxineus) was active in England in 2004 – 2005. Forestry 91:434–443. doi: 10.1093/forestry/cpx059

Yadav AN, Verma P, Kour D, et al (2017) Plant microbiomes and its beneficial multifunctional plant growth promoting attributes. Int J Environ Sci Nat Resour 3:555601. doi: 10.19080/IJESNR.2017.03.555601

Yan JF, Broughton SJ, Yang SL, Gange a. C (2015) Do endophytic fungi grow through their hosts systemically? Fungal Ecol 13:53–59. doi: 10.1016/j.funeco.2014.07.005

Zhao Y, Hosoya T, Baral H, et al (2012) Hymenoscyphus pseudoalbidus, the correct name for Lambertella albida reported from Japan. Mycotaxon 122:25–41. doi: 10.5248/122.25

Zugmaier W, Bauer R, Oberwinkler F (1994) Mycoparasitism of some Tremella species. Mycologia 86:49–56.

DIRECT EVIDENCE OF *HYMENOSCYPHUS FRAXINEUS* INFECTION PATHWAY THROUGH THE PETIOLE-SHOOT JUNCTION

Zuzana Haňáčková[1,2,*], Ondřej Koukol[2], Adéla Čmoková[2], Daniel Zahradník[1] and Ludmila Havrdová[1]

[1]*Department of Biological Risks, Silva Tarouca Research Institute for Landscape and Ornamental Gardening Publ. Res. Inst., Květnové náměstí 391, Průhonice 252 43, Czech Republic,*
[2]*Department of Botany, Faculty of Science, Charles University, Benátská 2, Prague 128 01, Czech Republic, *Corresponding author: Zuzana.Hanackova@vukoz.cz*

Haňáčková et al. 2017. Forest Pathology, 47/6: 1-6, doi: 10.1111/efp.12370.

SUMMARY

The symptoms of ash dieback caused by the fungus *Hymenoscyphus fraxineus* include wilting of the foliage followed by dieback of shoots, twigs and branches. Necroses in shoots are assumed to develop after infection through leaf petioles; however, clear evidence of this infection pathway has not yet been provided. Considering the multiple pathogen genotypes in dead ash petioles, we aimed to obtain a spatial overview of all *H. fraxineus* genotypes colonizing individual shoots and their corresponding petioles before leaf shedding in order to acquire precise information about the infection biology of *H. fraxineus* and its ability to cross the petiole-shoot junction. Individual genotypes of *H. fraxineus* were characterized by the analysis of microsatellites using DNA extracted directly from petiole segments or cultures isolated from the segments. We detected 150 different multilocus genotypes in 10 analysed shoots and their respective petioles; the highest number of genotypes was eight for a single petiole and three for a single shoot. The genotypes of most shoot lesions were identical to particular genotypes from the proximal segments of petioles, implicating the main pathway of shoot infections. To test whether the amount of colonized substrate or intraspecific competition have an effect on successful infection, genotypes that reached the most proximal end of the petioles were scored for the number of invaded petiole segments and for the number of *H. fraxineus* genotypes co-occurring in the segments. The extent of colonization of the scored genotypes and intraspecific competition with other *H. fraxineus* strains were not shown to be factors influencing pathogen success in entering the shoot. This study confirms that the majority of ash shoot infections

are caused by genotypes of *H. fraxineus* originating from petioles and shows that these are rare events in comparison with the quantity of genotypes colonizing the petioles of a particular ash shoot.

INTRODUCTION

Ash dieback, caused by the ascomycete fungus *Hymenoscyphus fraxineus*, is a serious disease of *Fraxinus excelsior* and certain other native and non-native *Fraxinus* species occurring in many parts of Europe (Baral & Bemmann, 2014; Gross, Holdenrieder, Pautasso, Queloz, & Sieber, 2014). General symptoms of the disease include wilting of the foliage followed by dieback of shoots, twigs and branches. The earliest visible symptoms are manifested as brown to blackish necrotic lesions on the leaflets, rachises and petioles (Gross et al., 2014; Hietala, Timmermann, Børja, & Solheim, 2013). Bark necrotic lesions can first be observed on shoots in August; however, most of them occur at the end or outside the vegetative period (Kirisits, Matlakova, Mottinger-Kroupa, Cech, & Halmschlager, 2009). Necroses in shoots spread through the pith and sapwood in a longitudinal direction with later radial expansion towards the bark (Matsiakh, Solheim, Nagy, Hietala, & Kramarets, 2016; Schumacher, Kehr, & Leonhard, 2010).

In ash dieback, each leaflet provides a possible entry point for the pathogen. Dead and fresh leaf petioles are colonized by multiple genotypes of the same fungal species (Gross, Zaffarano, Duo, & Grünig, 2012; Hayatgheibi 2013; Nguyen, Cleary, Enderle, Berlin, & Stenlid, 2016), and even an individual necrotic bark lesion may contain more than one genotype of *H. fraxineus,* as recorded by Bengtsson, Barklund, von Brömssen, and Stenlid (2014) and Haňáčková, Koukol, Havrdová, and Gross (2015). On the basis of the occurrence of bark necrotic lesions around leaf scars, it has been supposed that infection can enter a shoot via the leaf (Kirisits et al., 2009). To our knowledge, there has been no direct evidence of this feature up to now. Attempts to confirm the growth of *H. fraxineus* from a petiole into the shoot after artificial leaf inoculation were unsuccessful, supposedly due to the high doses of the pathogen mycelium used in those experiments, which may have led to premature leaf shedding (Cleary, Daniel, & Stenlid, 2013; Schwanda & Kirisits, 2016). An additional route into shoots could be through the lenticels (Husson, Caël, Grandjean, Nageleisen, & Marçais, 2012) or through seeds (Cleary, Arhipova, Gaitnieks, Stenlid, & Vasaitis, 2013).

Multiple infections in leaves and petioles contrast with the much lower incidence of necrosis development in shoots. Thus, the petiole-shoot junction might represent an important location determining resistance/tolerance against *H.*

fraxineus. The ability of the pathogen to cross this interface might be influenced by various factors such as 1) the virulence of individual strains, 2) the resistance of host genotypes and 3) competition with other fungi (other *H. fraxineus* strains, endophytes or secondary colonizers of the lesion) (Landolt, Gross, Holdenrieder, & Pautasso, 2016). Further possible factors include 4) time (the pathogen must reach the shoot before petiole dehiscence, which may be prematurely induced by a resistant host) (McKinney, Nielsen, Hansen, & Kjær, 2011) and (5) the available nutrients needed as an energy source for entry into the shoot (Dighton, 2007).
The objective of this study was therefore to obtain a spatial overview of all *H. fraxineus* genotypes colonizing individual shoots and their corresponding petioles in order to 1) provide direct evidence of the natural infection route into the shoots and 2) test whether the extent of petiole colonization or competition pressure from other strains of *H. fraxineus* might be responsible for their successful entry into shoots.

MATERIALS AND METHODS

Design of the experiment and sampling

The experiment was conducted at four sites in the northern part of the Czech Republic (Table 1); all localities were 4–9 km apart. The surveyed sites consisted of adult *F. excelsior* trees in lines along infrequently travelled side roads or in windbreaks. At each site, three trees were selected, and five current-year shoots (without visible bark lesions on the current or two-year-old shoot segments) with symptoms of ash dieback on the leaves were labelled at a height of 1.5–3 m on each tree in September 2014 (relatively late term due to a dry summer and a small amount of infection). Thereafter, individual petioles were tied with a nylon thread to the respective shoot and labelled with plastic tape with a code describing its position on the shoot. In total, 60 shoots and 471 petioles were prepared for the trial. In November 2014, after natural leaf fall, the abscised petioles still attached to the shoot by the thread were sampled and frozen at −80 °C until further analysis. The shoots were sampled in November 2014 and January 2015 when the first visible necroses occurred on the bark and were processed immediately.

Isolation of *H. fraxineus* and DNA extraction

In the laboratory, the shoots were surveyed for the extent and potential source of the necrotic lesions and cut into segments, each containing one leaf scar. *H. fraxineus* was isolated from shoot segments in the proximal direction from each leaf scar and from the two-year-old shoot in cases of apparent necrosis, according

to Haňáčková et al. (2015). The isolation of *H. fraxineus* from petioles was performed after scraping the epidermis from a petiole with a sterile scalpel (dampening of the petiole may be necessary for this procedure). Subsequently, the petiole was divided into basal and apical segments and further segments according to the number of leaflet pairs (each segment was ca. 5 mm long, each included leaflet scars except for the basal segment, see Figure 1). These segments were surface sterilized using the protocol of Kirisits, Dämpfle, Kräutler, and Woodward (2013) and then placed on ash leaf malt agar (Gross, Grünig, Queloz, & Holdenrieder, 2012). Outgrowing strains of *H. fraxineus* were transferred onto new dishes with malt extract agar. After the pathogen isolation (when the characters of a developing colony allowed its identification as *H. fraxineus*), the petiole segments were reused for DNA extraction.

Table 1 Characteristics of study sites and *Hymenoscypus fraxineus* populations

Study site	Locality	GPS	Elev.	Number of shoots with necrosis	Shoot code	Number of MLGs	Number of petioles with the same MLG	Index of association *IA*	p value *IA*	Allelic richness	Number of alleles per locus	Gene diversity *He*
A	Dolní Šebířov	50.63068N, 14.25052E	415	9						1,96	2,00	0,39
					A1_3	18	NA	-0,084	0,76			
					A1_4	20	2;2	0,332	0.01 *			
					A1_5	15	2;2;2	0,072	0,32			
					A3_2	14	2;2	-0,187	0,91			
B	Velká Javorská	50.66947N, 14.35631E	518	11						2,30	2,90	0,43
					B2_2	22	NA	0,206	0.04*			
					B2_5	7	NA	0,073	0,37			
					B3_2	9	NA	-0,244	0,82			
C	Janovice	50.63948N, 14.38449E	298	2						1,95	2,00	0,37
					C3_2	17	2	0,173	0,10			
F	Mukařov	50.64451N, 14.31067E	527	3						1,94	2,00	0,37
					F1_4	27	3;2	-0,020	0,54			
					F2_1	17	2	0,134	0,19			

NA, not available

* significant with $\alpha = 0.05$, insignificant after correcting α by the sequential Bonferroni procedure (Rice, 1989)

DNA from *H. fraxineus* isolates was extracted using the ZR Fungal/Bacterial DNA kit (Zymo Research, Orange, CA, USA). DNA extraction from petiole segments was performed by the NaOH extraction method (Wang, Qi, & Cutler, 1993) with the following modifications: the petiole segment was crushed with two wolfram beads for 5 min at 30 Hz in a MM200 mixer mill (Retsch, Haan, Germany), and then 40 µl of 3 M NaOH was added to the sample, and it was crushed for 2 min. After centrifugation, 20 µl of supernatant was transferred to a new tube and diluted with 200 µl of 100 mM Tris-HCl (pH 8.3).

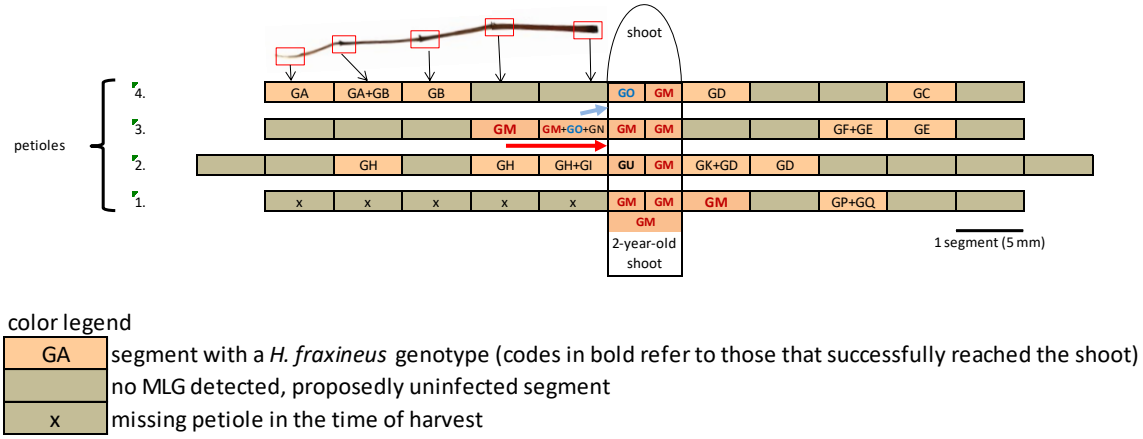

Figure 1 A colonization schema of the shoot A3_2 and its respective petioles infected by *Hymenoscyphus fraxineus* multilocus genotype (MLGs). Outer rows represent petioles and the inner column, the shoot. There are three sources of shoot infection, from which genotypes GM and GO probably entered through one petiole (the red and blue arrow) and genotype GU by direct infection. Genotype GM found in the 1st right petiole might have entered the petiole from the shoot

Microsatellite genotyping

Eleven microsatellite markers (Gross, Grünig, et al., 2012) were used for strain genotyping in two adjusted multiplex PCRs (Table S1). Fragment analysis was performed on an ABI 3130xl Genetic Analyzer (Life Technologies, Carlsbad CA, USA) at the Sequencing Laboratory of the OMICS Core Facility, BIOCEV (Vestec, Czech Republic), and fragments were further scored by GeneMarker software v. 2.2.0 (SoftGenetics, State College, PA, USA).

Data analysis

Colonization of each petiole by the distinguished multilocus genotypes (MLGs) was assessed manually, considering consecutive samples because of the expected

co-occurrence of several genotypes in one segment of the petiole. We did not disqualify any multiple allele records, but we compared the microsatellite profile from the total DNA to the profile of a strain isolated from the given segment, which allowed us to connect alleles to the isolated strain and estimate the allelic set of the other "hypothetical" genotype(s) that were not isolated. We also compared the strength of allele signals to differentiate genotypes from mixed DNA. Still, the number of genotypes in one segment may tend to be underestimated in cases of more than two genotypes. For the purpose of the analyses, multiple records of the same genotype in consecutive segments of a petiole were considered to result from the growth of the given genotype rather than from multiple infections. Distinguished genotypes that reached the most proximal segment of the petiole were characterized according to the number of colonized petiole segments (1-6 segments), the number of other MLGs co-occurring in the given segments (0-3 other MLGs) and the sum of all competitive interactions with other MLGs in the given segments (0-4 other MLGs). These three independent variables were used to explain the success of a given genotype in entering the shoot. Statistical evaluation of the data was performed using logistic regression due to the nature of the quantities involved:

$$\log\frac{p(\boldsymbol{x})}{1-p(\boldsymbol{x})} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 ,$$

where $p(\boldsymbol{x})$ is the probability of a pathogen penetrating the stem, x_1 is the number of segments colonized by the genotype, x_2 is the number of other *H. fraxineus* genotypes in competition with the observed genotype, x_3 is the sum of other genotypes competing with the observed genotype in each segment, and $\beta_0 - \beta_3$ are the respective regression coefficients. The statistical analyses were performed using the R plus statistical package (R Development Core Team 2014).

We were not able to measure the time of infection of a particular petiole, but we supposed that earlier infection would be correlated with a longer lesion in the petiole, i.e., expressed as the number of consecutive segments colonized by the genotype.

Analysis of genetic diversity

Population genetic characteristics such as allelic richness (normalized to the smallest population size, N=14), number of alleles and Nei's average gene diversity, *H*e (Nei, 1987), were calculated with the software SPAGEDI 1.4c (Hardy & Vekemans, 2002). Genotypes from all populations were analysed using the R package poppr v 1.0.3 (R Development Core Team 2014; Kamvar, Tabima, & Grunwald, 2014) to

compute the number of MLGs. To assess the probability of a MLG occurring more than once as the result of distinct reproductive events, P_{Sex} (in the case of shared MLGs between populations or subpopulations), the software Genclone v 2.0 (Arnaud-Haond & Belkhir, 2006) was used. The population in each shoot was also tested for the hypothesis of random mating using the index of association, I_A, calculated with the R package poppr v 1.0.3 (calculated with clone-corrected data; R Development Core Team 2014; Kamvar et al., 2014), which is expected to be zero if the alleles are distributed randomly among different MLGs (Brown, Feldman, & Nevo, 1980; Smith, Smith, O'Rourke, & Spratt, 1993).

RESULTS

Twenty-five shoots had developed necrosis (42% of the initially labelled shoots), with the highest number at site B (11 shoots infected) and the lowest at site C (2 shoots) (Table 1). Isolation of *H. fraxineus* was successful only from 10 shoots with visible necrosis at the time of harvesting. In total, genotypes from 79 petioles (426 segments) belonging to 10 shoots (104 segments) were characterized. The isolation of *H. fraxineus* from a petiole was not always successful, but when combined with genotyping from the total extracted DNA, 169 genotypes were detected, which were assigned to 150 unique *H. fraxineus* MLGs. Based on genotyping, we detected 1-3 different MLGs in a single shoot. Together, all petioles from an individual shoot were colonized by 7-27 MLGs (Table 1), with up to eight MLGs per single petiole; frequently, a single segment was colonized by two or three MLGs (Figure 1). Identical MLGs were detected in different petioles belonging to the same shoot (Table 1). We also detected 15 MLGs that were present in more than one shoot. In four cases, they came from two shoots from the same site; in eight cases, they originated from two different sites; and in three cases, the putatively same genotype was detected at three different sites. None of the MLGs shared between shoots had the probability of occurrence more than once because of random mating (P_{Sex}>0.05 in all cases). Subpopulations of two shoots had I_A values that were significantly different from zero ($p<0.05$, Table 1). However, the I_A for the total set of samples was not significant. The average gene diversity, H_e, varied from 0.37 to 0.43 at the study sites (average H_e=0.39).

The majority of shoots were colonized by a single MLG entering through the proximal end of the petiole. We also traced the further spread of these *H. fraxineus* genotypes (in seven cases) into the two-year-old tissue. Except for one shoot (A1_3, see Fig. S1), necrosis in the two-year-old tissue was caused by an MLG growing from the current-year shoot. In one case (shoot A3_2; Figure 1), multiple

MLGs were detected in a single shoot, and its respective petioles indicated the entry of two genotypes through one petiole in addition to the direct infection of the shoot by another genotype. For shoots A3_2, B2_2 and D1_4 (Fig. S1), there are two hypotheses explaining the detected MLGs: first, the source of infection is a petiole, but second, the infection originated in the wood, and the pathogen grew through the shoot into the petiole, i.e., reverse route of infection. The second assumption was considered due to the presence of identical genotype in basal segments of another petiole of the shoot A3_2, presence of dead tissue (supposedly older part of the lesion) in the lower part of the shoot B2_2 and due to damaged bud (supposedly infected or otherwise damaged already in summer) in close proximity to the shoot D1_4.

The statistical analyses showed that successful entry into the shoots was not affected by the extent of petiole colonization ($p=0.2954$), the competition pressure from other genotypes of *H. fraxineus* ($p=0.4963$) or the sum of these competitive interactions ($p=0.7260$). Even the colonization of all segments in a petiole did not ensure successful entry into the shoot (provided that the petiole was not thoroughly colonized after leaf fall). Similarly, the presence of a single strain in the petiole and thus a limited negative effect of competition did not prove to be an advantage for shoot entry.

DISCUSSION

Using field sampling of marked shoots and petioles at multiple sites coupled with microsatellite analysis, we were able to confirm the dominant infection route of *H. fraxineus* from a single leaf petiole into the shoot. Compared to previous studies aimed at population diversity on a larger scale (Burokiene et al., 2015) or a local scale with a single sampling effort (Nguyen et al., 2016), we were able not only to provide a survey of the genetic diversity of *H. fraxineus* on a local scale but also to trace particular genotypes from the infected petiole into the shoot, where they caused necrosis the following season. Our detailed analysis enabled us to distinguish up to eight different genotypes in a single petiole, which compares well to previous analyses (Gross, Grünig, et al., 2012). Scaling up our results to a whole tree suggests that a mature tree must host thousands of *H. fraxineus* individuals. In one case (shoot C3_2; Figure S2), the source of the genotype in the shoot could not be traced from any corresponding petiole, which suggested direct entry, likely through a lenticel (Bengtsson et al., 2014; Husson et al., 2012), infection from a lateral shoot or infection from small leaves (one pair of leaflets)

from the base of the current-year shoot. These small leaves could have been shed before the labelling in September because of faster colonization by the pathogen.
Systematic sampling of the tissue from several places on the shoot also confirmed that one lesion can contain multiple genotypes (Bengtsson et al., 2014), although this number is small. Most of the shoots analysed in this study were infected in a single infection event. Given the ratio between the total number of genotypes and the ones successfully entering the shoot (169:8–10), it is clear that the fungal spread through the petiole represents a strong bottleneck. However, the first bottleneck that we did not detect but may be anticipated is the entry of *H. fraxineus* from leaflets into petioles. Although microscopic observation provided clear evidence of the direct infection of petioles (Cleary, Daniel, et al., 2013), the larger surface area of leaflets most likely contrasts in infection extent with the narrow and slender petioles. Success in entering the shoot is likely not influenced by the specific genotype of *H. fraxineus*, since MLGs from bark lesions and petioles have been not genetically differentiated (Burokiene et al., 2015).
The repeated recording of a single genotype in different petioles of one shoot and even at different localities may be explained by several factors. First is the proximity of the leaves, i.e., they were exposed to the same infection load originating from a single nearby source (i.e., one ascus or apothecium). Although this reason appears to be rather improbable considering the huge mass of apothecia formed under each tree, we supposed that our two shoots with significant $I_A>0$ could serve as examples of infection from a single source. Second, some identical genotypes could be created by chance, considering the large quantities of ascospores (Dvorak, Rotkova, & Botella, 2015). Third, identical MLGs might have resulted from clonal reproduction. The probability of identical MLGs originating from different sexual reproduction events was not significant in our study. This occurrence was previously discussed by Burokiene et al. (2015) but may also be disregarded, as *H. fraxineus* is a strictly outcrossing fungus. Most recently, Fones, Mardon, and Gurr (2016) provided evidence of the germination of conidia on ash leaves, disproving the earlier view that conidia serve solely as spermatia (Gross et al., 2014), but this finding was based only on a laboratory experiment and needs to be confirmed with additional approaches. The most likely explanation is that the microsatellite markers used in our study have limited discriminatory power.
The MLGs that entered the shoots as well as those that reached the most proximal end of the petiole without penetrating the shoot were characterized by the extent of colonized petiole and the number of other *H. fraxineus* MLGs. None of the

tested factors explained the successful entry into the shoot. This result may reflect the limited roles of nutrient sources and competition with other *H. fraxineus* MLGs, which could also facilitate spread, in successful entry into the shoot. Important issues thus represent intrinsic factors, such as virulence or enzymatic abilities. One of the pitfalls of our study could be the effect of time, which we could not measure. Thus, the first genotype successfully entering a shoot could also quickly occupy entry spots in neighbouring petioles and prevent the infection of other genotypes. If so, this first genotype would have had to grow asymptomatically (Matsiakh et al., 2016; McKinney, Thomsen, Kjaer, & Nielsen, 2012) because most of the shoots appeared healthy immediately after leaf shedding, and only two shoots showed symptoms of the pathogen. It is known that the first lesions occur during late autumn and winter (Gross et al., 2014; Kirisits et al. 2009). Asymptomatic growth could also support our hypothesis that petioles can sometimes be entered by the pathogen from wood. A limited number of replicates, only 58 MLGs, were analysed because they reached the most proximal part of the petiole, which may also explain the non-significant result. Another factor that may affect spread through the petiole is competition from endophytic fungi that may be present in the petioles (Cross et al., 2016; Scholtysik, Unterseher, Otto, & Wirth, 2013). Evidence from *in vitro* experiments showed that particular fungi may limit the spread (Schulz, Haas, Junker, Andrée, & Schobert, 2015; Haňáčková, Havrdová, Černý, Zahradník, & Koukol, 2017) and even the germination of *H. fraxineus* (Schlegel, Dubach, von Buol, & Sieber, 2016). However, their protective effect *in vivo* remains unclear, and *H. fraxineus* appears to hold an advantage due to its early and immense sporulation pressure (Cross et al., 2016).

ACKNOWLEDGEMENTS

We would like to thank Liliya Fedusiv and Michal Samek for their help with sample isolation and Karel Černý for suggesting the idea of this study. We also thank Andrin Gross for helpful discussion and two anonymous reviewers for valuable comments. This study was supported by project NAZV QJ1220218 of the Ministry of Agriculture and by Institutional Support for Science and Research of the Ministry of Education, Youth and Sports of the Czech Republic.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article (https://doi.org/10.1111/efp.12370).

REFERENCES

Arnaud-Haond, S., & Belkhir, K. (2006). Genclone: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Molecular Ecology Notes, 7*, 15-17.

Baral, H-O., & Bemmann, M. (2014). *Hymenoscyphus fraxineus* vs. *Hymenoscyphus albidus* – A comparative light microscopic study on the causal agent of European ash dieback and related foliicolous, stroma-forming species. *Mycology, 5*, 228-290.

Bengtsson, S.B.K., Barklund, P., von Brömssen, C., & Stenlid, J. (2014). Seasonal pattern of lesion development in diseased *Fraxinus excelsior* infected by *Hymenoscyphus pseudoalbidus*. *PLoS ONE, 9*, e76429.

Brown, A. H., Feldman, M. W., & Nevo, E. (1980). Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics, 96*, 523-536.

Burokiene, D., Prospero, S., Jung, E., Marciulyniene, D., Moosbrugger, K., Norkute, G., Rigling, D., Lygis, V., Schoebel, C.N. (2015). Genetic population structure of the invasive ash dieback pathogen *Hymenoscyphus fraxineus* in its expanding range. *Biological Invasions, 17*, 2743-2756.

Cleary, M. R., Daniel, G., & Stenlid, J. (2013). Light and scanning electron microscopy studies of the early infection stages of *Hymenoscyphus pseudoalbidus* on *Fraxinus excelsior*. *Plant Pathology, 62*, 1294-1301.

Cleary, M. R., Arhipova, N., Gaitnieks, T., Stenlid, J., & Vasaitis, R. (2013). Natural infection of *Fraxinus excelsior* seeds by *Chalara fraxinea*. *Forest Pathology*, *43*, 83-85.

Cross, H., Sønstebø, J. H., Nagy, N. E., Timmermann, V., Solheim, H., Børja, I., Kauserud, H., Carlsen, T., Rzepka, B., Wasak, K., Vivian-Smith, A, Hietala, A. M. (2016). Fungal diversity and seasonal succession in ash leaves infected by the invasive ascomycete *Hymenoscyphus fraxineus*. *New Phytologist, 213*, 1405-1417.

Dighton, J. (2007). Nutrient cycling by saprotrophic fungi in terrestrial habitats. In C. P. Kubicek & I. S. Druzhinina (Eds.), *The Mycota IV. Environmental and Microbial Relationships* (pp. 287-300). Berlin, Germany: Springer.

Dvorak, M., Rotkova, G., & Botella, L. (2015). Detection of airborne inoculum of *Hymenoscyphus fraxineus* and *H. albidus* during seasonal fluctuations associated with absence of apothecia. *Forests, 7*, 1-13.

Fones, H. N., Mardon, C., & Gurr, S. J. (2016). A role for the asexual spores in infection of *Fraxinus excelsior* by the ash-dieback fungus *Hymenoscyphus fraxineus*. *Scientific Reports, 6*, 34638.

Gross, A., Grünig, C. R., Queloz, V., & Holdenrieder, O. (2012). A molecular toolkit for population genetic investigations of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. *Forest Pathology, 42*, 252-264.

Gross, A., Holdenrieder, O., Pautasso, M., Queloz, V., & Sieber, T. N. (2014). *Hymenoscyphus pseudoalbidus*, the causal agent of European ash dieback. *Molecular Plant Pathology, 15*, 5-21.

Gross, A., Zaffarano, P. L., Duo, A., & Grünig, C. R. (2012). Reproductive mode and life cycle of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. *Fungal Genetics and Biology, 49*, 977-986.

Haňáčková, Z., Havrdová, L., Černý, K., Zahradník, D., & Koukol, O. (2017). Fungal endophytes in ash shoots – diversity and inhibition of *Hymenoscyphus fraxineus. Baltic Forestry 2,*. 89-106.

Haňáčková, Z., Koukol, O., Havrdová, L., & Gross, A. (2015). Local population structure of *Hymenoscyphus fraxineus* surveyed by an enlarged set of microsatellite markers. *Forest Pathology, 45*, 400-407.

Hardy, O. J., & Vekemans, X. (2002). spagedi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes, 2*, 618-620.

Hayatgheibi, H. (2013). Studies on the microflora associated with the seeds of European ash (*Fraxinus excelsior*) and the infection biology of the pathogen *Hymenoscyphus pseudoalbidus* causing ash dieback. (Master thesis). Uppsala, Sweden: Swedish University of Agricultural Sciences.

Hietala, A. M., Timmermann, V., Børja, I., & Solheim, H. (2013). The invasive ash dieback pathogen *Hymenoscyphus pseudoalbidus* exerts maximal infection pressure prior to the onset of host leaf senescence. *Fungal Ecology, 6*, 302-308.

Husson, C., Caël, O, Grandjean, J. P., Nageleisen, L.M., & Marçais, B. (2012). Occurrence of *Hymenoscyphus pseudoalbidus* on infected ash logs. *Plant Pathology 61*, 889-895.

Kamvar, Z. N., Tabima, J. F., & Grunwald, N. J. (2014). Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ, 2,* e281.

Kirisits, T., Dämpfle, L., Kräutler, K., & Woodward, S. (2013). *Hymenoscyphus albidus* is not associated with an anamorphic stage and displays slower growth than *Hymenoscyphus pseudoalbidus* on agar media. *Forest Pathology, 43*, 386–389.

Kirisits,T., Matlakova, M., Mottinger-Kroupa, S., Cech, T.L., & Halmschlager, E. (2009). The current situation of ash dieback caused by *Chalara fraxinea* in Austria. In Doğmuş-Lehtijärvi T. (Ed.), *Proceedings of the conference of IUFRO Working Party 7.02.02* (pp. 97-119). Eğirdir, Turkey. May 2009. SDU Faculty of Forestry Journal, ISSN: 1302-7085, Serial: A, Special Issue.

Landolt, J., Gross, A., Holdenrieder, O., & Pautasso, M. (2016). Ash dieback due to *Hymenoscyphus fraxineus*: what can be learnt from evolutionary ecology? *Plant Pathology, 65*, 1056-1070.

Matsiakh, I., Solheim, H., Nagy, N. E., Hietala, A. M., & Kramarets, V. (2016). Tissue-specific DNA levels and hyphal growth patterns of *Hymenoscyphus fraxineus* in stems of naturally infected *Fraxinus excelsior* saplings. *Forest Pathology, 46*, 206-214.

McKinney, L. V., Nielsen, L. R., Hansen, J. K. & Kjær, E. D. (2011). Presence of natural genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease. *Heredity 106*, 788–797.

McKinney, L. V., Thomsen, I. M., Kjaer, E. D., & Nielsen, L. R. (2012). Genetic resistance to *Hymenoscyphus pseudoalbidus* limits fungal growth and symptom occurrence in *Fraxinus excelsior*. *Forest Pathology, 42*, 69-74.

Nei, M. (1987). *Molecular Evolutionary Genetics*. New York, NY: Columbia University Press.

Nguyen, D. T., Cleary, M. R., Enderle, R., Berlin, A., & Stenlid, J. (2016). Analyses of the ash dieback pathogen, *Hymenoscyphus fraxineus*, suggest role of tree species diversity on colonization and population structure differentiation. *Forest Pathology, 46*, 82-84.

Schlegel, M., Dubach, V., von Buol, L., & Sieber, T. N. (2016). Effects of endophytic fungi on the ash dieback pathogen. *FEMS Microbiology Ecology, 92*, fiw142.

Scholtysik, A., Unterseher, M., Otto, P., & Wirth, C. (2013). Spatio-temporal dynamics of endophyte diversity in the canopy of European ash (*Fraxinus excelsior*). *Mycological Progress, 12*, 291-304.

Schulz, B., Haas, S., Junker, C., Andrée, N., & Schobert, M. (2015). Fungal endophytes are involved in multiple balanced antagonisms. *Current Science, 109*, 39-45.

Schumacher, J., Kehr, R., & Leonhard, S. (2010). Mycological and histological investigations of *Fraxinus excelsior* nursery saplings naturally infected by *Chalara fraxinea*. *Forest Pathology, 40*, 419-429.

Schwanda, K., & Kirisits, T. (2016). Pathogenicity of *Hymenoscyphus fraxineus* towards leaves of three European ash species: *Fraxinus excelsior*, *F. angustifolia* and *F. ornus*. *Plant Pathology, 65*, 1071-1083.

Smith, J. M., Smith, N. H., O'Rourke, M., & Spratt, B G. (1993). How clonal are bacteria? *Proceedings of the National Academy of Sciences, 90*, 4384-4388.

R Development Core Team, (2014). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org/

Rice, W.R. (1989). Analyzing tables of statistical tests. *Evolution*, *43*, 223–225.

Wang, H., Qi, M., & Cutler, A. J. (1993). A simple method of preparing plant samples for PCR. *Nucleic Acids Research, 21*, 4153-4154.