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Advances in Ash Dieback Research,
- and Some Other Invasive Diseases of Trees

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Contents

Introduction Part 1: New Insights into Disease Development and Control of Ash Dieback

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Schulz, B. 2017. Introduction Part 1: New Insights into Disease Development and Control of Ash Dieback. *Baltic Forestry* 23(1):1-3.

Hymenoscyphus fraxineus, the causative agent of ash dieback, has been spreading from east to west across continental Europe and the British isles during the course of the last two decades. The pathogen was presumably introduced from Asia, where it colonizes *Fraxinus mandshurica* asymptomatically (Zhao et al. 2013). Proliferation of the disease is assumed to occur via ascospores, which develop on rachises of the previous year's leaves in early summer and infect young leaves (Gross et al. 2014). Molecular studies have shown that there is great molecular diversity of the individual strains of *H. fraxineus,* presumably due to its sexual mode of reproduction. Symptoms in *Fraxinus excelsior* include dieback of young shoots, necrotic lesions on the leaves and stems (Gross et al. 2014), and development of collar on the tree trunk (Langer 2017).

Whereas, sanitation measures can be useful in retarding disease progression in individual trees, especially in cities or parks (Thomsen, cited by McEwan 2015), there are presently no known measures for preventing disease due to *H. fraxineus* in forests or larger stands. Fungicides are effective against *H. fraxineus* (Hrabětová et al. 2016), but they obviously cannot be applied on a large scale, e.g. in forests. However, since individual trees of *F. excelsior* vary in their susceptibility to *H. fraxineus*, one approach to assure the survival of *F. excelsior* is to breed for relatively resistant phenotypes of the species (Enderle et al. 2015, Harper et al. 2016). Other potential approaches would employ biocontrol. For example, Schoebel et al. (2014) erudited the use of mycoviruses, which on the basis of their research suggested that they are not a promising option for controlling *H. fraxineus.* Initial experiments by Schlegel et al. (2016) are not optimistic regarding the use of endophytic fungi for biocontrol of ash dieback.

This special issue on *Hymenoscyphus fraxineus* presents results dealing with disease development and spread of *H. fraxineus* in species of *Fraxinus*, with physiology of the pathogen and with possible methods for controlling the disease.

Highlights of this issue

Langer (2017) found that *H. fraxineus* and *Neonectria punicea* are the primary pathogens responsible for emerging collar rot of *F. excelsior;* secondary pathogens include *Phytophthora and Armillaria* species. Using highly sensitive real-time PCR of symptomatic bark sampled during all four seasons, Matsiakh et al. (2017) showed that *H. fraxineus* is also the primary pathogen of bark lesions. One of the factors responsible for the success of this invasive pathogen may be its extreme phenotypic and molecular variability. Junker et al. (2017) found that each isolate of *H. fraxineus* has its own exoenzyme profile and that growth rates of the individual isolates also vary considerably. Stenlid et al. (2017) described the genomes of *H. fraxineus* and *H. albidus,* a native non-pathogenic sister species to *H. fraxineus,* showing that their genomes harbor similar and extensive Cell Wall Active Enzyme (CAZYme) repertoires and suggesting

that the prolonged saprotrophic growth phase on ash leaves of *H. fraxineus* and *H. albidus* has probably shaped their genomes.

Natural infection of *Fraxinus angustifolia* was found in Slovakia by Kádasi-Horáková et al. (2017) and of *F. excelsior* and *F. angustifolia* in Serbia by Keča et al. (2017). Results by Kirisits et al. (2017) demonstrated the relative resistance of *Fraxinus ornus* to *H. fraxineus.* In contrast, in Spain, Trapiello et al. (2017) found neither *H. fraxineus* nor other *Hymenoscyphus* species among the mycobiota of leaves of diseased *F. excelsior*. In New Zealand, where *F. excelsior* is an introduced species and *H. fraxineus* is not present, Power et al. (2017) showed that it has retained many of its native endophytes.

Other authors addressed potential biocontrol options for *H. fraxineus.* The option of using leaf litter of *Tilia* to accelerate decomposition of *F. excelsior* leaf litter was explored by Bartha et al. (2017). Most of the endophytic fungi isolated from healthy stems of *F. excelsior* inhibited growth of *H. fraxineus* in dual culture and thus may have biocontrol potential (Haňáčková et al. 2017). The group also showed that those with a presumed saprotrophic role *in planta* increased in the summer, those with a pathogenic role in the winter.

Čermáková et al. (2017) investigated mycoviruses in *H. fraxineus,* finding mycovirus HfMV1 and other putative double-stranded RNA mycoviruses in the isolates from central Europe.

And finally, Mitchell et al. (2017) investigated which tree or trees could assume the ecological functions of *Fraxinus excelsior* for organisms that in some way depend on it to complete their life-cycles, concluding that no one tree can substitute for the presumed future loss of *F. excelsior.*

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Collar Rots in Forests of Northwest Germany Affected by Ash Dieback

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Langer, G. 2017. Collar Rots in Forests of Northwest Germany Affected by Ash Dieback. *Baltic Forestry* 23(1): 4-19.

Abstract

The formation of collar rots in association with ash dieback was studied under different site conditions. The fungal community associated with lesions, necroses and stem collar rots, especially the occurrence of *Hymenoscyphus fraxineus* at these symptomatic plant tissues, was investigated. Filamentous fungi and *Phytophthora* spp. were isolated from affected tissues of stem collar rots of various developmental stages. Tissue samples of collar rots were collected from 32 ash trees in seven different forest plots located in Northwest Germany. Obtained isolates were assigned to morphotypes and identified based on mycelial morphology and by molecular methods. Primary agents causing collar rots were identified and the influence of site conditions was derived. The studied stem collar rots were assigned to five symptomatic categories: (0) without collar rots or lesions, (1) emerging collar rots, (2) larger collar rots without visible wood decay, (3) advanced collar rots with visible wood decay and (4) collar rots, necroses or lesions associated with dark sap oozing. In most of the studied collar rots that were collected in Schleswig-Holstein and Lower Saxony, *H. fraxineus* was isolated and assumed to be the primary agent. From samples of category 0 neither *H. fraxineus* nor other fungi or *Phytophthora* species were isolated. From collar rots of the other symptomatic categories, varying quantities of endophytic, saprotrophic and pathogenic species had been isolated. Overall, the number of isolated species was higher in advanced stages of collar rot. Most common species were *H. fraxineus* and *Neonectria punicea*, followed by *Diaporthe eres*, *Botryosphaeria stevensii*, *Gibberella* sp., *Fusarium solani* and *Cadophora* sp. However, collar rots in early stages were only associated with *H. fraxineus* and *N. punicea*. *Armillaria* or *Phytophthora* species were only isolated from advanced collar rots or occurred under special side conditions.

Keywords: Ash dieback, *Fraxineus excelsior*, Collar rot, Wood decay, *Hymenoscyphus fraxineus*, *Neonectria punicea*, *Armillaria*, *Phytophthora*, Endophytes, Plant pathogens, Fungi

Introduction

Meanwhile, ash dieback caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya is present in all parts of Germany where common ash (*Fraxinus excelsior* L.) is growing. Since 2009 a dramatic increase in the number of infested stands and a severe disease progression has been obvious (Langer 2017). Main disease symptoms were necroses on leaves and twigs, which lead to crown dieback after several years of multiple infections (e.g. Bakys et al. 2009, Kirisits et al. 2009, Skovsgaard et al. 2010, Gross et al. 2014, Langer et al. 2015a). Tree mortality is increasing in older age classes and often connected to stem collar necroses associated e.g. with *H. fraxineus* or *Armillaria* root rot (Metzler 2012, Langer 2015b, Langer 2017). Disease progression and mortality in pole and timber size trees were intensified by increasing numbers of collar rots (Metzler and Herbstritt 2014). *H. fraxineus* is able to colonize the bases of ash stems and to infect stem tissues (Husson et al. 2012) and causes, in conjunction with secondary fungi, basal lesions, necrosis and collar rots (Lygis et al. 2005, Skovsgaard et al. 2010, Bakys et al. 2011, Husson et al. 2012, Enderle et al. 2013, Langer et al. 2015b). Collar rots or necroses associated with ash dieback had been monitored in Germany (e.g. Metzler 2012, Langer 2017) as well as in other European countries (e.g. Bakys et al. 2011, Chandelier 2015, Marçais et al. 2016, Muñoz et al. 2016). Of 60 adult ash dieback-diseased trees (92-148 years old) that were monitored in Schleswig-Holstein, 82 % exhibited collar rot in the year 2012 (Langer et al. 2015a). Chandelier (2015) reported that only 41 % of studied necroses at the collar base of diseased ash trees were infected

by *Armillaria* spp., while most of them (98 %) were associated with *H. fraxineus*.

Collar rots seem to be typical after-effects of ash dieback and are common in young ash as well as in older ash. They can occur on trees with or without crown symptoms of ash dieback (Enderle et al. 2013, Muñoz et al. 2016). Muñoz et al. (2016) showed that susceptibility towards collar rots seems to be genetically determined. Collar rots may be primarily induced by *H. fraxineus* itself, opportunistic wood decaying fungi or other pathogens (Bakys et al. 2011, Langer 2017, Metzler 2012). For example, in a 16-years-old ash stand in Lower Saxony, 96% of trees were infected by ash dieback and 6.8 % of the trees exhibited collar rot. These collar rots and tissue lesions were partly associated with *H. fraxineus* and other fungi, such as *Neonectria punicea* (J.C. Schmidt) Castl. et Rossman, *Fusarium solani* (Mart.) Sacc., and *Botryosphaeria stevensii* Shoemaker, but *Armillaria* species were not observed in the diseased tissues (Langer et al. 2015a, Langer 2017). In contrast, *Armillaria* spp. were assigned to be primary causal agents of collar rot in diseased trees, e.g. *Armillaria gallica* Marxm. et Romagn. in Germany (Metzler and Herbstritt 2014) and in Denmark (Skovsgaard et al. 2010), *A. cepistipes* Velen. in Lithuania (Bakys et al. 2011) and *A. gallica* and *A. cepistipes* in Belgium (Chandelier 2015). The association of *Phytophthora* species with root and collar rots of mature ash trees (*F. excelsior*) were reported from Poland and Denmark (Orlikowski et al. 2011). Collar rots seem to play a major role in disease progression and imperil the tree stability. Therefore, salvage cuttings have been necessary in Northwest Germany since 2009 (Langer 2017).

The German temperate seasonal climate is influenced by the oceanic western and the continental eastern European climate. A distinct climatic gradient from western to eastern parts exists, with more continental conditions and decreasing precipitation in the east. The northwestern and northern parts of Germany exhibit an oceanic climate with mild winters and warm summers. Northwest Germany, comprising the federal states Schleswig-Holstein (SH), Lower Saxony (LS), Saxony-Anhalt (ST) and Hesse (HE), is mainly characterized by lowland, which was essentially shaped by the Saale und Weichsel glacial periods. The latter led to spatially highly differentiated site conditions. The climate is euatlantic at the coasts and atlantic to subatlantic from the Northern parts of Lower Saxony and Schleswig-Holstein to the low mountain range bordering the area in the South. The potential natural zonal vegetation is characterized by beech forests (Fagion sylvaticae, Bohn and Neuhäusel 2000/2003). The Northwest German Highlands comprise areas of the low mountain range located north of the Main River. In the year 2012, 2.4% of the total forest area in Germany was covered by *F. excelsior* (Enderle et al. 2017).

To elucidate the formation of collar rots associated with diseased ash trees, different development stages of lesions, necroses and complex damaged tissues with wood rot were studied in stands with different site conditions. The presented study aimed to identify the primary invaders causing collar rot, and the influence of site conditions on the formation of collar rots and necroses. The main aim of the study was to characterize the fungal community associated with stem collar necroses of ash trees that were diseased by ash dieback, especially the occurrence of *H. fraxineu*s at these symptomatic plant tissues.

Materials and methods

Forest stands

In total, seven different forest stands with ash trees (*F. excelsior*) were studied. The studied forest plots were located in three German federal states (SH, LS, HE). Except the forest stand in LS, which was a north-eastwards gently inclined slope in the foothill zone, all studied forest plots were flat and located in the planar zone. In all studied stands, *F. excelsior* was the dominant tree species, except Kuehkopf-Knoblochaue (HE), which was a mixed broad-leaved riparian forest dominated by *Fagus sylvatica* and *F. excelsior*. Ash dieback in SH was first observed in 2002 and laboratory-confirmed in 2005, in LS in 2006 and in HE in 2008. The presence of collar rots in the studied forest plots were recognized in different years: plots 1-4 in Satrup (SH): 2012, plot 5 in Nehmten (SH): 2007, plot 6 in Kuehkopf-Knoblochaue (HE): 2014 and plot 7 in Stroit (LS): 2007.

Studied material

In total, stem collars of 32 ash trees that were diseased by ash dieback were sampled, most of them in SH at forest plots 1 and 2. Of interest were mainly ashes with characteristic stem collar rots or necroses and moderate ash dieback symptoms in the crowns. In addition, two ash trees without distinctly obvious stem collar rots or necroses, which were diseased by ash dieback, were sampled at forest plot 2 (trees No. 17 and 21) and investigated. Information about studied samples (i.e. *F. excelsior* trees) and locations and site conditions is listed below:

No. 1-8: 25 years old, forest plot 1, SH, Mohrkirch, 54°40'17"N, 09°41'20"E, eutrophic and weak changing moist to stagnant fresh site, leg. U. Harriehausen and I. Krischock, 06.02.2013.

No. 9-20: 21 year old, forest plot 2, SH, Boeklund, 54°36'30"N, 09°36'15"E, well mesotrophic and weak changing moist to stagnant fresh site, leg. U. Harriehausen and I. Krischock, 06.02.2013.

No. 21-22: 20 years old, forest plot 2, SH, Boeklund, 54°36'30"N, 09°36'15"E, eutrophic and fresh to stock fresh site, leg. U. Harriehausen, 04.01.2013.

No. 23-24: 20 years old, forest plot 3, SH, Obdrup, 54°41'32"N, 09°33'18"E, well mesotrophic and fresh to stock fresh site leg. U. Harriehausen, 04.01.2013.

No. 25: 65 years old, forest plot 4, SH, Obdrup, 54°41'25"N, 09°33'12"E, eutrophic and weak changing moist to stagnant fresh site, leg. U. Harriehausen, 04.01.2013.

No. 26-27: 20 years old, forest plot 5, SH, Nehmten, 54°05'15"N, 10°23'11"E, well mesotrophic and and fresh to stock fresh site, leg. I. Krischock, 07.02.2013.

No. 28-29: 15-20 years old, forest plot 6, HE, Kuehkopf-Knoblochaue, 49°50'15"N, 08°23'54"E, eutrophic and moist, alluvial site, leg. A. Noltensmeier, 03.06.2014.

No. 30-32: 15-20 years old, forest plot 7, LS, Stroit, 51°53'52"N, 09°51'42"E, eutrophic and fresh site, leg. P. Gawehn and M. Pfeffer, 21.01.2015.

Sampling Method

Trees were felled with a chainsaw. The affected stem base of each tree was collected and washed with running water. Stem parts with collar lesions, necroses and rots were dissected in longitudinal and cross-sections and the bark was removed. All stages of preparation were documented by photography.

Classification of Stem collar rots

The studied stem collar rots were assigned to the following symptomatic categories: (0) necrosis or collar rots not distinctly visible on the tree surface; no wood discolouration due to stem collar rot (Figure 1), (1) emerging collar rots, collar necroses or lesions distinctly visible on the tree surface but small, only several cm^2 in diameter; wood discolouration only visible as a small dark brownish, fan-like pattern in a cross section of the stem (Figure 2), (2) large collar rots, necroses and bark lesions (a single or several meshing necroses), were visible on the stem base surface; wood discolourations were visible as dark brownish, larger fan-like patterns and/ or other discolorations in a cross section of the stem (Figure 3), (3) advanced collar rots with very large necroses and bark lesions (a single or several meshing necroses); partly large portions of the wood and the root collar were discolourated and wood rot was visible (Figure 4), and (4) one or several collar necroses or lesions distinctly visible on the tree surface and associated with dark sap oozing from the margin of the necrosis; browning of the outer layer of the sapwood; wood discolourations were mainly peripheral, in cross-sections not visible as dark brownish fan-like patterns but diffuse or with greenish line of demarcation (Figure 5).

Agar media

Four different agar media (MEA. MYP, PDA, CJ) were used for the isolation in the detailed study of forest plots 1 and 2, while MYP was standard medium for all samples and CJ was standard medium for the isolation of *Phytophthora*.

Malt extract agar (MEA), modified according to Langer (1994): 20 g malt extract (Merck 1.05391.0500), 15 g agar (Fluka 05040-1KG), 1000 ml Aqua dest.

Malt yeast pepton (MYP), modified according to Langer (1994): 7 g malt extract (Merck 1.05391.0500), 0.5 g yeast extract (Fluka 70161-100G), 1 g pepton (Merck 1.07272.0500), 15 g agar (Fluka 05040-1KG), 1000 ml Aqua dest.

Potato dextrose agar (PDA): 39 g PDA FLUKA (70139-500G), 1000 ml Aqua dest.

Carrot juice agar (CJ): modified according to Kröber (1985): 50 ml cold pressed carrot juice, 18 g agar (Fluka 05040-1KG), 1000 ml Aqua dest.

Isolation of filamentous fungi and Phytophthora

For the isolation of fungi and *Phytophthora*, tissue pieces (wood chips) were extracted from discoloured areas in the wood. Per studied tree sample, 16 to 188 tissue pieces (77 tissue pieces on average) were extracted with sterile tools under sterile conditions. The number of tissue samples depended on the size of the collar rots and necroses. Tissue pieces were incubated on different agar media at room temperature (ca. 22°C) and daylight. Usually, three wood chips were placed on an agar medium in a 90 mm Petri dish. The Petri dishes were checked visually for developing colonies over a period of four weeks. Emerging mycelia were sub-cultured separately on MYP. Representative isolated fungal strains were kept in MYP slants at 4°C.

Identification of isolated organisms

Isolated strains were assigned to mycelial morphotypes (MT, Table 1) and identified by mircomorphological characters or based on DNA sequence similarities. For the identification of fungi, a ZEISS Axiostar plus microscope was used and standard procedures for fungi described in Lee and Langer (2012) were followed. In addition to standard literature recommended by Oertel (2003) for determination of fungi and forest diseases, the following literature was used: e.g. Booth (1971), Gerlach and Nirenberg (1982), Butin (2011), von Arx (1981) and Domsch et al. (1980). The names of fungal species follow Index Fungorum (www.indexfungorum.org) and Mycobank (www.mycobank.org).

If possible, at least one representative strain of each morphotype was used for molecular identification, involving DNA extraction from mycelium, polymerase chain reaction (PCR) amplification of ribosomal DNA, and DNA

Figure 1 Collar rot category 0: ash tree 17, a) stem base, b) stump surface, c) longitudinal-section of stem base, with numbered isolation loci, d) cross-section of the stem base without distinctly visible wood discolouration due to collar rots, e) central discolouration with numbered isolation loci, e) *Neonectria punicea*, isolated from tree 3, cultured three weeks on MYP

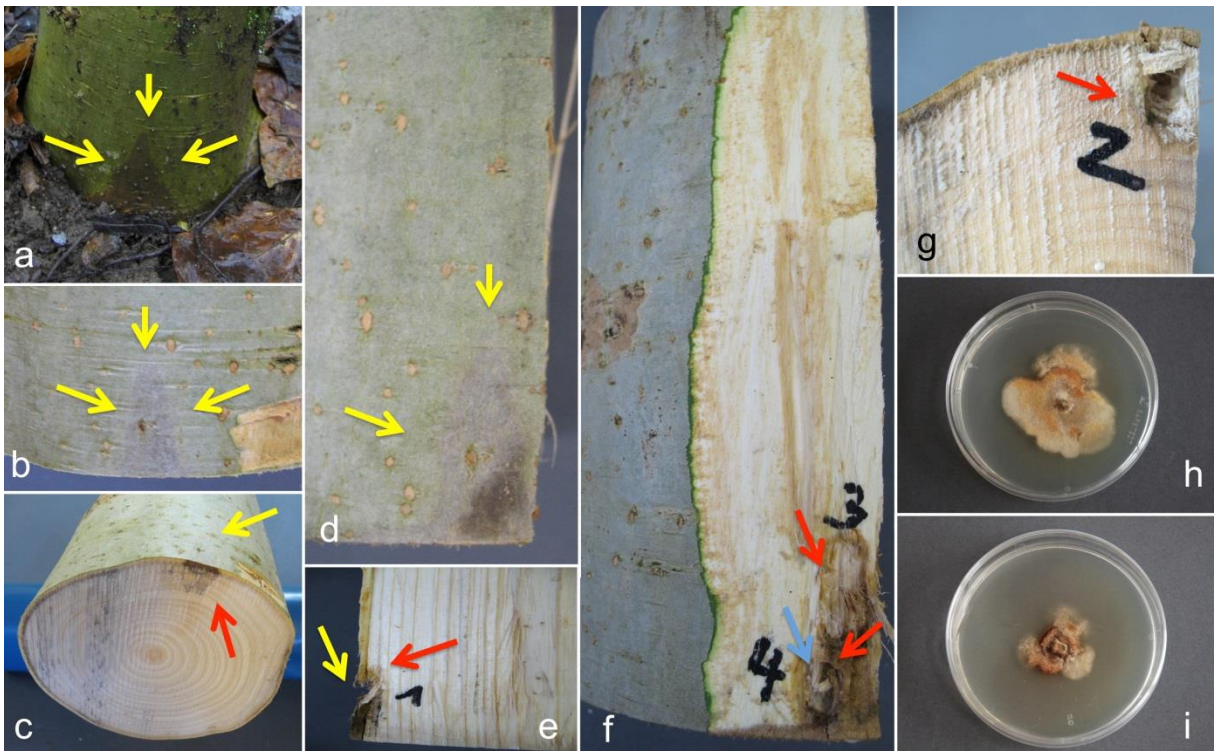

Figure 2 Collar rot category 1: Ash tree 18, a) stem base of the living tree, b) stem surface with collar rot after washing, c) cross-section of the stem base with distinctly visible wood discolouration due to the collar rot d) part of stem base with distinctly visible wood discolouration due to collar rot, e) longitudinal-section of stem base, with numbered isolation locus, f) stem surface with removed bark and visible wood discolouration due to the collar rot, g) cross-section of the stem base with distinctly visible wood discolouration due to collar rot, h, i) *Hymenoscyphus faxineus*, cultured three weeks on MYP, h) isolated from tree 2 and i) isolated from tree 4. Yellow arrows indicate the collar rot; red arrows indicate isolation loci of *H. fraxineus*; blue arrow indicates an isolation locus of *Neonectria punicea*

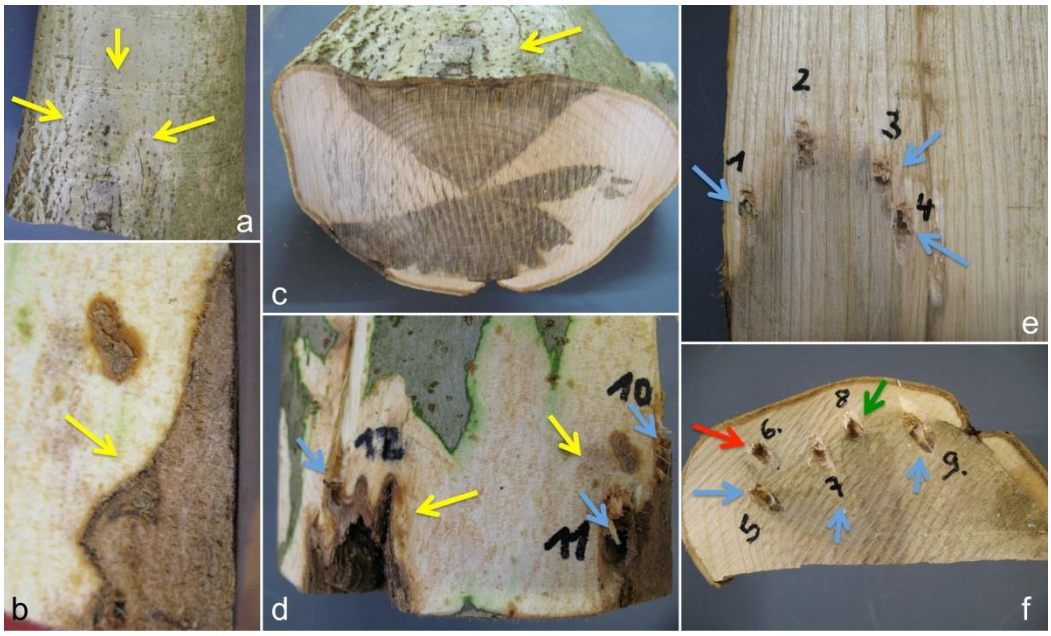

Figure 3 Collar rot category 2: Ash tree 13, a) stem base after washing b) part of a stem collar with removed bark and visible wood discolouration due to the collar rot, c) cross-section of the stem base with distinctly visible, fan-like wood discolourations due to the collar rots d) part of the stem base with removed bark and visible wood discolouration and lesions due to the collar rots, e) longitudinal-section of stem base, with numbered isolation loci, f) cross-section of stem base halve with distinctly visible wood discolouration due to collar rot. Yellow arrows indicate the collar rot; red arrows indicate an isolation locus of *H. fraxineus*; blue arrows indicate isolation loci of *Neonectria punicea;* green arrow indicates the isolation locus of *Cadophora sp*.

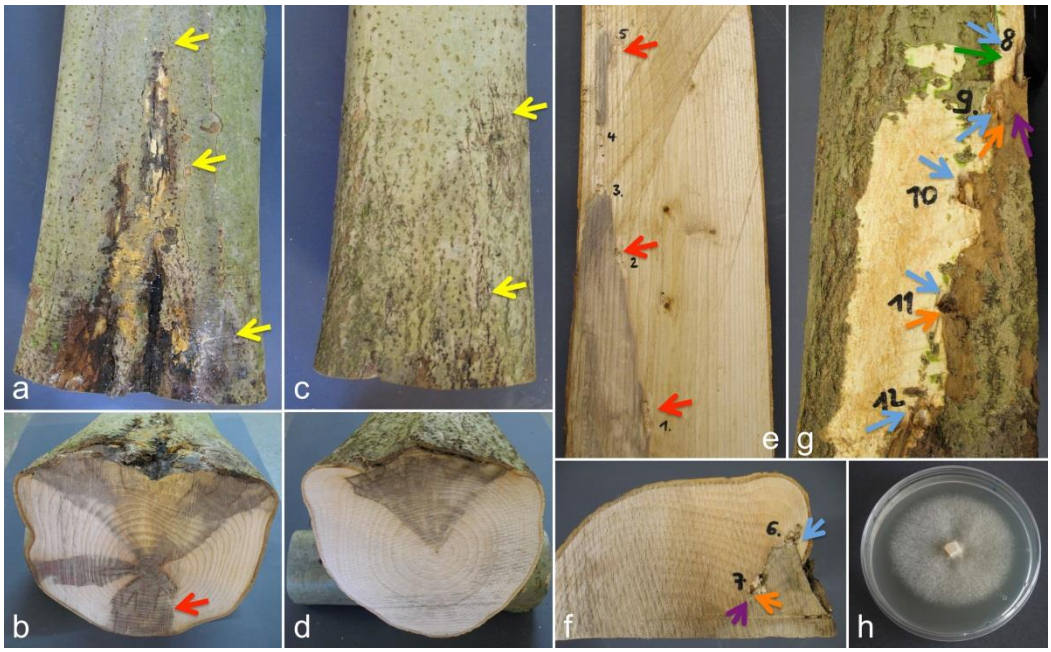

Figure 4 Collar rot category 3: a-b) Ash tree 2, a) stem base after washing, b) cross-section of stem base with four, distinctly visible, fan-like wood discolourations due to collar rots; c-g) ash tree 5, c) stem base after washing d) cross-section of stem base with distinctly visible, fan-like wood discolouration due to collar rot, e) longitudinal-section of stem base, f) cross-section of stem parts with distinctly visible, fan-like wood discolouration due to collar rot g) stem surface with removed bark and visible wood discolouration due to the collar rot, h) *Flammulina velutipes*, (Ac. no: KU712226). Yellow arrows indicate the collar rot; red arrows indicate isolation loci of *H. fraxineus*; blue arrows indicate isolation loci of *Neonectria punicea*, green arrow indicates the isolation locus of *F. velutipes*, orange arrows indicate isolation loci of *Diaporthe eres*, violet arrows indicate isolation loci of *Botryosphaeria stevensii*

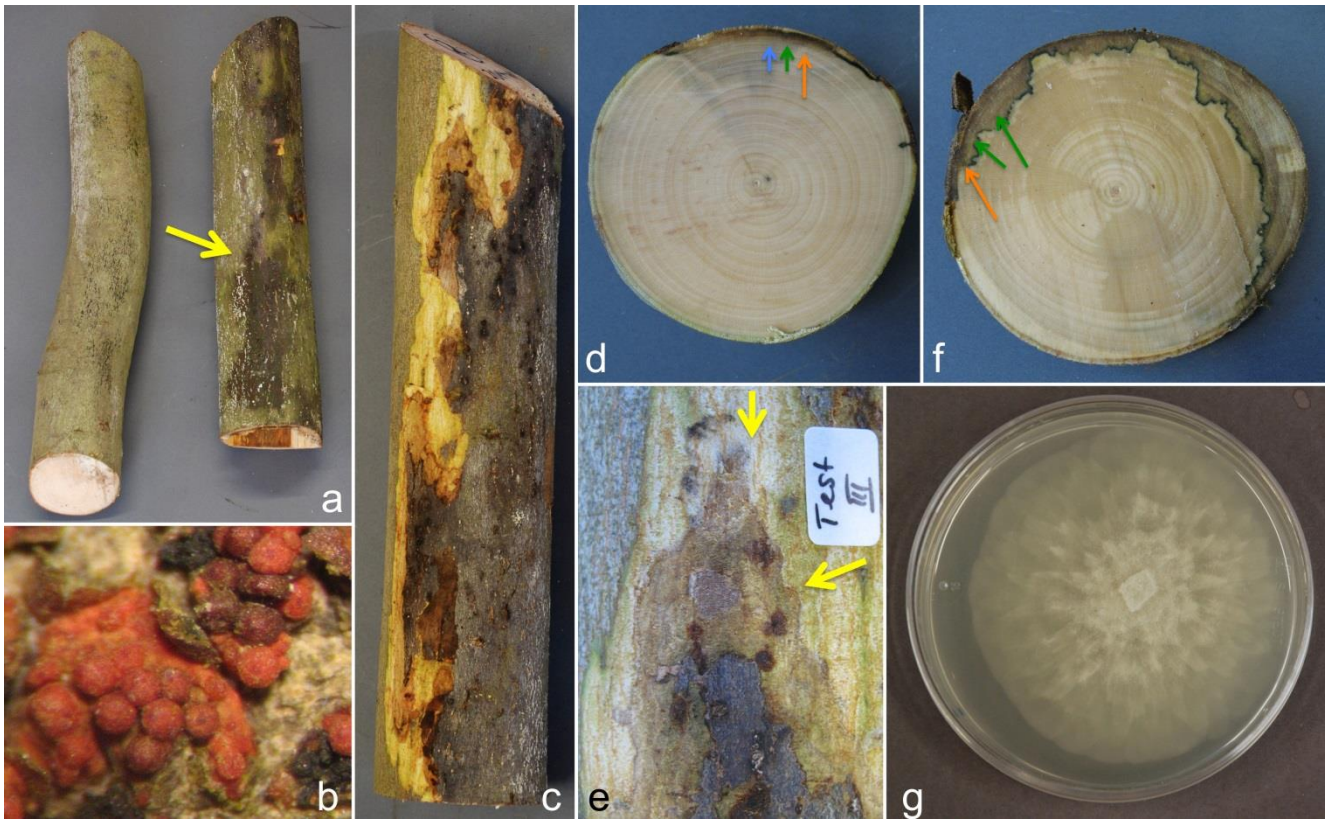

Figure 5 Collar rot category 4: a) left: sample 28, right: sample 29. Figure b, c, f, e) sample 29: b) *Neonectria punicea*, ascocarps, c) collar rot with oozing sap and partly removed bark, e) brownish necrosis due to the *Phytophthora*-infection, and f) cross-section of stem base with wood discolouration and greenish line of demarcation. Figure d, g) sample 28: d) a cross-section of stem base with diffuse wood discolourations due the *Phytophthora*-infection and g) *P. plurivora*. Yellow arrows indicate the collar rot; blue arrow indicates isolation locus of *N. punicea;* green arrows indicate the isolation loci of *Gibberella sp.*, orange arrows indicate isolation loci of *Fusarium solani*

sequencing. The databases at GenBank (http://www.ncbi.nlm.nih.gov/genbank/, Altschul et al. 1997) were used to define the identity of sequences. Intraspecific ITS similarity for the sequenced fungi of 98-100% was used at species level.

To proof *H. fraxineus*, isolated suspicious strains were transferred to MYP medium. The mycelia were microscoped during the following days, in order to provide evidence of the typical conidiophores and conidia of *H. fraxineus*.

Molecular methods

DNA isolation, PCR and sequencing were performed by the laboratory of Prof. E. Langer, University Kassel by order and for account of the NW-FVA. From each morphotype, 1-2 mg culture tissue was suspended in 100 µl TE buffer in a 1.5 ml-tube. A microwave (600 W) was used twice for 1 min each, including a pause of 30 s, to break up cells. Tubes were cooled to -20°C for 20 min. and then centrifuged at 10000 rpm for 5 min. A 100 times diluted portion of the supernatant was used for PCR. Primer pairs for amplification of ITS1, 5.8S and ITS2 region were ITS1F/ITS4 or ITS1/ITS4 (Gardes and Bruns 1993, White et al. 1990). The PCR was performed with 45 µl Master mix from QIAGEN, Hilden, Germany and 5 µl of extracted DNA. PCR was carried out with the primer pairs with initial denaturation at 94°C for 3 min, followed by 29 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 60 s. Final elongation was performed at 72°C for 7 min. PCR products were separated on 1% agarose gel stained with GelRed fluorescence dye (Biotium, Hayward, CA, USA) followed by a cleaning with QIAquick PCR Purification Kit (QIAGEN). Sanger sequencing of purified products (Sanger et al. 1977) was commissioned at GATC Biotech (Cologne, Germany). Editing of DNA sequences and alignment were performed with MEGA6 (Tamura et al. 2013) followed by submission to GenBank (Table 1).

Results

Stem collar rots

Two of the 32 studied trees had no stem collar rots while 12.5% of trees showed stem collar rots of the symptomatic category 1, 37.5% exhibited stem collar rots of the symptomatic category 2, 37.5% showed stem collar rots of the symptomatic category 3, and 6.25% revealed stem collar rots of the symptomatic category 4. Stem collar rots of the symptomatic category 4 were only found at the moist riparian forest plot 6.

35 morphotypes and a few additional, nonrecurring taxa, which were not obtained as culture or not identified further, were isolated from the necroses and collar rots (Table 1). *H. fraxineus* (MT 35) was obtained on all tested agar media. Usually, anamorph-stages of this fungus were observed within one or two days after transfer of mycelia that was growing from wood chips in the original Petri dishes to MYP agar.

From collar rots of different symptomatic categories, varying numbers of species associated with wood discolouration, rot and lesions were isolated (Table 2). In the studied tissues of ash stems without distinctly visible necroses (category 0, Figure 1), neither *H. fraxineus* nor other fungi or *Phytophthora* species could be isolated. In the studied emerging collar rots (category 1), only *H. fraxineus* and / or *Neonectria punicea* had been detected and no wood decaying fungi nor *Phytophthora* species were present. In more advanced collar necrosis (category 2), *Botryosphaeria stevensi*, *Fusarium solani* (MT 4), *Gibberella* sp. (MT 5) and *Cadophora* sp. (MT 6) were species additional to *H. fraxineus* and *N. punicea*, which had been isolated at least in two of the studied trees. Advanced collar necroses or rots of category 3 were often associated with *B. stevensii*, *Diaporthe eres* Nitschke (MT 3), *Gibberella sp.* (MT 5), the ascomycete MT 16 and *H. fraxineus* and *N. punicea*. The collar necroses of category 4 were caused by *Phytophthora plurivora* T. Jung & T.I. Burgess and only a few additional secondary pathogens like *N. punicea*, *F. solani* or *Gibberella* sp. were isolated.

Frequency of isolated organisms

Most frequent species were *N. punicea* (MT 2, isolated from 77.4 % of 31 studied trees) and *H. fraxineus* (MT 35, 77.4 %) followed by *D. eres* (MT 3, 29 %), *B. stevensii* (MT 1, 25.8 %), *Fusarium solani* (MT 4, 22.8 %), *Gibberella* sp. (MT 5, 22.8 %), *Trichoderma* sp. (MT 27, 19.3 %) and *Cadophora* sp. (MT 6, 16.1 %). Other obtained species were only isolated from single up to 4 trees: *Diaporthe* sp. (MT 3.1), *Phomopsis* sp. (MT 3.2), *Xylaria polymorpha* (Pers.) Grev. (MT 7), *Flammulina velutipes* (Curtis) Singer (MT 8), Basidiomycete sp. (MTs 9 and 22) *Trichothecium roseum* (Pers.) Link (MT 10), *Ascocoryne* sp. (MT 14), *Alternaria* sp. (MT 20), Ascomycete sp. (MTs 13, 17, 24, 25, and 28), *Lophiostoma corticola* (Fuckel) E.C.Y. Liew, Aptroot & K.D. Hyde (MT 29), *Mortierella* sp. (MT 30), *Eurotium* sp. (MT 31), *Coprinellus micaceus* (Bull.) Vilgalys, Hopple & Jacq. Johnson (MT 32), and *Armillaria* sp. (MT 33) all with a frequency of 3.2 %; *Neofabraea* sp. (MT 12), Ascomycete sp. MT 19, *Ilyonectria* sp. (MT 23), *Epicoccum nigrum* Link (MT 26), and *Phytophthora plurivora* (MT 36) with a frequency of 6.5 %; *Valsa cypri* (Tul.) Tul. & C. Tul. (MT 11) with a frequency of 9.7 % and Ascomycete sp. (MT 16) with a frequency of 12.9 %.

Discussion and conclusions

The presented study was the first research on ash dieback and stem collar rots in north-western Germany. A large number of young and adult ash trees that were diseased with ash dieback and associated with collar rots were observed. In most of the studied collar rots and necroses (67%, n = 30) that were collected in Schleswig-Holstein and Lower Saxony, *H. fraxineus* was isolated and assumed to be the first colonizer in most studied tissues. This assumption was motivated by the results that: 1) In the studied tissues of ash stems with emerging collar rots (category 1), only *H. fraxineus* and / or *N. punicea* had been detected, but wood decaying fungi, typical secondary fungi or *Phytophthora* species were not present. *N. punicea* and *H. fraxineus* were the most frequently isolated species. On average, the more advanced the collar rot was, the more species had colonized the tissue. 2) It was proven for *H. fraxineus* to cause symptomatic necroses of ash bark and cambium (Bakys et al. 2009) on stems and occasionally also on roots or root collars (Gross et al., 2014). Several studies confirmed a high pathogenicity of *H. fraxineus* towards *F. excelsior* (Bakys et al. 2009, Kowalski & Holdenrieder 2009, Husson et al. 2011, Kräutler et al. 2015, Gross and Sieber 2016, Kowalski et al. 2017). Moreover, it was proven that *H. fraxineus* is able to infect intact shoots of ash (Kräutler et al. 2015). 3) *Neonectria* species are known to be endophytes (Ceccarelli 2010, Sieber 2007). Nevertheless, they are also able to cause necroses and initialize complex diseases as secondary plant pathogens (Bressem 2002, Sieber 2007, Hirooka et al. 2013).

Hence, *H. fraxineus* is supposed to be the first colonizer of collar tissues, in which only a few other fungal species (typical secondary pathogens or endophytes like *N. punicea*) had been detected and in which no wood rotting fungi were present (categories 1-2). The hypothesis that the collar lesions and rots of ash trees affected by ash dieback are often caused primarily by *H. fraxineus* is supported by the presented results, Langer et al. (2015b and 2017) and by Chandelier (2015).

N. punicea, which is a species with *Cylindrocarpon* Wollenw. anamorphs, was the most common species

Table 1 Isolated Morphotypes and NCBI GenBank Blasting information for isolated strains and the closest blast matches (Frequency = Frequency of occurrence in % of studied 31 trees; A = anamorph)

MT	Frequency [%	Forest plot	Tree	Taxon name	NCBI GenBank BLAST check 05./08.02.2015					
					GenBank accession No.	GenBank taxon	Sequence ID	[%] ITS similarity	[%] Query coverage	Source
1	25.8	1	6	*Botryosphaeria stevensii,* A: *Diplodia mutila*	KU712211	*Diplodia mutila*	KF766158	99	92	Slippers et al. (2013)
2	77.4	2	14	*Neonectria punicea,* A: *Cylindrocarpon album,* syn. *Neonectria confusa*	KU712212	*Neonectria punicea*	HM534901	99	99	Jacklitsch and Voglmayr (2011)
		1	5		KU712213	*N. punicea*	Jf268768	99	100	Zhao et al. (2011)
						N. confusa	KM515889	99	98	Lombrad et al. (unpublished)
						Neonectria sp.	JF268760	100	99	Luo and Zhuang (2010)
						Neonectria sp.	FJ560437	100	99	
3	29.0	1	5	*Diaporthe* eres	KU712214	*Diaporthe eres*	EU571099	100	97	Kacergius and Jovaisiene (unp.)
		1	13		KU712215	*D. cotoneastri*	KC843328	100	100	Udayanga et al. (2014)
						D. eres	JF430493	100	99	Petrovic et al. (unp.)
		5	27		KU712216	*D. cotoneastri*	KC843328	100	97	Udayanga et al. 2014
						D. eres	JF430493	100	97	Petrovic et al. (unp.)
		5	26		KU712250	*Diaporthe cotoneastri*	KC145903	100	99	Johnston and Park (unp.)
						D. eres	JF430493	99	99	Petrovic et al. (unp.)
3.1	3.2	2	9	*Diaporthe* sp. MT 3.1	KU712217	*Diaporthe viticola*	KC145904	99	99	Johnston and Park (unp.)
						D. viticola	KC145906	100	96	
3.2	3.2	5	26	*Phomopsis sp.* MT 3.2	KU712245	*Phomopsis columnaris*	KC145883	99	99	Johnston and Park (unp.)
						Phomopsis sp.	KF428571	100	92	Bonito et al. (unp.)
4	22.6	1	4	*Fusarium solani*	KU712218	*Fusarium solani*	FJ478128	99	97	Jiang et al. (unp.)
		5	26		KU712219	*F. solani*	FJ478128.	100	99	
5	22.6	5	10	*Gibberella* sp. MT 5	KU712220	*Fusarium lateritium*	AF310980	100	100	Schuett et al. (unp.)
6	16.1	1	1	*Cadophora* sp. MT 6	KU712221	*Cadophora luteo-olivacea*	HM116747	99	100	Johnston et al. (2010)
		2	13		KU712222	*Cadophora* sp.	HM116752	100	100	Johnston et al. (unp.)
						C. malorum	KT358982	93	100	Diaz et al. (unp.)
		2	15		KU712223	*C. luteo-olivacea*	HM116747	99	99	Johnston et al. (2010)
7	3.2	1	2	*Xylaria polymorpha*	KU712224	*Xylaria polymorpha*	KF897015	99	89	Ma and Lei (unp.)
						X. polymorpha	GU322460	98	93	Hsieh et al. (2010)
		1	2		KU712228	*X. polymorpha*	KF897015	99	89	Ma and Lei (unp.)

Table 1. (Continued)

MT	Frequency [%	Forest plot	Tree	Taxon name	NCBI GenBank BLAST check 05./08.02.2015					
					GenBank accession No.	GenBank taxon	Sequence ID	[%] ITS similarity	[%] Query coverage	Source
8	3.2	1	5	*Flammulina velutipes*	KU712226	*Flammulina velutipes* var. *longispora*	AF051700	99	96	Hughes et al. (unp.)
						F. velutipes	KM668876	99	93	Senik et al. (2015)
9	3.2	1	7	Basidiomycete MT 9, not further identified , no ITS product						
10	3.2	1	2	*Trichothecium roseum,* no ITS product						
11	9.7	1	8	*Valsa cypri* A: *Cytospora pruinosa*	KU712230	*Valsa cypri*	KT004557	99	89	Kowalski et al. (unp.)
						Cytospora pruinosa	EU552121	95	99	Marincowitz et. al. (2008)
		2	10		KU712235	*V. cypri*	KT004557	99	88	Kowalski et al. (unp.)
						Cytospora pruinosa	EU552121	95	100	Marincowitz et. al. (2008)
12	6.5	2	15	*Neofabraea* sp. MT 12	KU712232	*Neofabraea malicorticis*	AF141189	99	98	Abeln et. al. (2000)
						N. inaequalis	KR859081	99	98	Chen et. al. (2015)
						N. alba	KJ396074	99	100	Cameldi et al. (unp.)
		2	15		KU712233	*N. malicorticis*	AF141189.	99	99	Abeln et. al. (2000)
			12		KU712234	*N. malicorticis*	AF141189	99	99	
13	3.2	1	8	Ascomycet sp. MT 13, not amplified						
14	3.2	2	10	*Ascocoryne sp.* MT 14	KU712235	*Ascocoryne sarcoides*	GQ411510	100	99	Fukami et. al. (2010)
15	3.2	2	15	Basidiomycete MT 21	KU712239	*Phlebia* sp.	KP135360	100	93	Floudas and Hibbett (2015)
		2	15		KU712238					
16	12.9	1	7	Ascomycete MT 16	KU712236	Helotiales sp.	GU934595	99	99	Bakys et al. (2011)
						Neobulgaria sp.	KR072504	88	100	Arhipova et al. (2011)
						Neobulgaria pura	HM051080	88	98	Wu et al. (unp.)
		2	27		KU712237	Helotiales sp.	GU934595	99	100	Bakys et al. (2011):
						N. pura	HM051080	89	98	Wu et al. (unp.)
17	3.2	2	12	Ascomycete MT 17	KU712240	*Phaeomollisia piceae*	LN714584	99	96	Vetrovsky et al. (unp.)
						Phialocephala sp.	FJ903362	99	88	Arhipova et al. (2011)
						Phialoceph. fortinii	AB671499	94	99	Kiyuna et al. (2012)
19	6.5	2	20	Ascomycete sp. MT 19, *Sclerostagono-spora cycadis*	KU712246	*Sclerostagonospora cycadis*	KR611890	99	99	Crous et al. (2015)
20	3.2	2	12	*Alternaria* sp., not amplified						

Table 1. (Continued)

MT	Frequency [%	Forest plot	Tree	Taxon name	NCBI GenBank BLAST check 05./08.02.2015					
					GenBank accession No.	GenBank taxon	Sequence ID	[%] ITS similarity	[%] Query coverage	Source
22	3.2	5	27	Basidiomycete MT 22, *Psathyrella panaeoloides*	KU712241	*Psathyrella panaeoloides*	KC992894	99	97	Larsson and Oerstadius (unp.)
23	6.5	5	27	*Ilyonectria* sp. MT 23	KU712242	*Ilyonectria robusta*	JF735264	100	99	Groenewald et al. (2012)
		5	26		KU712243	*I. europaea*	JF735294	100	99	
						I. robusta	JF735264	99	98	
24	3.2	7	32	Ascomycete sp. MT 24, not amplified						
25	3.2	7	32	Ascomycete sp. MT 25, not amplified						
26	6.5	2	19	*Epicoccum nigrum,* not amplified						
27	19.3			*Trichoderma* sp. MT 27, not obtained in culture, not amplified						
28	3.2	1	1	Ascomycet sp. MT 28	KU712244	*Sydowia polyspora*	KF993419	100	93	Garzoli (unp.)
						Pyrenochaeta acicola	KT309815	100	88	Johnston and Park (unp.)
29	3.2	2	10	*Lophiostoma corticola*	KU712227	*Lophiostoma corticola*	KT004559	100	95	Kowalski et al. (unp.)
30	3.2	5	27	*Mortierella* sp. MT 30	KU712228	*Mortierella verticillata*	KF944471	100	96	Zhao (unp.)
31	3.2	7	31	*Eurotium sp.* MT 31	KU712229	*Eurotium* sp.	KF367488	100	100	Oliveira et. al. (2015)
32	3.2	1	6	*Coprinellus micaceus*	KU712252	*Coprinellus micaceus*	EU436684	99	100	Miles et al. (2012)
33	3.2	5	26	*Armillaria* sp. MT 33	KU712248	*Armillaria gallica*	KP960530	99	99	Tizzani et al. (unp.)
						A. cepistipes	AB510862	99	99	Hasegawa et al. (2010)
					KU712247	*A. cepistipes*	GU934598	99	100	Bakys et al. (2011)
						A. bulbosa	EU784165	99	100	Brock et al. (2012)
						A. gallica	KP162313	99	100	Guo (unp.)
35	71	1	2	*Hymenoscyphus fraxineus*	KU712251	*Chalara fraxinea*	GU797159	100	99	Rytkonen et al. (2011)
36	6.5	6	28	*Phytophthora plurivora*, two strains, identified by Marco Thines, Frankfurt via sequencing analysis						

associated with the studied collar necroses, except for *H. fraxineus*. It was isolated at all sampled forest plots, in 77.4 % of the collar rots and even if *H. fraxineus* was not found in the studied tissues. The native species *N. punicea* is distributed in Europe (Austria, France, Germany, Scotland, Slovakia and Switzerland), in North America (United States), and in Asia (China, Japan). So far, ascocarps were found on dead woody substrates of *Acer macrophyllum*, *Acer sp.*, *Frangula alnus*, *Fagus grandifolia*, *F. sylvatica*, *Prunus* × *yedoensis*, *Quercus crispula*, *Rhamnus fallax*, *Rhamnus* sp., and *Ulmus* sp. (Hirooka et al. 2013). Until now, *N. punicea* was not rated as wood-inhabiting fungus in stems of *F. excelsior* (Lygis et al. 2005) and was only mentioned by Langer et al. (2015b) and Langer (2017) as species associated with stem collar rots in north-western Germany. *Neonectria* spp. have not been found to be associated with visually healthy shoots, necrotic shoots (Bakys et al. 2009) or with rotting roots (Bakys et al. 2011) of ash trees that were diseased by ash dieback in Lithuania. Moreover, *Neonectria* spp. have

Table 2. Isolated Organisms from stem collar rots (n = 32 studied *F. excelsior* trees)

Collar rot category	Forest plot	Sample / tree	*H. fraxi-neus*	*Armillaria*	*Phy-tophthora*	Other fungi (Morphotypes)	Wood-decaying species	Species in total	Species on average
0	1	17	-	-	-	-	0	0	0
0	2	21	-	-	-	-	0	0	
1	2	11	-	-	-	2	0	1	1.8
1	2	14	+	-	-	2	0	2	
1	2	18	+	-	-	2	0	2	
1	3	24	+	-	-	2	0	2	
2	1	1	+	-	-	2, 6, 28	0	4	5.1
2	1	3	+	-	-	2	0	2	
2	1	4	+	-	-	1, 2, 4	0	4	
2	2	9	+	-	-	2, 3.1	0	3	
2	2	12[1]	+	-	-	6, 12, 17, 19, 20, 1X	0	7	
2	2	13	+	-	-	2, 6	0	3	
2	2	14	+	-	-	2, 6, 11, 3X	0	7	
2	2	20	+	-	-	1, 2, 19, 27	0	5	
2	3	23	+	-	-	2, 5, 26, 27, 3X	0	8	
2	2	22	+	-	-	2, 5, 3X	0	6	
2	7	30	+	-	-	4, 4X	0	6	
2	7	31	-	-	-	1, 3, 4, 31, 2X	0	6	
3	1	2	+	-	-	2, 7, 10	1	4	6.6
3	1	5	+	-	-	1, 2, 3, 8, 27	1	6	
3	1	6[2]	+	-	-	1, 2, 3, 27, 32	1	6	
3	1	7	-	-	-	1, 2, 3, 9, 16, 1X	1	7	
3	1	8[2]	-	-	-	1, 2, 3, 11, 13, 16	0	6	
3	2	19	+	-	-	2, 5, 16, 26, 27	0	6	
3	2	10	+	-	-	2, 3, 5, 11,14, 29	1	7	
3	2	15	+	-	-	2, 3, 5, 6, 12, 15, 2X	1	9	
3	4	25	+	+	-	na	1	na	
3	5	26	-	+	-	1, 2, 3, 3.2, 23, 27, 1X	1	8	
3	5	27	-	-	-	3, 4, 16, 22, 23, 30	2	6	
3	7	32	+	-	-	2[3], 4, 24, 25, 2X	0	7	
4	6	28	-	-	+[5]	2, 4, 5	0	4	3,5
4	6	29[2,4]	-	-	+[5]	4, 5	0	3	

+ = isolated; - = not isolated / detected; X = species unidentified or strains not obtained in pure culture; na = not applicable, or not tested; [1]) Stem base with an additional central discoloration of the wood beside the collar necrosis; [2]) stem collar had a secondary infestation with bark beetles; [3]) Ascocarps and anamorph-stages were visible on the tree surface; [4]) anamorph stages were visible on the tree surface; [5]) MT 36 = *Phytophthora plurivora* T.

not been detected as foliar endophytes of *F. excelsior* in a floodplain forest in eastern Germany (Scholtysik et al. 2013) nor as endophyte of *F. excelsior* in New Zealand (Chen 2011).

In collar rots of advanced developmental stages that were not colonized by *H. fraxineus*, mainly wood decaying fungi (e. g. *X. polymorpha, F. velutipes, or Armillaria* sp.), *Ilyonetria* sp. or *Phytophthora* species have been suggested to be the causing agents. The infection with *Phytophthora* was considered to be a primary attack, because species of this genus can actively infect healthy plant tissue. *P. plurivora*, isolated from trees in Hesse, is an aggressive soil-borne plant pathogen, with worldwide distribution and a wide host range of tree species (Jung and Burgess 2009).

Except *P. plurivora* and *Armillaria* sp., most of the isolated fungi were assumed to be secondary colonizers of ash tissue necroses and were less frequent than *H. fraxineus* and *N. punicea*. Common species on dying stems and twigs of ash, such as *Cytospora pruinosa* (Fr.) Sacc. (teleomorph *Valsa cypri*), *Diaporthe eres*, *Diplodia mutila* (Fr.) Mont. (teleomorph *B. stevensii*), *Fusarium avenaceum* (Fr.) Sacc., *F. lateritium* Nees and *F. solani* (Kowalski et al. 2017) were frequently isolated, but only in advanced collar necroses or rots (categories 2-4).

The third common isolated species was *D. eres* with a frequency of 25.8%. Kowalski et al. (2017) suggested that this plant pathogen has significantly lower capacity than *H. fraxineus, B. stevensii* or *V. cypri* to cause necroses on *F. excelsior*. *D. eres* was often reported as common endophyte in symptomless stems and twigs of *F. excelsior*, but is considered as a weak pathogen (Kowalski et al. 2017). *D. eres* is known from more than 60 host species, e.g. *Fraxinus* in the Netherlands, in Scotland, and in Germany (Gomes et al. 2013). It is synonymizied with *Phomopsis cotoneastri* Punith. and its current name is *P. velata* (Sacc.) Traverso according to Index fungorum. Other *Phomopsis*-like species (MT 3.1 and 3.2) were associated with samples of category 1 necroses. *Diaporthe viticola* Nitschke, which is the closest blast match for the isolated MT 3.1, was isolated of initial necroses from shoots in declining ash (Bakys et al. 2009). It is known to colonize various hosts, especially grapevine, in which it causes the cane spot disease (Gomes et al. 2013). *Phomopsis* spp. were consistently found in healthy and necrotic shoots of declining ash in Lithuania (Bakys et al. 2009).

B. stevensii was isolated from the necrotic tissues of 25.8% of 31 studied trees (categories 1-3). According to studies of (Kowalski et al. 2017), *B. stevensii* was the second most pathogenic fungus, after *H. fraxineus*. It was one of the most frequently isolated species from healthy and necrotic ash shoots (Bakys et al. 2009), but not detected in rotting ash roots (Bakys et al. 2011). Furthermore, it was frequently associated with dieback and canker diseases of oak, but also occurred on several other deciduous or coniferous trees (Correia et al. 2004).

F. solani and *Gibberella* sp. were both isolated from the studied necrotic tissues (categories 2-4) with a frequency of 22.6%. *F. solani*, *F. avenaceum* and *F. latericium* were common species on dying stems and twigs of ash in Poland (Kowalski et al. 2017). However, in other studies, *F. solani* and *F. avenaceum* were not obtained from the root system of declining *F. excelsior* (Bakys et al. 2011), from healthy or necrotic shoots (Bakys et al. 2009) or from healthy leaves of ash (Scholtysik et al. 2013). *F. solani* and strains similar to MT 5 were demonstrated to be secondary pathogens on *Acer* (Langer et al. 2013). *F. latericium*, however, which is the closest blast match for the isolated *Gibberella* species (MT 5), was isolated in initial and advanced necroses from shoots in declining ash (Bakys et al. 2009) and as a foliar endophyte in ash (Scholtysik et al. 2013). *F. avenaceum* and *F. lateritium* are known as common endophytes in symptomless stems and twigs of *F. excelsior*. The intensity of induced necroses caused by *F. avenaceum* was low and those caused by *F. solani* or *F. lateritium* were statistically similar to the control (Kowalski et al. 2017).

Valsa cypri and *Neofabraea* sp. were associated with few samples of necroses of categories 2-3. *Valsa* Fr. species are linked to *Cytospora* Ehrenb. Fr.-anamorphs have a world-wide distribution and are saprobes or pathogens on various woody hosts (Spielman 1985). *V. cypri* is growing on Oleaceae, e.g. *Fraxinus* sp. (Spielman 1985). It was also isolated from visually healthy ash shoots (Bakys et al. 2009). According to Kowalski et al. (2017), *V. cypri* is the tested third most pathogenic species after *H. fraxineus*, to cause necrotic lesions on ash. Species of *Neofabraea* H.S. Jacks. are known to belong to the fungal community of *F. excelsior* (Chen 2011), but had not been isolated from healthy or necrotic ash tissues by Bakys et al. (2009, 2011). *Neofabraea vagabunda* (Desm.) Rossman is causing coin canker of ash (*Fraxinus* spp.) in North America (Putnam and Adams 2005).

Sporadically, various ascomycetes, e.g. *Cadophora* sp, *Epicoccum nigrum*, *Ascocoryne* sp., *Lophiostoma corticola* (Fuckel) E.C.Y. Liew, Aptroot & K.D. Hyde and *Ilyonectria* sp., were isolated from advanced collar rots. A species of the plant pathogenic genus *Cadophora* was found in roots of declining *F. excelsior*, too (Bakys et al. 2011). *E. nigrum* was among the most frequently isolated fungi from healthy and necrotic shoots of declining ash (Bakys et al. 2009) and healthy ash leaves (Scholtysik et al. 2013). *L. corticola* was also isolated from healthy ash shoots in New Zealand (Chen et al. 2011). *Ilyonectria radicicola* (Gerlach & L. Nilsson) P. Chaverri & Salgado was obtained from root rots of ash trees that were diseased by dieback (Bakys et al. 2011) and was among the most common isolated species in that study.

Moreover, wood decaying fungi, such as the ascomycetes *Xylaria polymorpha*, *Ascocoryne sp.* and MT 16 and some basidiomycetes, e.g. *Armillaria sp*., *Flammulina velutipes*, *Coprinellus micaceus*, were obtained from advanced rots only. *Xylaria* species are belonging to the endophytic fungal community of *F. excelsior* (Chen 2011, Scholtysik et al. 2013) and were among the most common isolated species in the studied rotten ash roots in Lithuania (Bakys et al. 2011). *Armillaria* species were very often associated with root and collar rots of dieback diseased trees, as mentioned before (e.g. Bakys et al. 2011). Usually, as opportunistic pathogens, *A. gallica* and *A. cepistipes* are able to attack stressed trees (e.g. Entry et al. 1986, Lygis et al. 2005, Skovsgaard et al. 2010) and to cause white rot. In general, *A. cepistipes* has a pronounced saprophytic life cycle (Wahlstroem 1992), but it was apparently the most pathogenic of all tested fungi obtained from rotting ash roots in Lithuania (Bakys et al. 2011). *F. velutipes*, which causes white rot, is a typical stump-rotting basidiomycete and frequently occurs as a secondary damaging agent. *Coprinellus disseminatus* (Pers.) J.E. Lange and other white rotting basidiomycetes, such as *Phanerochaete* P. Karst. or *Pholiota* (Fr.) P. Kumm. species, were isolated by Bakys et al. from decaying ash roots (2011) and necrotic shoots (2009).

Summing up, collar necroses and collar rots are typical but not obligate after-effects of ash dieback. They can be caused by opportunistic wood decaying fungi on diseased trees or by primarily causing agents like *Phytophthora* species or the pathogen of ash dieback itself. In more advanced necroses and rots (categories 2-3), several species were isolated in addition to the causing agent *H. fraxineus*, such as typical bark and wood-decaying or wood-inhabiting fungi, which are known to belong to the fungal community of ash, e.g. *A. cepistipes, Coprinus disseminatus, Phlebia sp., Psathyrella candolleana* (Fr.) Maire, *Alternaria alternata* (Fr.) Keissl., *Ascocoryne sarcoides* (Jack.) Grov. & Wils., *B. stevensii, Diaporthe sp., E. nigrum, F. latericium, Gibberella avenacea* R. J. Cook, *Lophiostoma* sp., *Phialophora* species, *Phomopsis* sp., and *Xylaria* ssp. (Lygis et al. 2005, Chen 2011) or leaf-inhabiting endophytic fungi of *F. excelsior*, e.g. *Alternaria sp., E. nigrum* Link, *F. lateritium, L. corticola, Phomopsis* sp. and *Xylaria* sp. (Scholtysik et al. 2013).

Collar necroses on ash at different sites are caused by various pathogens, e.g. *H. fraxineus* in Schleswig-Holstein and Lower Saxony (ibid), *Armillaria gallica* in Germany (Baden-Wuerttemberg (Metzler and Herbstritt 2014) and in Denmark (Skovsgaard et al. 2010) and *A. gallica, A. cepistipes* and *Phytophthora* in Belgium (Chandelier 2015). An association of *Phytophthora* species with root and collar rots of mature ash trees, as documented in Hesse, was also described for Denmark and Poland, where the pathogen led to a decline of *F. excelsior* (Orlikowski et al. 2011). The studied stem collar necroses from Hesse were not associated with *H. fraxineus*. These affected trees grew in a floodplain forest, a riparian life zone of back water of the River Rhine with eutrophic, moist soils. The forest stands in north-western Germany, where *Armillaria* was isolated from collar rots in diseased ash trees, are characterized by eutrophic to well mesotrophic and weak changing moist to stagnant fresh or fresh to stock fresh soils. The association of collar rots with *Armillaria* spp. is not obligatory linked with an attack by *H. fraxineus* to the stem collar. Like other wood decaying fungi, *Armillaria* spp. can be soil-borne, opportunistic pathogens on ash trees that are weakened (e.g. by ash dieback).

For *H. fraxineus, A. alternata, B. stevensii, D. eres, E. nigrum, Phomopsis* sp. and *V. cypri*, the ability to cause symptomatic necroses in bark and cambium was proven (Bakys et al. 2009, Kowalski et al. 2017). It is also known that *A. cepistipes* can cause discolorations in functional sapwood of ash (Bakys et al. 2011). It remains unknown, if *N. punicea* itself is able to primarily cause wood discolourations or stem collar necroses. Further studies seem to be necessary to solve this problem.

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Assessment of Seasonal Patterns in Tissue-specific Occurrence of *Hymenoscyphus fraxineus* in Stems of *Fraxinus excelsior*

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Abstract

Dieback of European ash (*Fraxinus excelsior* L.), a disease caused by the ascomycete *Hymenoscyphus fraxineus* (previously referred to as *H. pseudoalbidus* or *Chalara fraxinea*), was first observed in Poland in the early 1990ies, and is currently present almost throughout the entire distribution area of European ash. The characteristic symptoms of the disease include dead shoots with necrotic lesions in the bark and discoloration of xylem and pith but the seasonal dynamics of pathogen spread in shoot tissues remain poorly understood. To investigate whether the internal spread of the fungus involves season-specific patterns, saplings with necrotic bark lesions in 1-2 -year-old stem regions were collected during 2014-2015 at time intervals in spring, summer, autumn and winter at several localities in western Ukraine and at two localities in south-eastern Norway. Tissue-specific presence of *H. fraxineus* was determined by a highly sensitive quantitative real-time PCR assay that is specific to DNA of *H. fraxineus*. The relatively high proportion of bark samples positive for *H. fraxineus* in the saplings collected during spring provides support to a model that *H. fraxineus* can be a primary causative agent of bark lesions and that other fungi may eventually replace it in old infection areas.

Key words: ash dieback, *Hymenoscyphus fraxineus*, bark, phloem, pith, qPCR (Real-Time PCR).

Introduction

The virulent fungal pathogen, *Hymenoscyphus fraxineus*, causing 'chalara ash dieback,' disease of ash trees in northern and central Europe has been reported from countries across Europe: Czech Republic, Denmark, Finland, Germany, Slovakia, Austria, Hungary, Poland, Romania, Slovenia, Switzerland, France, Estonia, United Kingdom, Ukraine and Norway (Zubrik and Kunca 2007, Halmschlager and Kirisits 2008, Jankovsky and Holdenrieder 2009, Szabo 2009, Drenkhan and Hanso 2009, Engesser et al. 2009, Ioos et al. 2009, Kowalski and Holdenrieder 2009, Davydenko et al. 2011, Matsiakh and Kramarets 2014, Timmermann et al. 2014).

According to the current model, shoot infection by *H. fraxineus* is caused by fungal mycelium that originates from ascospores germinating on the leaf surfaces. Following the infection of leaf tissues, the mycelium spreads through the petiole into shoots and twigs, and causes characteristic bark lesions and crown dieback (Gross et al. 2014, list some other references also). Within the stem, it is proposed that further colonization and infection occurs by an axial and radial expansion through the pith and the sapwood (Schumacher et al. 2010). The authors proposed that this leads to eventual necrotization of cambium and bark and this in turn triggers the development of many secondary fungi that are commonly associated with necrotic bark lesions of ash. In our recent study, we combined histologi-

cal observations with a highly sensitive qPCR assay specific to *H. fraxineus* DNA, and profiled stem tissues associated with regions with necrotic bark lesions and adjacent asymptomatic tissues in naturally infected saplings of *F. excelsior*: outside bark lesion areas pathogen DNA was detected in pith and sapwood, but not in the radially corresponding bark, and pathogen growth fronts were characterized by abundant presence of fungal mycelium in the starch-rich perimedullary zone of the pith during the summer season (Matsiakh et al. 2016).

The present work was designed to increase our understanding of seasonal growth dynamics of the fungus in shoot tissues. For this purpose, *H. fraxineus* specific qPCR assay was used to profile the presence of pathogen DNA in pith, xylem, phloem and outer bark in naturally infected stems of ash saplings that were collected from Ukraine and Norway at different seasons.

Materials and Methods

Experimental material

The experimental material originated from forest stands in continental climate in Ukraine and Norway and was collected at time intervals during 2014-2015. Stems of living 5-10 -year-old saplings of European ash with necrotic lesions in 1-2 -year-old stem regions were collected at vil. Nyvytsi (Lviv region) and vil. Ishkivtsi (Ternipol region) in western Ukraine and at Norderås, Ås (Akershus) and Årungen, Ås (Akershus) in south-eastern Norway.

Sampling

Up to 1-m-long stem segments enclosing regions with necrotic bark lesions, and up to 10 cm healthy bark on both sides of the lesions, were cut from a total of 21 saplings sampled throughout the year and transported to laboratory. The vertical orientation of the stem piece was marked. In laboratory, the stem lesions were split longitudinally with a knife and the split lesion halves were scanned with a photocopy machine to obtain an image of tissue discoloration. A total of 55 five-mm-long stem segments were sampled in areas with necrotic bark and a total of 131 such stem segments were sampled in adjacent areas with healthy bark. The position of each stem segment sampled was marked in the scanned image. These stem segments were further divided into radially corresponding tissue samples and outer bark, inner bark (i.e. phloem), xylem and pith samples were separately subjected to DNA isolation.

DNA extraction and real-time PCR

All tissue samples were weighed (up to 30 mg fresh weight) and placed in a 2 mL Eppendorf tube with a lid reinforced with glue. The samples were then pulverised with the aid of liquid Nitrogen and steel beads in a Retsch mill (max speed, 1.5 min). DNA isolation was performed with DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The real-time PCR detection of *H. fraxineus* DNA was performed using Takyon Low Rox Probe MasterMix dTTP Blue w/o UNG for probe Assay Low ROX (Eurogentec, Seraing, Belgium), and the forward primer Cfrax-F 5′-ATTATATTGTTGCTTTAGCAGGTC-3, reverse primer Cfrax-R 5′-TCCTCTAGCAGGCACAGTC-3′ and probe C-frax-P5′-FAM- CTCTGGGCGTCGGCCTCG-BHQ1-3′ designed and tested for species specificity by Ioos et al. (2009). The primer and probe concentrations were 300 nM and 100 nM, respectively (Ioos et al. 2009).

Pathogen DNA amount standard curves with known DNA concentrations were prepared from fungal pure cultures (Hietala et al. 2013). For both the experimental and standard curve samples, 3 µl of the DNA solution was used as the template for each 20-µl PCR reaction. Each singleplex reaction was repeated twice. PCR cycling parameters were 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 65°C for 25 s (target DNA) or 60°C for 60 s (reference DNA). Fluorescence emissions were detected with an Applied Biosystems ViiA™ 7Systems (Applied Biosystems, Foster City, CA, USA). Standard curves were constructed based on the relationship of cycle threshold (Ct) values and known DNA concentrations: the Ct values were plotted against log-transformed DNA amounts, and linear regression equations were calculated for the quantification of DNA pools by interpolation in unknown samples.

Statistical analyses

Sampling time and tissue type –specific differences in the presence of *H. fraxineus* DNA were tested by ANOVA and Tukey HSD post hoc tests in SPSS 22.0 (IBM Inc., Armonk, NY, USA), and considered statistically significant at $P < 0.05$.

Results

No significant differences were observed in the occurrence of *H. fraxineus* between the Norwegian and Ukrainian shoot material in the different tissue types, neither within a bark lesion area nor outside this region. Therefore, the material was pooled together for subsequent statistical analyses. Of the stem segments analysed from regions with necrotic bark lesions, 27, 80, 89 and 84% were positive for *H. fraxineus* DNA in the outer bark, phloem, sapwood and pith, respectively (Table 1). Regarding stem segments analysed from regions from adjacent regions with healthy bark, 22, 31, 43 and 48% were positive for *H. fraxineus* DNA in the outer bark, phloem, sapwood and pith, respectively (Table 1). Except for outer bark (p 0.35), the differences in the tissue-type specific occurrence of *H.fraxineus* DNA between regions with necrotic bark and regions with healthy bark were statistically highly signifi-

Table 1. Presence of *Hymenoscyphus fraxineus* DNA in different stem tissues of naturally infected *Fraxinus excelsior* saplings during the seasons (specified as n, number of trees or stems with positive detections of the pathogen and quantified as percentage % of total trees analysed; ns, not sampled). Samples were taken either in a stem region with a necrotic bark lesion or in adjacent regions with healthy bark. Samples collected in spring are from the study of Matsiakh et al. (2016)

		Within bark lesion area					Outside bark lesion area				
Season	Sapling	Total	Outer bark	Inner bark	Xylem	Pith	Total	Outer bark	Inner bark	Xylem	Pith
Spring	NOR1	3	ns	2	3	3	5	Ns	0	0	1
	NOR2	2	ns	2	2	2	2	Ns	2	2	2
	NOR3	2	ns	2	2	2	2	Ns	1	0	0
	NOR4	4	ns	4	4	4	3	Ns	1	0	1
	NOR5	0	ns	ns	ns	ns	8	Ns	1	4	5
	NOR6	3	ns	3	3	3	13	Ns	6	6	8
	UKR1	4	ns	4	3	4	8	Ns	2	2	4
	UKR2	4	ns	3	4	3	6	Ns	2	2	1
	UKR3	2	ns	2	2	2	8	ns	3	2	3
	UKR4	4	ns	4	4	4	4	ns	0	2	1
	Sum (n)	28		26	27	27	59		18	20	26
	Sum (%)	100		93	96	96	100		31	34	44
Summer	NOR7	2	2	2	2	2	7	2	2	2	5
	NOR8	1	1	1	1	1	9	8	2	6	3
	UKR5	4	2	1	2	4	3	0	1	1	2
	UKR6	6	2	3	6	3	4	0	1	0	1
	UKR7	2	0	1	1	1	6	3	1	3	2
	UKR8	1	1	1	1	0	9	4	3	8	6
	Sum (n)	16	8	9	13	11	38	17	10	20	19
	Sum (%)	100	50	56	81	69	100	45	26	53	50
Autumn	NOR9	3	1	3	2	2	5	0	1	3	2
	UKR9	0					9	6	6	6	6
	UKR10	4	4	4	4	4	4	2	4	3	3
	Sum (n)	7	5	7	6	6	18	8	11	12	11
	Sum (%)	100	71	100	86	86	100	44	61	67	61
Winter	NOR10	2	1	1	2	2	9	2	0	5	5
	UKR11	2	1	1	1	0	7	2	1	0	2
	Sum (n)	4	2	2	3	2	16	4	1	5	7
	Sum (%)	100	50	50	75	50	100	25	6	31	44
All seasons	Sum (n)	55	15	44	49	46	131	29	40	57	63
	Sum (%)		27	80	89	84		22	31	44	48

cant ($p < 0.01$). To obtain a sufficient number of replicates for considering the significance of seasonal differences, the samples collected from autumn and winter were pooled together. No tissue type showed significant seasonal changes in the presence of *H. fraxineus* DNA, neither within a bark lesion area nor outside this region (Fig. 1). Within a bark lesion area, the greatest differences in the occurrence of *H. fraxineus* DNA were observed between spring and summer for inner bark (p 0.09) and pith (p 0.08).

Discussion

To explore seasonal growth dynamics of *Hymenoscyphus fraxineus* in shoots of European ash, we used *H. fraxineus* DNA – specific qPCR to assess tissue type - specific presence of the pathogen in stems of naturally infected saplings of European ash that had been collected from Ukraine and Norway during spring, summer, autumn and winter. According to the current model that is based on tissue microscopy and pathogen DNA profiling, *H. fraxineus* spreads axially in shoot tissues by growing in the starch-rich cells in the perimedullary pith and in xylem, while the necrotic bark lesions arise in association with radial spread of pathogen mycelia to phloem through the rays that bridge pith and xylem with inner bark (Schumacher et al. 2010, Matsiakh et al. 2016). The now recorded generally higher occurrence of *H. fraxineus* in pith

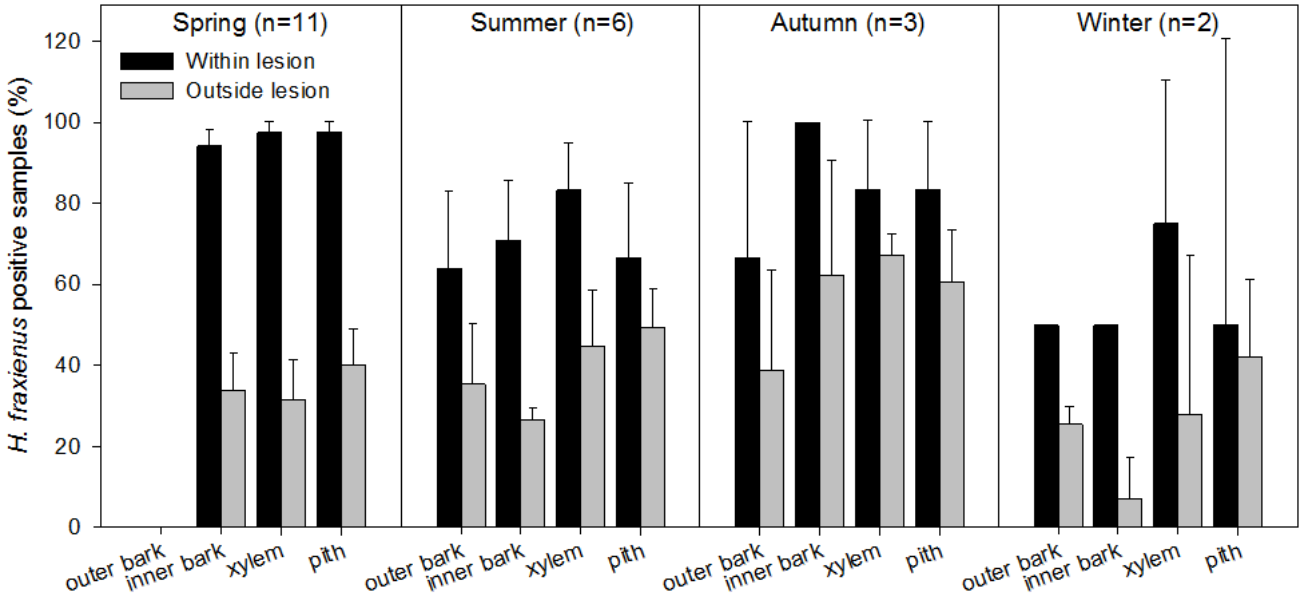

Figure 1. Occurrence frequency of *Hymenoscyphus fraxineus* DNA (% mean ± *SE*) in different stem tissues of naturally infected *Fraxinus excelsior* saplings collected in Ukraine and Norway during the seasons in 2014-15. The analysed samples were collected either from a region showing a necrotic bark lesion ("Within lesion") or from adjacent regions with healthy bark ("Outside lesion"). No samples were taken from the outer bark in the samples collected in spring that are derived from our previous study (Matsiakh et al. 2016).

and xylem in asymptomatic regions adjacent to stem areas with necrotic bark lesions is in line with this model. However, it is noteworthy that healthy outer bark showed the presence of *H. fraxineus* DNA throughout the year. Detection of *H. fraxineus* by fungal isolation and diagnostic PCR assays in collar lesions at ash stem base suggests that the fungus is able to cause direct infection of ash stem (e.g. Chandelier et al. 2016, Marçais et al. 2016). Could some of the now obtained positive signals from outer bark be derived from quiescent endophytic thalli established directly by ascospores, thalli that can resume growth and challenge shoot tissues from outside under certain circumstances? In any case, given the relatively small material now examined from autumn and winter sampling, the generally high occurrence frequency of *H. fraxineus* in phloem in late spring is coherent with the data of Bengtsson et al. (2014), who visually monitored seasonal expansion of bark lesions and observed that they could expand throughout the year but that the high season of lesion expansion initiated in spring. Based on the literature available, Gross et al. (2014) proposed a model according to which shoot bark is initially colonized by *H. fraxineus* but secondary fungi can replace *H. fraxineus* in these lesions and even contribute to lesion enlargement in the tissue. Taken together, the findings of Bengtsson et al. (2014) and the current study may suggest that *H. fraxineus* can indeed be a primary causative agent of bark lesions.

Does all this point out that *H. fraxineus* is above all a pioneer colonizer that feeds primarily on easily accessible non-structural carbohydrates present in the ash shoot and is eventually replaced by endophytes that are better equipped to feed on cell-wall components? To rigorously consider this scenario, a tissue-specific sampling scheme coupled with fungal community profiling by next-generation sequencing and qPCR verification of tissue colonization level by *H. fraxineus* and fungi competing for this niche would be required.

Conclusions

1. qPCR assays specific to *Hymenoscyphus fraxineus* DNA provide a sensitive method for monitoring tissue and season-specific growth of this pathogen in ash stems. 2. No significant differences were observed in the occurrence of *H. fraxineus* between the Norwegian and Ukrainian shoot material in the different tissue types, neither within a bark lesion area nor outside this region.

2. The differences in the tissue-type specific occurrence of *H. fraxineus* DNA between regions with necrotic bark and regions with healthy bark were statistically highly significant.

3. The relatively high proportion of bark samples positive for *H. fraxineus* in the saplings collected during spring provides support to a model that *H. fraxineus* can be a primary causative agent of bark lesions and that other fungi may eventually replace it in old infection areas.

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Each Isolate of *Hymenoscyphus fraxineus* is an Individualist as Shown by Exoenzyme and Growth Rate Profiles

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Abstract

Isolates of *Hymenoscyphus fraxineus,* the causal agent of ash dieback, were analyzed for growth rates and production of the exoenzymes β-glucosidases, amylases, cellulases, lipases, laccases, phenoloxidases, peroxidases and tyrosinases. None of the 176 isolates from diverse locations in Germany, Poland and Sweden secreted all of these enzymes. Growth rates also varied considerably, under the conditions studied between 0.03 and 0.28 cm / day. Additionally, we found inter- and intrapopulation diversity regarding exoenzyme profiles and growth rates among and between the isolates from the Elm forest and a tree nursery in Ellerhoop, both locations in northern Germany. There were no correlations between number of enzymes an isolate produces and growth rate, nor between either growth rate or number of enzymes and virulence, virulence having been determined in a previous study. These phenotypic results concur with previous genetic analyses that have found significant inter- and intrapopulation variability at the molecular level. Only considerable sexual reproduction can explain these results. We conclude that each isolate of *H. fraxineus* is an individualist.

Keywords: Exoenzymes, growth rate, ash dieback, *Hymenoscyphus fraxineus,* inter- and intrapopulation variation

Introduction

Hymenoscyphus fraxineus is the causal agent of ash dieback (Kowalski 2006, Kowalski and Holdenrieder 2009, Queloz et al. 2011, Gross et al., 2014a) and is endangering the existence of *Fraxinus excelsior* in Europe. The pathogen was first observed in the 1990s in Poland (Kowalski 2006, Kowalski and Holdenrieder, 2009) and has been rapidly spreading across Europe, arriving in Great Britain in 2012 (http://www.forestry.gov.uk/chalara). The pathogen is assumed to be native to Asia (Zhao et al. 2012), where *H. fraxineus* does not cause disease on native species of *Fraxinus* and was probably introduced once by at least two individuals (Gross et al. 2014b). The disease manifests itself in wilting of young shoots, and necroses on leaves, twigs and branches (Gross et al. 2014a). Within the host tissues, the hyphae grow both inter- and intracellularly (Figure 1a and 1b) as previously shown by Schumacher et al. (2009), apothecia developing on the rachises of the previous year's leaves (Figure 1c and 1d). The disease spreads primarily via ascospores (Gross et al. 2014a).

The isolates of *H. fraxineus* can differ considerably in their macroscopic morphologies (Kowalski and Bartnick 2010) and in their molecular identities, analyses having shown significant intra- and interpopulation diversity. Rytkoenen et al. (2012) reported the identities of 14 haplotypes among 32 isolates from three countries, i.e. Finland, Estonia and Latvia. Kraj and Kowalski (2014) and Nguyen et al. (2015) found significant genetic intrapopulation variability among *H. fraxineus* isolates from Poland and Germany, respectively; Haňáčková et al. (2015) also found interpopulation variability between isolates from

the Czech Republic, Switzerland and Norway. In the same leaflets of *F. excelsior* from Britain, Cross et al. (2016) found two genets of *H. fraxineus.* The analyses of Bengtsson et al. (2012) revealed that there has been a high degree of gene flow, suggesting that *H. fraxineus* is a sexually out-crossing species.

Virulence factors of phytopathogens include phytotoxins and exoenzymes (Agrios 1997). Viridiol is a phytotoxin of *H. fraxineus* and when applied to leaves of *F. excelsior* causes necroses (Andersson et al 2010, Cleary et al. 2014). The lactone, 3,4 dimethylpentan-4-olid, another phytotoxin of *H. fraxineus*, inhibits germination and causes necroses on germinating seedlings (Citron et al. 2014). In culture, concentrations of viridiol and the lactone vary considerably among the various isolates (Junker et al. 2014; Citron, Junker, Schulz and Dickschat, unpublished). Hymenosetin is an antibacterial toxin that is also only produced by some isolates of *H. fraxineus* (Halecker et al. 2014). Considering the fact that isolates of *H. fraxineus* can vary morphologically and molecularly, as well as in concentrations of the secondary metabolites they produce, we here determined to what extent the growth rates of 176 isolates of *H. fraxineus* differed and if the exoenzyme profiles of the individual isolates of *H. fraxineus* varied. Subsequently, we determined whether there are correlations between exoenzyme profiles and another phenotypic characteristic, i.e. growth rate, but also of exoenzyme profiles and growth rates with virulence factors of *H. fraxineus* (disease symptoms following inoculation into *F. excelsior* and synthesis of the phytotoxin viridiol) as determined in previous research. Additionally, we were interested in determining whether there is inter- and/or intrapopulation variability of exoenzyme profiles and growth rates among isolates of *H. fraxineus*, and in particular among and between those from the locations Ellerhoop and Erkerode.

Material and Methods

Isolates

The 176 strains of *H. fraxineus* used in this study had been isolated from diseased *F. excelsior* growing in various locations in Germany, Sweden and Poland (Supplementary Table 1). Larger numbers of isolates from one single location were either from the Elm forest (23 isolates; 52.204514, 10.719210°) or from a tree nursery in Ellerhoop (100 isolates; Landwirtschaftskammer Schleswig-Holstein; 53.716928, 9.770588°), two locations in northern Germany. C398 – C548 were isolated at the Julius Kühn-Institute Braunschweig and identified by Jörg Schumacher between 2007-2009, with the exception of the three strains from Poland (C428-C430), which were provided by Tadeusz Kowalski and C401 from Sweden, provided by Rimvys Vasaitis (Schumacher et al. 2009). Those from the Elm forest near Erkerode were isolated and identified by Siegfried Draeger and authors of this paper in 2009. The studies reported here were conducted in 2010.

Growth rates

The growth rates of all 176 isolates were determined following inoculation of a culture plug (ca. 0.5 x 0.5 cm) onto potato – carrot agar medium (20 g l^{-1} cooked and mashed potatoes, 20 g l^{-1} cooked and mashed carrots, 12 g l^{-1} agar) and incubation at 20° C for 25 days or until the culture had reached the boundaries of the Petri dish (9 cm). Tests were run in triplicate. The growth rate was determined to be the average of the colony radius (measured from the boundary of the inoculated culture plug) in four directions / number of days of culture, i.e. cm/day.

Exoenzymes

The capability of the isolates to secrete exoenzymes that could be utilized for infection and colonization of *F. excelsior* was analyzed using established methodologies for cellulases, β-glucosidases, amylases, lipases, laccases, phenoloxidases, peroxidases and tyrosinases. Positive controls were *Chaetomium globosum* for cellulases and laccases, *Schizophyllum commune* for β-glucosidases, *Xylaria hypoxylon* for peroxidases, *Phialocephala fortinii* for polyphenoloxidases, *Penicillium chrysogenum* for amylases and *Aspergillus niger* for lipases. The control isolates were from our institute's culture collection and had been isolated and identified by Siegfried Draeger from our institute.

The activities of cellulases and β-glucosidases, both involved in the degradation of cellulose, were determined according to the methods of Thorn (1993) and Ng and Zeikus (1980), and Falbe and Regitz (1996) and Nakatsubo (2001), respectively. Starch is degraded by amylases, the activity of which was determined using a starch-nitrite agar medium according to Krainsky (1914). A medium containing Tween-20 (Sierra, 1957) was used to determine the activity of lipases. Laccases and peroxidases are necessary for the degradation of lignins; the methods of Pointing (2000) were used for measuring their activities. The activities of phenoloxidases and tyrosinases, which are also involved in the degradation of lignin as well as other phenolic compounds, were determined according to Bavendamm (1928) and Lyr (1958), respectively. Tests were run in duplicate. Since the tests were conducted on agar media, an objective quantification of dry weight and activity were not possible. Thus, a color change indicated a positive reaction and the respective exoenzyme was evaluated as active.

Statistical analyses

Normality was tested using a Shapiro-Wilk Test (Shapiro and Wilk, 1965). Since the isolates from Ellerhoop were not normally distributed, a Mann-Whitney U test was used to test for significant differences between growth rates in Erkerode vs. Ellerhoop (Mann and Whitney, 1947). Correlation between number of enzymes and growth rate and of both of these parameters with virulence and viridiol concentration were computed using Spearman's rank correlation coefficient or Spearman's rho, a non-parametric parameter of rank correlation for the ranking of two variables (Spearman, 1904). A perfect Spearman correlation of +1 or −1 occurs when each of the variables is a perfect monotone function of the other. Virulence in seedlings of *F. excelsior* and concentration of viridiol in culture extracts of *H. fraxineus* had been determined previously (Junker et al., 2014). All statistical analyses were performed using R version 3.3.2 (R Core Team 2016).

Results

Growth rates of H. fraxineus have inter- and intrapopulation variability

The growth rates of the *H. fraxineus* isolates varied considerably (Supplementary Table 1), ranging from 0.03 to 0.28 cm / day. Exemplary for the interpopulation variability of growth rates are the differences in growth rates of isolates from Erkerode and from Ellerhoop. Whereas the growth rates of the 23 isolates from the Elm Forest near Erkerode varied between 0.03 and 0.19 cm / day with an average of 0.09 cm / day, the growth rates of the 100 isolates from 3-year old seedlings that had all been isolated from a tree nursery in Ellerhoop, Germany, ranged from 0.05 to 0.28 cm / day with an average of 0.15 cm / day (Supplementary Table 1, Figure 2).

To compare the interpopulation variance between growth rates of *H. fraxineus* isolates from Ellerhoop and those from Erkerode, normality was tested using a Shapiro-Wilk Test (Shapiro and Wilk, 1965). The growth rates of *H. fraxineus* isolated from ash in Erkerode followed a normal distribution ($p = 0.3795$), while the growth rates of *H. fraxineus* isolated from ash in Ellerhoop did not ($p = 0.0008666$). We, hence, performed a Mann-Whitney *U* test (Mann and Whitney, 1947) and found that the growth rates of *H. fraxineus* isolates from Ellerhoop (average 0.15 ± 0.06 cm / day) were significantly higher ($p = 1.332 \times 10^{-5}$) than the growth rates of isolates from Erkerode (average 0.09 ± 0.04 cm / day; Figure 2).

Exoenzyme profiles of isolates of H. fraxineus vary

The proportion of the isolates that secreted the eight exoenzymes tested varied between 17 % for cellulases and 98 % for tyrosinases (Figure 3). Lipases (95 %) and β-glucosidases (91 %) were the second and third most frequently secreted enzymes. None of the isolates produced all eight of the tested enzymes. All of the isolates had at least two of the enzymes characteristic for fungi that colonize woody hosts (Dashtban et al 2009; Supplementary Table 1). However, they differed considerably in their individual exoenzyme profiles, as demonstrated by the 100 isolates from Ellerhoop, e.g. the enzyme profiles of C461, C477, C484 and C506 (Figure 4). Whereas, C461 secreted all the enzymes with the exception of peroxidases and C484 had all but peroxidases and lipases, C506 produced only lipases and tyrosinases. Others such as C477 secreted four enzymes – tyrosinases, laccases, lipases and amylases (Figure 4). As exemplified in Figure 4, there was also no obvious link between morphology and exoenzyme profile.

In spite of the fact that lipases, tyrosinases and β-glucosidases were the most frequently secreted enzymes of isolates from both the locations Ellerhoop and Erkerode, the proportion of isolates that secreted the exoenzymes varied between these locations (Figure 5). For example, none of the isolates from Erkerode secreted cellulases in contrast to 15 % of those from Ellerhoop. Whereas only 9 % of the Erkerode isolates secreted polyphenoloxidases, 33 % of those from Ellerhoop did.

Correlations between parameters involved in infection

Table 1 shows the parameters and isolates used to calculate correlation coefficients between growth rates and exoenzyme profiles with virulence of *H. fraxineus*, using data from our previous results (disease symptoms following inoculation of *F. excelsior* with *H. fraxineus* and concentration of viridiol in cultures extracts of *F. excelsior*, Junker et al., 2014); Table 2 shows the calculated Spearman coefficients. With a Spearman coefficient of rho = -0.1935709 and p-value = 0.5466, it is clear that there was no correlation between growth rate and disease symptoms in *F. excelsior* following inoculation with *H. fraxineus.* There was also no correlation between growth rate and synthesis of viridiol by *H. fraxineus*, as shown by a coefficient of rho = -0.01967954 and p-value = 0.9516. Nor was there a correlation between number of enzymes / isolate and virulence, the Spearman coefficient between number of enzymes / isolate and disease symptoms in *F. excelsior* following inoculation with *H. fraxineus* being rho = 0.03194128 and p = = 0.9215, and between number of enzymes / isolate and synthesis of viridiol by *H. fraxineus* being rho = -0.2839943 and p = 0.371.

Discussion and Conclusions

Exoenzymes can be virulence factors of pathogenic fungi, enabling infection and colonization of the plant host (Agrios 1997). As tree pathogens, the isolates of *H. fraxineus* secreted exoenzymes for degrading lignin: 98 %

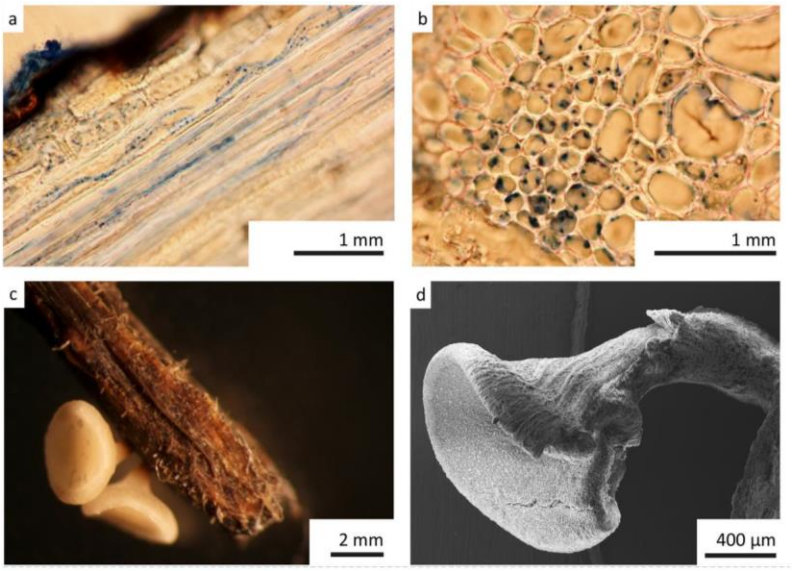

Figure 1. a) and b): inter- and intracellular growth of *Hymenoscyphus fraxineus* in *Fraxinus excelsior,* c) and d): Apothecia of *H. fraxineus* on rachises of *F. excelsior*

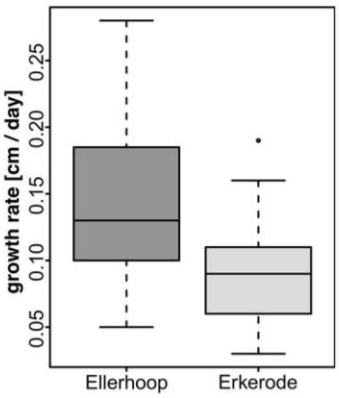

Figure 2. Box-plots displaying the interquartile range (50 ± 25%) of the growth rates of 100 *H. fraxineus* isolates from Ellerhoop and 23 *H. fraxineus* isolates from Erkerode. The thick horizontal lines in each box mark the median (50%) and the whiskers extend to the furthest data points within the 1.5 times interquartile range; the circle above the right box-plot marks the outlier EL 101 with a growth rate of 0.19 cm / day

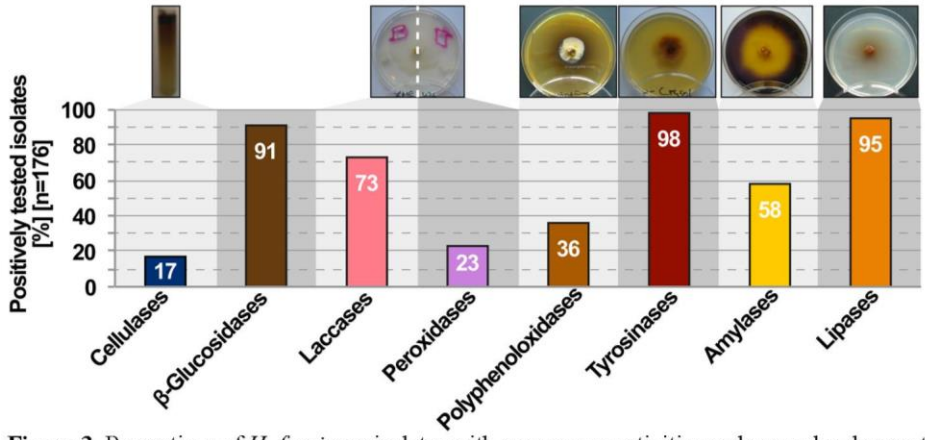

Figure 3. Proportions of *H. fraxineus* isolates with exoenzyme activities and examples demonstrating positive detection of exoenzymes on special media. Culture was for about 21 days at 20°C. Positive reaction (dark brown) with β-glucosidase is not shown

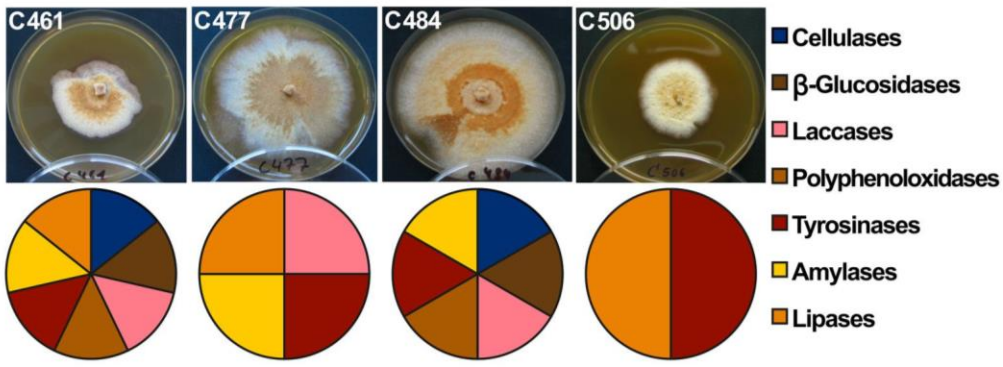

Figure 4. Exoenzyme profiles and morphologies of isolates of *H. fraxineus* demonstrate the diversity of exoenzyme profiles and culture morphology. Exoenzyme activity was evaluated as present or absent. All isolates are from the same origin, i.e. Ellerhoop, Germany. Culture was for two to three weeks at 20°C on biomalt medium.

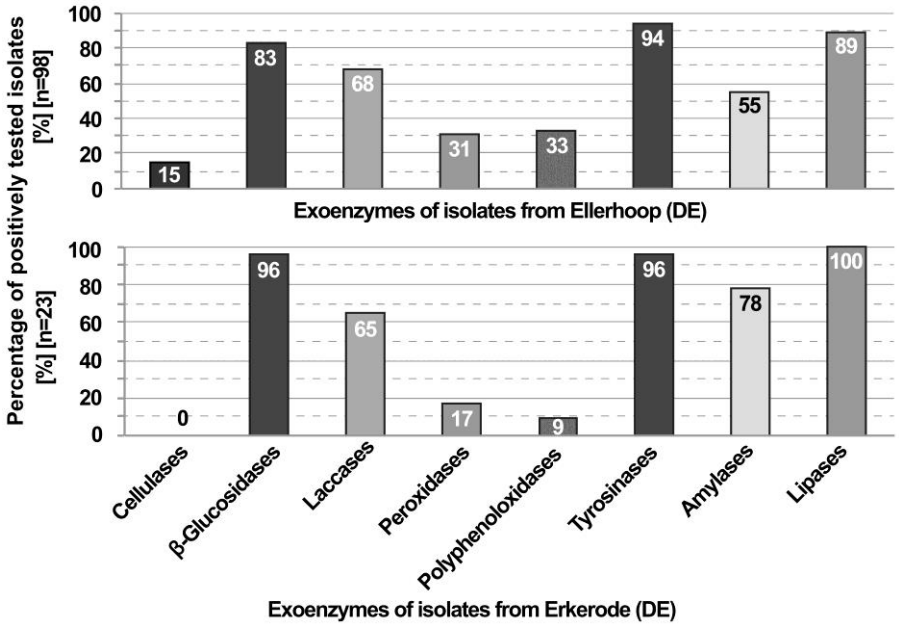

Figure 5. Proportions of *H. fraxineus* isolates isolated from *Fraxinus excelsior* from a tree nursery in Ellerhoop and from the Elm forest near Erkerode that produce each of the tested exoenzymes

Table 1. Strains of *Hymenoscyphus fraxineus* for which Spearman's coefficients were computed for number of enzymes / isolate and growth rate with virulence in seedlings and with concentration of viridiol. For coefficients see Table 2 and the main text

	Enzymes								no. enzymes	Growth rate [ø cm/day]	[1]Virulence in seedlings	[2]Viridiol [µg/mg fungal culture extract]
	Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase				
C 403	-	+	+	-	+	+	+	+	6	0.13	2	0
C 411	-	+	+	-	-	+	+	+	5	0.15	1	77.2
C 421	+	+	+	-	-	+	+	+	6	0.09	1	43.5
C 428	-	+	+	-	-	+	+	+	5	0.13	2	2.33
C 429	+	+	+	-	+	+	+	+	7	0.22	1	0
C 444	+	+	-	-	+	+	-	+	6	0.28	2	80.6
C 472	-	+	+	-	+	+	+	+	6	0.23	2	0
C 489	-	-	+	+	+	+	-	+	5	0.08	3	0.02
C 492	-	+	+	-	+	+	+	+	6	0.22	3	0.55
C 502	-	+	+	-	-	+	+	+	5	0.28	0	0
C 509	+	-	+	-	-	+	-	+	4	0.24	2	24.9
EL 120	-	+	+	-	-	+	+	+	5	0.16	1	3.82

Activity of exoenzymes was either present (+) or absent (-).Previously determined (Junker et al., 2014): [1]Virulence of *H. fraxineus* in seedlings of *F. excelsior* (0 = no disease symptoms, 1 = mild disease symptoms, 2 = moderate disease symptoms, 3 = severe disease symptoms) and [2]Concentration of viridiol in culture extracts of *H. fraxineus*

of the isolates produced tyrosinases, 73 % laccases, 36 % polyphenoloxidases and 23 % peroxidases. Most of the isolates (95 %) secreted lipases, which are required for degradation of the leaf cuticle. Lipases could be a necessity for *H. fraxineus,* because initial infection presumably occurs primarily through the leaves and petioles (Cleary et al. 2013, Gross et al. 2014a). Even though most of the isolates secreted tyrosinases and lipases, the enzyme profiles of the individual isolates differed considerably (Supplementary Table 1). It is probable that other enzymes for which we did not test were involved and that isolates such as C506, which only secreted lipases and tyrosinases, attain their nutrients primarily from sugars present in the vascular bundles and might secrete, for example, invertases.

Table 2. Spearman correlation coefficients (rho-value) between number of exoenzymes / isolate and growth rates of *H. fraxineus* and virulence parameters involved in the infection of *F. excelsior* by *H. fraxineus*

Parameter	Growth rate	[1]Virulence: Infection ash seedlings	[1]Viridiol concentration
Number of exoenzymes	0.01895	0.03194128	-0.2839943
Growth rate	n.a.	-0.1935709	-0.01967954

Number of exoenzymes was for the individual isolates.
[1]Previously determined for isolates of *H. fraxineus* (Junker et al., 2014): Virulence evaluated as disease symptoms following inoculation of axenically cultured ash seedlings with isolates of *H. fraxineus,* and concentration of viridiol in culture extracts of *H. fraxineus.* n.a.=not applicable.

Fungi that grow predominantly in the apoplast can survive only on the nutrients present there (Boyle et al. 2001).

With respect to growth rate, our results corroborate with those of previous studies, (Kowalski 2006, Kowalski and Bartnik 2010, Kirisits et al. 2013). The growth rates of the 176 isolates tested here at 20° C on potato – carrot medium averaged 0.14 cm / day, though with considerable variation, i.e. between 0.03 and 0.28 cm / day, showing that the range of growth rates is even broader than previously measured. Kirisits et al. (2013) measured the growth rates of four isolates of *H. fraxineus* at 20° C, though on a malt extract medium. Nevertheless, although their measured growth rates were relatively low in comparison to ours, i.e. between 0.03 and 0.08 cm / day with an average of 0.05 cm / day, their values fit within the range that we measured and might have covered a broader range if they had included more isolates.

The production of a broad spectrum of exoenzymes should enable degradation of a broad range of nutrients and thus improve growth on complex substrates. Indeed, in the case of the four exemplary isolates (Figure 4) such a correlation is suggested. On potato – carrot medium, the growth rate of C461 with seven exoenzymes was fastest at 0.23 cm / day, that of C484 which produces six exoenzymes was 0.17 cm / day, that of C477 with four exoenzymes was 0.11 cm / day and that of C506 with only two exoenzymes was slowest with 0.05 cm / day. We, however, found that the number of exoenzymes per isolate did not correlate with growth rate (ρ = 0.01895). This fact is underscored by counter-examples of isolates that produce seven of the eight tested exoenzymes with growth rates of only 0.07 cm / day (e.g. C423, C532; Supplementary Table 1). In contrast, C409 produced only two of the exoenzymes, but had a growth rate of 0.20 cm / day. Thus, the results of this study revealed no correlation between number of exoenzymes an isolate secretes and growth rate.

Gross et al. (2014a) suggested that there may be a correlation between growth rate and virulence, the slower growing isolates being more virulent. However, we found no correlation between growth rate with our previous results regarding virulence, i.e. disease symptoms following inoculation into axenically cultured *F. excelsior* seedlings and synthesis of the phytotoxin viridiol by *H. fraxineus*.

Of interest is the finding that the two populations from the Elm forest near Erkerode and the tree nursery in Ellerhoop differ both in their growth rates and in their exoenzyme profiles. For example, none of the 23 isolates from Erkerode produced cellulase. And, the average growth rate of those from Erkerode was significantly lower than that of the isolates from Ellerhoop (Figure 2). Thus, there is interpopulation variation between the isolates from Erkerode and Ellerhoop, both located in northern Germany. Nevertheless, there is diversity within each of these populations. For example, not all of the isolates from Ellerhoop produced viridiol and not all of them were pathogenic when inoculated into axenically cultured seedlings of *F. excelsior* (Junker et al. 2014; Table 1). Junker et al. (2014) had also previously shown that there is no correlation between virulence and concentration of viridiol in the culture extract.

Previously, it was known that isolates of *H. fraxineus* can vary in morphology (Kowalski, 2006, Kowalski and Bartnik 2010), growth rate (Kowalski and Bartnik 2010, Kirisits et al. 2013, Gross et al. 2014a), virulence (Junker et al. 2014), and synthesis of phytotoxins and secondary metabolites, e.g. viridiol (Junker et al. 2014), hymenosetin (Halecker et al. 2014, Halecker, personal communication), and the lactone, 3,4 dimethylpentan-4-olid (Citron et al. unpublished). Results presented here revealed that there is also great variability in the individual exoenzyme profiles, providing further evidence for the uniqueness of each of the 176 isolates of *H fraxineus*. Additionally, we have shown that there is no correlation between number of exoenzymes / isolate and growth rate, between number of exoenzymes / isolate and the two parameters for virulence, and between growth rate and the two parameters for virulence (virulence as determined by Junker et al., 2014; Table 2).

Our results demonstrate diversity of the studied phenotypic parameters for individual isolates, but also inter- and intrapopulation variability. Only considerable sexual reproduction can explain these results, corroborating with genetic analyses that have found significant inter- and intrapopulation variability at the molecular level (Bengtsson et al. 2012, Rytkoenen 2011, Kraj and Kowalski 2014, Nguyen 2015, Haňáčková et al. 2015, Cross et al., 2016). These findings underpin the necessity to study isolates both from diverse as well as from confined sampling sites in future studies. It will be a challenge to recognize whether certain phenotypic characteristics other

than those studied here and / or molecular markers are correlated with virulence. Whether or not such correlations are found, on the basis of phenotypic diversity we can nevertheless conclude: Each isolate of *H. fraxineus* is an individualist.

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Supplementary material

Supplementary Table 1. Strains of *Hymenoscyphus fraxineus* with their origins, exoenzymes and growth rates. Activity of exoenzymes was either present (+) or absent (-). The isolates were from Germany, unless otherwise noted

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
C 398	Hamburg	2007	*F. excelsior* (received from the University of Hamburg)	J. Schumacher	-	+	+	+	+	+	-	+	0.14
C 399	Hamburg	2007	*F. excelsior* (received from the University of Hamburg)	J. Schumacher	+	+	+	-	-	+	-	-	0.14
C 400	Hamburg	2007	*F. excelsior* (received from the University of Hamburg)	J. Schumacher	-	+	-	-	+	+	-	+	0.27
C 401	Uppsala, Sweden	2007	*F. excelsior*	R. Vasaitis	-	+	-	-	-	+	+	+	0.15
C 402	Hamburg	2007	*F. angustifolia*, shoot	G. Hilfert	+	+	+	-	-	+	+	+	0.14
C 403	Hamburg	2007	*F. angustifolia*, shoot	J. Schumacher	-	+	+	-	+	+	+	+	0.13
C 404	Tree nursery Grelck, Haltenbeck, Pinneberg	2007	*F. excelsior*, 3 year old seedlings 1/2	J. Schumacher	-	+	+	-	-	+	-	+	0.16
C 405	Tree nursery Grelck, Haltenbeck, Pinneberg	2007	*F. excelsior*, 3 year old seedlings 1/2	J. Schumacher	-	+	+	-	+	+	-	+	0.21
C 406	Tree nursery Grelck, Haltenbeck, Pinneberg	2007	*F. excelsior*, 3 year old seedlings 1/2	J. Schumacher	-	+	+	-	-	+	+	-	0.08

Supplementary Table 1. (Continued)

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
C 407	Tree nursery Grelck, Haltenbeck, Pinneberg	2007	*F. excelsior*, 3 year old seedlings 1/2	J. Schumacher	-	+	-	+	-	+	-	-	0.16
C 408	Uelzen	2007	*F. excelsior*, branch with necroses	J. Schumacher	-	+	-	-	-	+	+	+	0.15
C 409	Uelzen	2007	*F. excelsior*, branch with necroses	J. Schumacher	-	-	-	-	-	+	-	+	0.20
C 410	Uelzen	2007	*F. excelsior*, branch with necroses	J. Schumacher	-	+	+	-	-	+	+	+	0.13
C 411	Uelzen	2007	*F. excelsior*, branch with necroses	J. Schumacher	-	+	+	-	-	+	+	+	0.15
C 412	Uelzen	2007	*F. excelsior*, branch with necroses	J. Schumacher	+	+	+	-	-	+	-	-	0.16
C 413	Uelzen	2007	*F. excelsior*, branch with necroses	J. Schumacher	-	+	+	-	-	+	+	+	0.11
C 414	Uelzen	2007	*F. excelsior*, branch with necroses	J. Schumacher	-	+	-	-	+	+	-	+	0.20
C 415	Uelzen	2007	*F. excelsior*, branch with necroses	J. Schumacher	-	+	+	-	+	+	-	+	0.05
C 416	Uelzen	2007	*F. angustifolia*, branch with necroses	J. Schumacher	+	-	-	-	+	+	-	+	0.17
C 417	Uelzen	2007	*F. angustifolia*, branch with necroses	J. Schumacher	+	+	+	-	+	+	-	+	0.18
C 418	Uelzen	2007	*F. angustifolia*, branch with necroses	J. Schumacher	-	+	+	-	+	+	+	+	0.23
C 419	Uelzen	2007	*F. angustifolia*, branch with necroses	J. Schumacher	-	+	+	-	+	+	-	+	0.16
C 420	Seeth Ekholt, Pinneberg	2008	*F. excelsior*, 1 year old seedling, shoot - transition brown to green, necroses	J. Schumacher	+	+	+	-	+	+	-	+	0.11
C 421	Seeth Ekholt, Pinneberg	2008	*F. excelsior*, 1 year old seedling, shoot - transition brown to green, necroses	J. Schumacher	+	+	+	-	-	+	+	+	0.09
C 422	Nursery Torsten Perrau, Rellingen	2008	*F. excelsior*, 1 year old seedling, shoot - transition brown to green, necroses	J. Schumacher	-	+	-	-	+	+	-	+	0.12
C 423	Nursery Torsten Perrau, Rellingen	2008	*F. excelsior*, 1 year old seedling, shoot - transition brown to green, necroses	J. Schumacher	-	+	+	+	+	+	+	+	0.07
C 424	Nursery Torsten Perrau, Rellingen	2008	*F. excelsior*, 1 year old seedling, thin brown shoot, necroses	J. Schumacher	-	+	+	-	-	+	-	+	0.13
C 425	Nursery Torsten Perrau, Rellingen	2008	*F. excelsior*, 1 year old seedling, thin brown shoot, necroses	J. Schumacher	-	+	+	-	-	+	+	+	0.17
C 426	Nursery Torsten Perrau, Rellingen	2008	*F. excelsior*, 4 year old seedling, brown wood after removal of bark	J. Schumacher	-	+	+	-	+	+	-	+	0.16

Supplementary Table 1. (Continued)

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
C 427	Nursery Torsten Perrau, Rellingen	2008	*F. excelsior*, 4 year old seedling, brown wood after removal of bark	J. Schumacher	-	+	+	-	-	+	+	+	0.13
C 428	Eastern Poland, Forest district Lublin, Forestry Mircze	2006	*F. excelsior*, necroses	J. Schumacher	-	+	+	-	-	+	+	+	0.13
C 429	Eastern Poland, Forest district Lublin, Forestry Mircze	2006	*F. excelsior*, necroses	T. Kowalski	+	+	+	-	+	+	+	+	0.22
C 430	Eastern Poland, Forest district Lublin, Forestry Mircze	2006	*F. excelsior*, necroses	J. Schumacher	-	+	+	-	-	+	+	+	0.05
C 431	Tree nursery Albert Brandt, Prisdorf, Hamburg	2008	*F. excelsior*, young seedlings	J. Schumacher	-	+	+	-	-	+	-	+	0.12
C 432	Ölper forest, Braunschweig	2008	*F. excelsior*, natural regeneration	J. Schumacher	-	+	+	+	+	+	+	+	0.24
C 433	Ölper forest, Braunschweig	2008	*F. excelsior*, natural regeneration	J. Schumacher	+	+	+	-	+	+	-	+	0.13
C 434	Elm Forest, near Königslutter	2008	*F. excelsior*, plantation	J. Schumacher	-	+	+	-	-	+	+	+	0.10
C 435	Elm Forest, near Königslutter	2008	*F. excelsior*, plantation	J. Schumacher	+	+	+	-	-	+	+	+	0.11
C 436	Richtenberg near Rostock	2008	*F. excelsior*	J. Schumacher	-	+	+	-	+	+	-	+	0.23
C 437	Richtenberg near Rostock	2008	*F. excelsior*	J. Schumacher	-	+	-	-	+	+	+	+	0.25
C 438	Hamburg (Billwerder)	2008	*Fraxinus* sp.	J. Schumacher	-	+	+	-	+	+	-	-	0.13
C 439	Berlin	2008	*F. excelsior*	J. Schumacher	-	+	-	+	-	+	-	-	0.22
C 441	Haiming, SE-Baveria	2008	*F. excelsior*, young trees	J. Schumacher	-	+	+	-	+	+	-	+	0.19
C 442	Haiming, SE-Baveria	2008	*F. excelsior*, young trees	J. Schumacher	+	+	+	-	+	+	-	+	0.10
C 443	Haiming, SE-Baveria	2008	*F. excelsior*, young trees	J. Schumacher	-	+	+	-	+	+	-	+	0.21
C 444	Tree nursery Hobohm, Nauen	2009	*F. excelsior*, "Westhof's Glory", grafting of a piece of stem	J. Schumacher	+	+	-	-	+	+	-	+	0.28
C 445	Tree nursery Hobohm, Nauen	2009	*F. excelsior*, young seedling, shoot tip, necrotic	J. Schumacher	-	+	+	-	+	+	-	+	0.15
C 446	Nauen, Am Kuhdamm	2009	*F. excelsior*, old tree population, sucker	J. Schumacher	-	+	+	-	-	+	+	-	0.19
C 447	Nauen, An den Rohrwiesen, Havelland	2009	*F. excelsior* "Westhof's Glory", 3 year old graft, necroses of cortex and sapwood	J. Schumacher	-	+	+	-	-	+	+	+	0.15
C 448	Cottbus	2008	*F. excelsior* "Pendula"	J. Schumacher	-	+	+	-	-	+	+	+	0.05

Supplementary Table 1. (Continued)

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
C 449	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings from a tree nursery	J. Schumacher	-	+	-	+	+	+	-	+	0.23
C 450	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.12
C 451	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings from a tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.13
C 452	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.12
C 453	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	+	-	+	+	+	0.07
C 454	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	+	+	-	+	0.23
C 455	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	-	+	0.17
C 456	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	+	-	+	0.15
C 457	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	-	-	-	+	-	+	0.20
C 458	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	-	-	-	+	-	-	0.07
C 459	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	-	-	+	+	+	0.13
C 460	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.16
C 461	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	-	+	+	+	+	0.23
C 462	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	-	+	0.12
C 463	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.18
C 464	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	+	+	+	-	+	0.14
C 465	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	-	-	+	+	+	+	0.20
C 466	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.09
C 467	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	-	+	+	+	+	0.11
C 468	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	+	+	0.21
C 469	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	-	+	-	+	0.12
C 470	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	+	+	-	+	0.17
C 471	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	-	+	0.13
C 472	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	+	+	0.23

Supplementary Table 1. (Continued)

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
C 473	Ellerhoop	2008	*F. excelsior*3 year old seedlings, tree nursery	J. Schumacher	-	-	-	-	+	+	-	-	0.13
C 474	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	+	-	+	+	+	0.18
C 475	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.25
C 476	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.13
C 477	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	-	-	+	+	+	0.11
C 478	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.12
C 479	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	+	-	+	-	+	0.07
C 480	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	-	-	+	-	+	-	+	0.24
C 481	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	-	+	+	+	+	0.15
C 482	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	+	-	+	-	+	0.10
C 483	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	-	-	+	-	+	0.11
C 484	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	-	+	+	+	-	0.17
C 485	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	-	-	+	-	+	0.10
C 486	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	-	-	-	+	+	+	0.07
C 487	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	-	+	+	+	-	+	0.22
C 488	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	+	-	+	+	+	0.16
C 489	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	+	+	+	-	+	0.08
C 490	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.09
C 491	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.24
C 492	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	+	+	0.22
C 493	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	-	-	+	-	+	0.12
C 494	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	+	-	-	+	+	0.08
C 495	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	-	+	0.17
C 496	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	+	+	+	0.07
C 497	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	+	+	+	+	+	0.28

Supplementary Table 1. (Continued)

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
C 498	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	-	+	0.21
C 499	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	+	-	+	0.18
C 500	Ellerhoop	2008	*F. excelsior*, forest plantation	J. Schumacher	-	+	-	-	-	+	-	+	0.14
C 501	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	-	+	-	-	+	0.18
C 502	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.28
C 503	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	-	+	0.22
C 504	Ellerhoop	2008	F. excelsior, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.16
C 505	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.12
C 506	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	-	-	-	+	-	+	0.05
C 507	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	+	+	-	+	+	+	0.15
C 508	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	+	+	+	0.27
C 509	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	-	+	-	-	+	-	+	0.24
C 510	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	-	+	+	+	+	0.12
C 511	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.12
C 512	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	+	+	+	0.27
C 513	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	-	+	+	0.11
C 514	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	-	+	0.19
C 515	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	+	+	-	-	0.06
C 516	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	+	+	+	0.09
C 517	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.12
C 518	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	-	+	+	+	0.12
C 519	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	-	+	+	+	0.12
C 520	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.09
C 521	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	+	+	+	0.20
C 522	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	-	+	+	0.12

Supplementary Table 1. (Continued)

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
C 523	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	-	-	+	0.18
C 524	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	-	+	+	-	0.15
C 525	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	-	+	-	+	+	+	0.10
C 526	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	+	+	+	+	0.10
C 527	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	-	-	-	0.24
C 528	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.08
C 529	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	+	+	-	+	+	-	0.13
C 530	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	-	+	+	-	0.08
C 531	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	-	+	-	+	0.13
C 532	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	+	+	+	+	0.07
C 533	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	-	+	+	+	0.07
C 534	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	+	-	+	-	+	0.18
C 535	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	+	+	0.09
C 536	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	-	0.25
C 537	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	-	-	-	-	+	+	+	-
C 538	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.14
C 539	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	-	0.10
C 540	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	-	+	+	-	0.13
C 541	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	+	-	+	+	+	0.22
C 542	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	-	+	+	+	0.17
C 543	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	+	+	-	+	+	+	+	+	0.18
C 544	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	-	-	+	+	+	+	0.14
C 545	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	+	+	+	-	+	0.08
C 546	Ellerhoop	2008	*F. excelsior*, 3 year old seedlings, tree nursery	J. Schumacher	-	+	+	-	+	+	+	+	0.13
C 547	Tree nursery Gera, Auenboden	2009	*F. excelsior*, 2-3 year old seedling	J. Schumacher	+	+	+	-	-	+	-	+	0.20

Supplementary Table 1. (Continued)

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
C 548	Tree nursery Gera, Auenboden	2009	*F. excelsior*, 2-3 year old seedling	J. Schumacher	-	+	+	-	-	+	+	+	0.25
Es 89a	Ellerhoop	2009	*F. excelsior,* seedling, tree nursery	S. Draeger	-	+	-	+	-	+	+	+	0.21
Es 60c	Ellerhoop	2009	*F. excelsior,* seedling, tree nursery	S. Draeger	-	+	+	-	+	+	-	+	0.09
Es 20d	Ellerhoop	2009	*F. excelsior,* seedling, tree nursery	S. Draeger	-	+	+	-	-	+	-	+	0.07
EL 100	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	-	+	-	-	+	-	+	0.13
EL 101	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	+	+	0.19
EL 102	Elm Forest, near Erkerode	2009	*F. excelsior*, , sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	-	-	-	+	+	+	0.06
EL 103	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	-	-	+	+	+	+	0.13
EL 104	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	-	-	-	+	+	+	0.06
EL 105	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	+	+	0.03
EL 106	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	+	+	+	+	+	0.07
EL 107	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	-	+	0.08
EL 108	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue e	S. Draeger	-	+	-	+	-	+	+	+	0.13
EL 109	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	+	-	-	+	+	0.09
EL 110	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	-	-	-	+	+	+	0.11
EL 111	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	-	+	0.03
EL 112	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	-	+	0.09
EL 113	Elm Forest, near Erkerode	2009	*F. excelsior* sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	-	-	-	+	+	+	0.09

Supplementary Table 1. (Continued)

Strain no.	Origin			Identified by	Enzymes								Growth rate [ø cm / day]
					Cellulase	β-Glucosidase	Laccase	Peroxidase	Polyphenol-oxidase	Tyrosinase	Amylase	Lipase	
EL 114	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	-	-	-	+	+	+	0.08
EL 115	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	+	+	0.05
EL 116	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	+	+	0.08
EL 117	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	+	+	0.04
EL 118	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	+	-	+	+	+	0.09
EL 119	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	-	-	-	+	+	+	0.06
EL 120	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	+	+	0.16
EL 121	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	+	+	0.10
EL 122	Elm Forest, near Erkerode	2009	*F. excelsior*, sapwood from transition healthy to necrotic tissue	S. Draeger	-	+	+	-	-	+	-	+	0.11

Genomes of *Hymenoscyphus fraxineus* and *Hymenoscyphus albidus* Encode Surprisingly Large Cell Wall Degrading Potential, Balancing Saprotrophic and Necrotrophic Signatures

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Abstract

In Europe, an epidemic is currently occurring on common ash (*Fraxinus excelsior*). The disease, commonly known as ash dieback, is the result of a biological invasion by the causal Helotialean fungus *Hymenoscyphus fraxineus* Baral, Queloz, Hosoya. This study describes the genomes of *H. fraxineus* and *H. albidus*, a native non-pathogenic sister species to *H. fraxineus*. The *Hymenoscyphus* sp. genomes harbour similar and extensive Cell Wall Active Enzyme (CAZYme) repertoires, they appear better at degrading cellulose than e.g. *Botrytis* but has similar pectin-degrading capacities. *In planta*, the pathogenic *H. fraxineus* showed higher gene expression than *H. albidus* of two of the pectin degrading enzymes, consistent with a higher disruption of primary cell walls and possibly leading to a stronger host reaction. Based on SignalP and Phobious annotations, we identified 2160 and 2006 secreted genes in *H. fraxineus* and *H. albidus*, respectively. This is almost twice as many as for most other Helotialean fungi. Two small secreted proteins were transcribed in *H. fraxineus*, one being a cerato-platinin like protein with a putative role in pathogenicity. No small secreted proteins were detected in the *H. albidus* transcriptome. It has been suggested that fungal metallopeptidases, can target and degrade non-structural defense proteins *in planta*. We found that the *Hymenoscyphus* genomes encode more metallopeptidases than other Helotialean species. In conclusion, the prolonged saprotrophic growth phase on shed ash leaves of *H. fraxineus* and *H. albidus* has probably shaped the genomes. Both genomes are highly similar and have CAZYme profiles similar to saprotrophic fungi. The relatively small differences between the two *Hymenoscyphus* spp. in gene expression are likely indicative of their differential interaction patterns with the host tree *F. excelsior*.

Keywords: Ash dieback, *Hymenoscyphus fraxineus, Hymenoscyphus albidus, Fraxinus excelsior*, cell wall degrading enzymes, small secreted proteins, metalloproteinases, invasive species

Introduction

European ash (*Fraxinus excelsior*) has been the subject of a sever die back during the last decades (Kowalski 2006, Bakys et al. 2009, Gross et al. 2014). The disease is caused by an introduced pathogen *Hymenoscyphus fraxineus* that in its origin is an endophyte or weakly pathogenic fungus (Cleary et al. 2016). The life cycle includes spore infection on leaves, or occasionally bark tissue, followed by growth via the petiole into twigs (Gross et al. 2014). Unchecked growth in the inner bark and xylem result in dieback of the crown, which ultimately can lead to mortality of the whole tree. Sporulation occurs on fallen leaf rachises typically the year after initial leaf infection and spores are produced in large amounts particularly after rainy periods in late summer. In Europe, a close relative *H.albidus* has long been present on ash leaves and fallen rachises without pathogenic growth in host woody tissue.

Sequencing the whole genome of an organism is invaluable for its comprehensive molecular characterization and has been drastically facilitated by the advent of high-throughput sequencing techniques. Important progress is currently made in our understanding of the genomic organization and the genetic repertoire of a wide range of fungi through the release and subsequent mining of genome sequences. Evolutionary and bioinformatic analyses of pathogen and non-pathogen genomes represent novel ways with which to decipher mechanisms controlling associations with the host tree and shed light on the acquisition of the genetic toolkit during fungal evolution.

The rapid spread of *H. fraxineus* following the introduction into Europe and the high susceptibility of *Fraxinus excelsior* poses the question as to if there are any specific parts of the genetic setup in the fungus that could explain its success. In order to shed light on this we have sequenced and assembled the genomes of *H. fraxineus* and *H. albidus* and performed comparative investigations of their genetic content. With the aim of putting this information into further perspective we also included a set of previously sequenced closely related ascomycete genomes of species with diverse ecological strategies. All *Hymenoscyphus* genomes were *de novo* assembled and genes in all studied genomes were annotated using the same methodology. Here we report on the gene content of cell wall degrading enzymes secreted proteins and metalloproteases.

Materials and Methods

Cultivation of fungal isolates and DNA extraction for genome sequencing

The *Hymenoscyphus* isolates (*H. fraxineus* isolate nf4 and *H. albidus* isolate 111/1/4) were cultivated in in liquid malt extract 1.75% and 0.25% peptone at 22°C for 4 weeks. DNA was extracted from mycelium samples according to CTAB/chloroform methods and samples were subsequently treated with RNase A (Sigma). After quality check, DNA was sent to SciLifeLab, Uppsala, Sweden, where sequencing libraries with a target insert size of 500bp were prepared. The libraries were sequenced using a paired end protocol with 100 sequencing cycles from each end on an Illumina HiSeq 2000 sequencer. Moreover, mate pair sequencing was done on an ABI SOLiD sequencing instrument (Life Technologies) using Exact Call Chemistry. For *H. fraxineus* two libraries with insert sizes of about 3 kb and 8 kb were prepared and sequenced, and for *H. albidus* a single library with an insert size of about 3 kb was prepared and sequenced. The mate pair libraries were sequenced with 60bp from each end (Elfstrand et al. in prep).

All data deposition numbers are available on request from Mikael Brandström Durling (Mikael.Durling@slu.se).

Annotation and evolution of carbohydrate active enzymes

To annotate all carbohydrate active enzymes (CAZymes) in the genomes, we used HMMER 3.0 (Finn et al. 2011) with Hidden Markov Models (HMMs) from dbCAN (Yin et al. 2012). The sizes and evolution of the different CAZyme families were compared using CAFE, following the same procedure as for the more general gene family evolution analysis.

Annotation of short secreted proteins (SSPs)

We defined Short Secreted Proteins (SSPs) as proteins with at most 150 amino acids, and having a signal peptide for secretion present. We also required that the protein did not contain any predicted trans-membrane domains. Signal peptide predictions were done with SignalP (Petersen et al. 2011) and Phobius (Kall et al. 2004). Transmembrane domains were annotated with Phobius. From the set of inferred SSPs we extracted the secreted peptides, by discarding the annotated signal peptide, and used these sequences for further analysis of the secretome. Furthermore we characterized the content of conserved motifs among the secreted peptides within each species using MEME (Bailey et al. 2015) searching for motifs of 3 to 13 bp occurring zero or one time per sequence. The discovered motifs were then searched for in the peptide set where they were discovered, as well as in all other species in the study. Tests for enrichment of individual motifs were done using the Fisher's exact test.

Gene models of other Helotialean taxa and evolutionary comparisons

To enable comparative genomic analysis of the focal species in a phylogenetic framework, we used published Helotiealean fungi for comparisons; *Ascocoryne sarcoides*, *Botrytis cinerea, Glarea lozoyensis, Marsonnina brunnea* f. sp. *multigermtubi, Sclerotinia sclerotiorum* and *Sclerotinia*

borealis (Amselem et al. 2011, Gianoulis et al. 2012, Mardanov et al. 2014, Youssar et al. 2012, Zhu et al. 2012), as well as the outgroup *Blumeria graminis* (Spanu et al. 2010). Since the gene annotations of these species were produced with a variety of different annotation pipelines and vary in age, we re-annotated them with MAKER following the same procedure as we used for the *Hymenoscyphus* species and utilising the available EST or RNASeq evidence that was available in conjunction with the genome sequences.

To test for expansions and contractions among the gene families, we used CAFE version 3.1 (De Bi et al. 2006). We used default settings in CAFE and let the program estimate the underlying birth-death ration. CAFE requires an ultrametric phylogenetic tree describing the relationship between the species. We constructed a maximum likelihood tree of the species based on the genes *rpb, tef1, tub1* and *tubA* using the timetree method of MEGA4 (Tamura et al. 2007), assuming the substitution rate as a proxy for the divergence times in the tree.

Analysis of RNASeq data

Expression of fungal genes in mixed tissues was determined by RNA sequencing. RNASeq data was filtered with Nesoni to trim off adaptor sequences, and to discard low quality sequences (same parameters as for the genome sequencing) before further processing. We used the Tophat-Cufflinks-Cummerband workflow as outlined in Trapnell et al. (Trapnell et al. 2012) to estimate expression as FPKM and to test for differential expression. Tophat and Cufflinks were provided with the MAKER gene predictions to hint the alignments, and the parameters were adjusted to better fit fungal data by allowing introns of 5-5000 bp and disabling coverage based merging of transcripts.

Experimental conditions, plant material, inocula

To test *in planta* expression of selected candidate genes, inoculations were conducted on 2-year-old bare-root seedlings of *F. excelsior* (30–50 cm in height) obtained from a commercial nursery in Sweden (Cleary et al. 2013). Seedlings were grown in 20 cm diameter plastic pots filled with potting media consisting of 60% light peat sieved, 25% black peat and 15% sand (Hasselfors Garden, Örebro, Sweden) in a greenhouse with a 16 h photoperiod of 20/15°C (day/night). To prepare inocula, sterile woody plugs were added to 2-week old 2% malt extract agar cultures of both *H. fraxineus* and *H. albidus*. After 4 weeks, fully colonized woody plugs were used for inoculating plants. A minimum of eight replicate plants of *F. excelsior* were wound-inoculated with either *H. fraxineus* or *H. albidus*, and sealed with Parafilm™. Control treatments were non-wounded. Plants were harvested after 7, 14, 28, and 42 days. Stems were dissected longitudinally and the extent of necrosis measured. Phloem tissue was collected from the margin of defined lesions, frozen in liquid nitrogen and then stored at -80°C until further processing.

RNA extraction and RT-QPCR ANALYSIS

Total RNA was extracted from the phloem samples representing different time points and treatments using a standard protocol (Chang et al. 1993). Again, the RNA was treated with DNaseI (Sigma) to remove the genomic DNA. RNA quality and concentration was measured on the Bio-Analyzer 2100 using RNA Nano Chips (Agilent). Dynabeads mRNA Purification Kit (Invitrogen) was used to extract polyA+RNA. mRNA amplification was performed using the MessageAmpIII kit (Ambion). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) from purified aRNA (1µg) of either *H. fraxineus* or *H. albidus* samples taken at 7, 14, and 28 dpi with an extended RT-reaction for 50 min.

The cDNA was diluted 1:1 in deionizer water, and an aliquot of cDNA equivalent to 25 ng of aRNA was used per 20 µL of PCR reaction using SSoFast EVAGreen Supermix (Bio-Rad) with a final concentration of 0.5 µM of each primer. Primers were designed from isotig sequences using the Primer3 software (http://primer3.wi.mit.edu/) with a melting temperature (Tm) between 58°C and 60°C, and amplicon length between 95-150 bp (Table 1). The thermal-cycling condition parameters, run on a iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad), were

Table 1. Primer sequences for the gene expression analysis

Gene	Forward primer 5' -3'	Reverse primer 5' -3'
UBC	CCTCGGACTCTCCATACTCG	GATAGATTCTGGTGGTGAAGTT
RPL17	AAGCAATCAACGGATGGAAG	CGGCATAACGTCTCATAGGAACA
HYFRA_T0002081	AGCTTGGTTTCACCGAGAGA	ATCACGGTTTGGTGTGGTTT
HYFRA_T0004626	TCCCAAGATGGATCTGGAAC	AGCATCCCAAAGGTACATGC
HYFRA_T0002445	AACGTCTGGCTCTTCTTCCA	ATGGGCAGTTCACCATGACA
HYFRA_T0000350	GGAGCCGACTACTTCACCAC	ATTCTTGGGGAGGTGCTTTG
HYFRA_T0005905	TAAGGCTGCTGAGTCGTCAA	AAGTTCCGGTGTGTGAGTCC
HYFRA_T0000722	TGCTCATGCTGTCGCTACTT	CTGGGAGCTTCTGTCTCCAC

as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 58 or 60 °C for 20 s. Each run was followed by a melt curve analysis to validate the specificity of the reaction. A linear plasmid standard curve was used to measure the PCR efficiency and primer pairs with efficiency lower than 95% were discarded. Three biological replicates for each time point and two technical replicates were prepared for each sample. Transcript abundance was normalized to the reference genes *UBC* and *RPL17*. The relative expression was calculated using the delta–delta CT-method (Livak & Schmittgen 2001). Kruskal-Wallis- and Mann-Whitney U-tests were performed on the RT-qPCR data using the GraphPad Prism5 software (GraphPad Inc.).

Results and discussion

Carbohydrate active enzymes

We annotated carbohydrate active enzymes (CAZymes) in all studied genomes using the dbCAN database (Yin et al. 2012) of Hidden Markov Models. A total of 688 CAZymes in 137 gene families and 659 CAZymes in 133 gene families were found in *H. fraxineus* and *H. albidus*, respectively. At least 186 *H. fraxineus* and 183 *H. albidus* CAZymes were associated with plant cell wall degradation (PCWD) (Table 2 and 3). The proportion of PCWD CAZymes in *Hymenoscyphus* spp. (1.5% of all predicted proteins) was similar to other Helotialean fungi. The necrotrophic pathogens *S. sclerotiorum* and *B. cinerea* have been suggested to be especially suited to degrade pectin as their genome harbours many pectin-degrading, but few cellulose degrading CAZymes (Amselem et al. 2011, Blanco-Ulate et al. 2014). Our analysis suggested that *H. fraxineus*, *H. albidus* and *M. brunnea* possess significant pectin-degrading capacities, but also that *H. fraxineus* and *H. albidus* has a higher number of cellulose degrading enzymes than *B. cinerea*, *S. borealis*, *S. sclerotiorum* and *M. brunnea* (Table 2).

It has been argued that the number of genes encoding for cellulolytic, hemicellulolytic and pectinolytic activities in a fungal genome is a reflection of the fungal strategy for degrading plant tissues (Amselem et al. 2011, Lyu et al. 2015, Ohm et al. 2012, Zhao et al. 2014). The broad host range necrotrophic fungi *B. cinerea* and *S. sclerotiorum* have been suggested to have a higher number of gene copies and therefor a larger capacity to degrade pectin than hemicellulose or cellulose compared to other Plant Cell Wall (PCW)-degrading pathogens, while the genomes of saprotrophic fungi such as *Neurospora crassa* and *Trichoderma reesei* indicate a preference for cellulose and hemicellulose over pectin (Amselem et al. 2011). In general it can be observed, that the two Hymenoscyphus species have a reportoir of cell wall degrading enzymes that is somewhat intermediate to those of the saprotrophic and necrotrophic/hemibiotrophic members of Helotiales. The PCW-degrading enzyme profiles in the genomes of *H. albidus* and *H. fraxineus* suggest that these fungi have an obvious and very similar potential to degrade plant cell walls, similar to that of the closely related species *G. lozoyensis*. However, this PCW-degrading capacity appears to differ from the profiles of the broad range necrotrophic pathogens *B. cinerea* and *S. sclerotiorum* (Amselem et al. 2011) in that *H. albidus* and *H. fraxineus* have a larger arsenal of PCWD enzymes and more potential to utilize cellulose and hemicelluloses as a carbon source, reminiscent of the PCW-degrading enzyme profiles described for several saprotrophs (Amselem et al. 2011).

In our analysis, we tested for expansion or contraction of specific CAZyme families using CAFE, assuming the same birth-death rate of family members as was estimated from the analysis of the full proteome gene families. We found four CAZyme families with significant expansions and one contraction in the ancestor to *Hymenoscyphus* (Figure 1). The higher number of cellulose degra-

Table 2. Summary table of genome content of cell wall degrading enzymes for nine Helotialean species of different ecological strategies. The number of genes and the percentage of genes out of the full gene space in the species are indicated for four categories of carbohydrate substrate

		CAZymes per PCW component								
	Trophic strategy	Cellulose		Hemicellulose		Hemicellulose w. pectic sidechains		Pectin		Total
		N	*%*	N	*%*	N	*%*	N	*%*	
A. sarcoides	Saprotroph	20	*15*	74	*55*	18	*13*	22	*16*	134
G. lozoyensis	Saprotroph	39	*20*	99	*51*	26	*13*	31	*16*	195
H. albidus	Endophytic fungus	30	*16*	92	*50*	22	*12*	39	*21*	183
H. fraxineus	Necrotropic pathogen	31	*17*	95	*51*	21	*11*	39	*21*	186
M. brunnea	Hemibiotrophic pathogen	22	*15*	70	*46*	21	*14*	38	*25*	151
B. cinerea	Necrotropic pathogen	22	*14*	75	*47*	17	*11*	44	*28*	158
S. borealis	Necrotropic pathogen	17	*12*	69	*50*	15	*11*	38	*27*	139
S. sclerotiorum	Necrotropic pathogen	20	*14*	71	*51*	16	*11*	33	*24*	140
*B. graminis**	Biotrophic pathogen	3	*14*	15	*71*	2	*10*	1	*5*	21

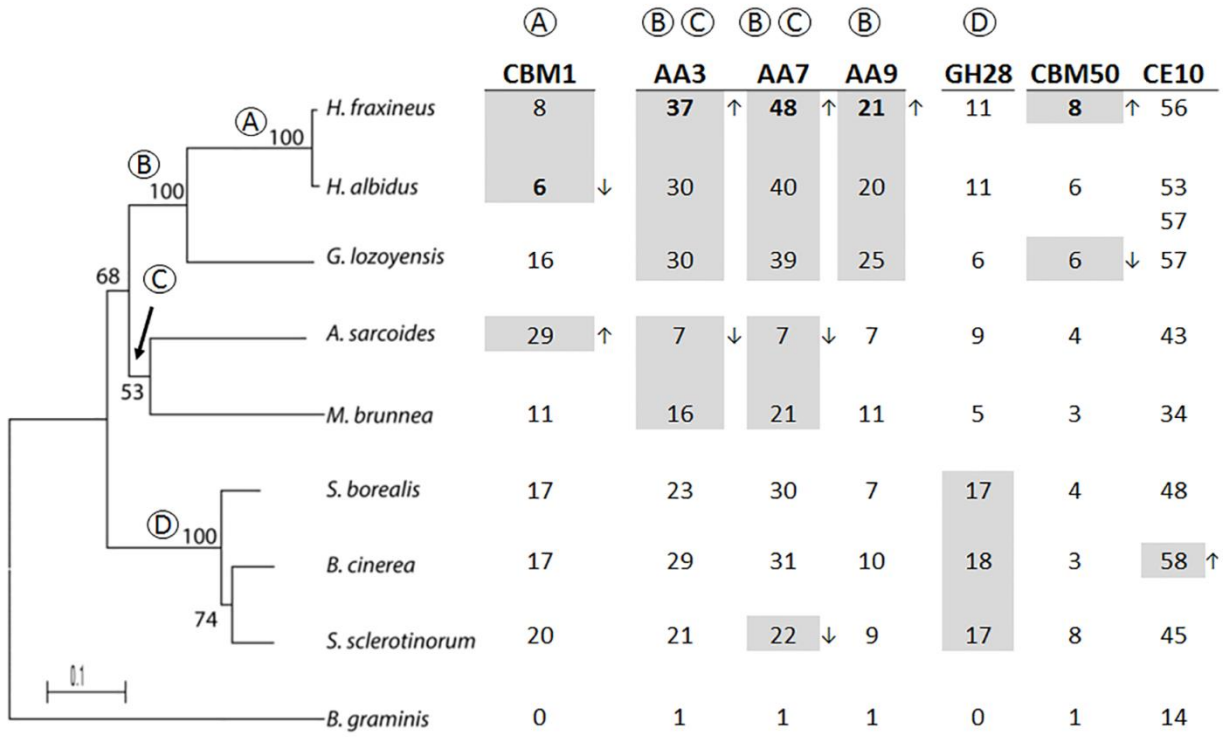

Figure 1. Expansions and contractions of CAZY gene families in the helotiales: The number of genes identified in CAZYme gene families with significant expansions and contractions in the helotiales. CAZYme gene families with significant (Viterbi *P*-value < 0.05) expansions or contractions in specific taxa or ancestral species are shaded in grey. The arrows indicate expansion (↑) or contraction (↓) for individual species along the tree. Expansion or contractions in ancestral species are indicated by encircled A, B, C and D at the node in question and above the columns of the CAZYme gene families. Numbers in bold font indicate expansions / contractions in *Hymenoscyphus*

ding enzymes in *H. fraxineus, H. albidus* and *G. lozoyensis* was traced to a significant expansion (p = 0.001) of the CAZY class auxiliary activities (AA) family 9 (formerly glycoside hydrolase (GH) family 61) in the *H. albidus, H. fraxineus* and *G. lozoyensis* branch (Figure 1)**.** Despite expansions of particular **c**ellulose-degrading CAZY classes in *Hymenoscyphus* spp., the number of cellulose binding domain (CBM) family 1-containg genes were significantly lower in *Hymenoscyphus* spp. compared with other Helotialean fungi (Figure 1).

Although the total numbers of predicted hemicellulose-degrading PCWD enzymes were larger in the *H. albidus, H. fraxineus* and *G. lozoyensis* genomes compared to other members of the Helotiales, the proportion of hemicellulose-degrading PCWD enzymes among the CAZYmes remained essentially constant, around 50%, among all Helotialean fungi **(**Table 2**).** Likewise the number of enzymes, targeting hemicellulose with pectic side chains was higher in these three species than in e.g. *B. cinerea,* although the percentual proportion was similar (Table 2). This was primarily a result of a larger GH43 family in *Hymenoscyphus* spp. and *G. lozoyensis*.

Overall, *H. albidus* and *H. fraxineus* possessed similar numbers of pectin-degrading and pectin-modifying CAZYs as the necrotrophic Helotialean fungi, although *H. albidus* and *H. fraxineus* both contained 11 GH28 genes while the closely related *G. lozoyensis* only had six. This indicates that the *H. albidus* and *H. fraxineus* have pectin-degrading potentials similar to that of *Sclerotinia* spp. and *B. cinerea*.

Gene expression was significantly higher *in planta* for *H. fraxineus* compared to *H. albidus* for two proteins involved pectin degrading activity PL3 and GH 28 (Figure 2a and b). There was no significant difference in CE 12 a gene involved in breakdown of cellulose (Figure 2c). The difference in expression of pectinolytic genes would fit well with the much higher level of damage caused by *H. fraxineus* when growing in *F. excelsior*. The enzymatic activity as such will degrade the primary cell walls and may in addition provoke host cell reactions by providing cell wall fragments acting as elicitors of host defense reactions (Boller and Felix 2009).

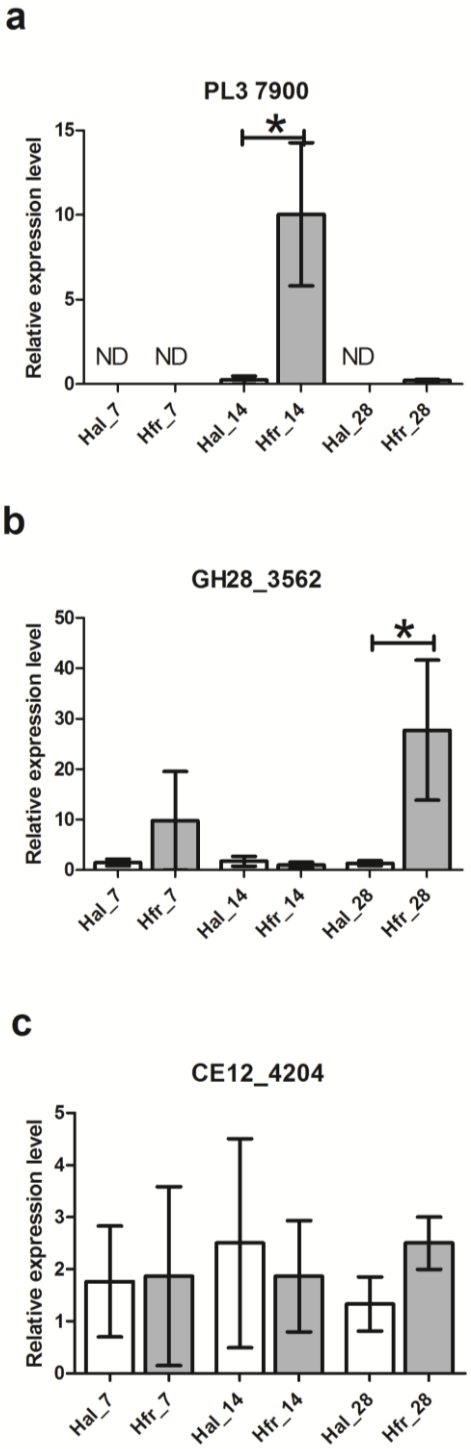

Figure 2. Expression of three *Hymenoscyphus* cell wall degrading enzymes in interactions with *Fraxinus excelsior*. Relative gene expression levels in *H. fraxineus* (shaded bars) and *H. albidus* (open bars) at 7, 14 and 28 dpi in *F. excelsior* bark: Pectin lyase PL3_7900 (A); Pectin degrading GH28_3562 (B); Cellulose degrading CE 12_4204 (C). Error bars indicate SE. Horizontal bars indicate pair-wise comparisons and asterisks indicate significances (* $p < 0.05$, ** $p < 0.01$, Mann-Whitney U-test)

The two *Hymenoschyphus* species both contained 17 GH18 genes, predicted to encode chitinases. GH18 genes can be divided into the defined GH18 subgroups GH18_A, GH18_B, GH18_C and ENGases (Seidl-Seiboth et al. 2014), and both *Hymenoscyphus* spp. possessed seven, three, four and two members of the respective class (Table 3). Subgroup GH18_C (killer toxin-like chitinases) was significantly expanded ($p = 0.002$) in the clade containing *Hymenoscyphus* spp. and *G. lozoyensis* compared to the other Helotialean species.

Table 3. Summary table of genome content of chitin degrading enzymes of Glucoside hydrolase family GH18 for nine Helotialean

	GH18_A	GH18_B	GH18_C	ENGase	GH18_ALL
H. albidus	7	3	4	2	17
H. fraxineus	7	3	4	2	17
G. lozoyensis	4	2	7	2	15
A. sarcoides	4	5	1	1	11
M. brunnea	5	1	0	1	7
S. borealis	7	4	5	2	18
B. cinerea	3	4	1	2	10
S. sclerotinorum	4	5	3	2	14
B. graminis	7	1	0	1	9

When examining the PCW-degrading enzyme profiles of the genomes of *H. albidus* and *H. fraxineus*, it appears that both fungi have a similar potential to degrade plant cell walls. Both species and the most closely related Helotialean fungus, the saprotrophic *G.* appear to utilize a large battery of LPMOs (AA09) to degrade cellulose (Morgenstern et al. 2014) The AA09 are oxidative enzymes that boost the enzymatic conversion of recalcitrant polysaccharides, in particular cellulose. This number of AA09 enzymes is clearly larger for *Hymenoscyphus* and *Glarea* than for other members of the Helotiales. Expansions of the LPMOs has been observed among saprotrophs, and some necrotrophs, but not among hemibiotrophs in *Dothideomycetes* (Ohm et al. 2012). This finding is an indication of the importance of saprotrophic part of the life cycle of *Hymenoscyphus* spp. Similarily, the hemicellulose degrading potential is also higher for the *Hymenoscyphus* spp. and *G. lozoyensis* than for the true necrotrophic/hemibiotrophic helotialean fungi, again suggesting an important role for the saprotrophic degradation of plant cell walls.

The interest in the LPMO enzymes stemmed from their ability to stimulate biomass hydrolysis of cellulose by giving an oxidative enzyme boost to the enzymatic conversion of recalcitrant polysaccharides. A putative additional

role for AA9 LPMOs in host invasion has also been suggested for an AA9 enzyme of the phytopathogen *Pyrenochaeta lycopersici*, following tomato root infections (Valente et al. 2011). Strong induction of *P. lycopersici* AA9 *Plegl1* transcription at 96 h after infection of tomato roots coincides with the switch from biotrophic to necrotrophic fungal growth (Goodenough et al. 1976, Valente et al. 2011). Similar importance in necrotrophic growth may be present for *H. fraxineus*.

Obviously the genomic CAZYme arsenal could be used differently by fungi. Consequently, our observation of such a pronounced similarity in genomic PCW-degrading enzyme profiles between *H. albidus* and *H. fraxineus*, and also with the saprotroph *G. lozoyensis*, does not prove that *H. albidus* and *H. fraxineus* use similar strategies in their interactions with *F. excelsior*. Instead we think that the similarity in genomic PCW-degrading enzyme profiles of *H. albidus* and *H. fraxinues* may be attributable to the prolonged phase of saprotrophic growth, decomposing leaves and rachises on the ground. The adaption to saprotrophic growth including frequent interactions with other fungi on the forest floor has further support in the expansion in the GH18 C group chitinases in *Hymenoscyphus* (Table 3). This group of enzymes has previously been interpreted to have a function similar to a killer toxin and to be involved in antagonistic interactions in saprotrophic life styles.

The predicted secretomes of H. albidus *and* H. fraxineus

Many plant-associated fungi interact with their hosts through the secretion of various proteins known as effectors, which alter host-cell structure and function. Based on SignalP and Phobious annotations done through InterPro Scan, we identified 2160 and 2006 secreted genes in *H. fraxineus* and *H. albidus*, respectively. This is similar to the number found in *G. lozoyensis*, but almost doubled as compared to the other Helotealian species**.** We further identified 308 and 294 short secreted proteins (SSPs) less than 150 amino acids in length in *H. fraxineus* and *H. albidus*, respectively. This represented more than a two-fold increase in SSP gene number as compared to *G. lozoyensis*, and a three to six-fold increase in comparison to the other Helotialean species of this study and *B. graminis*.

Genes of effector families often share common sequence motifs, such as the RXLR motif of oomycte effector proteins or the YxC effectors of *B. graminis* (Spanu et al. 2010). We mined the SSP sets of all studied species for conserved sequence motifs using MEME (Bailey et al. 2015) from the MEME package and then used FIMO to annotate all occurrences of the discovered motifs in SSPs and all other genes. We validated the approach by running it on *B. graminis* where we recovered the YxC motif, which shows a significant 10-fold increase in occurrence among the *B. graminis* SSPs (Fisher's exact test, $p < 10^{-9}$) but not overrepresented among *Hymenoscyphus* SSPs. Next, we analysed the occurrence of motifs in *Hymenoscyphus* spp. SSPs and found three motifs each in *H. fraxineus* and *H. albidus* that were significantly ($p < 0.0001$) 2-3 fold overrepresented (Figure 3). Two of the motifs, "CxDC" and "$CxSx_4I$" were shared between the species. The latter two motifs often occurred in tandem within the SSP genes where they occurred.

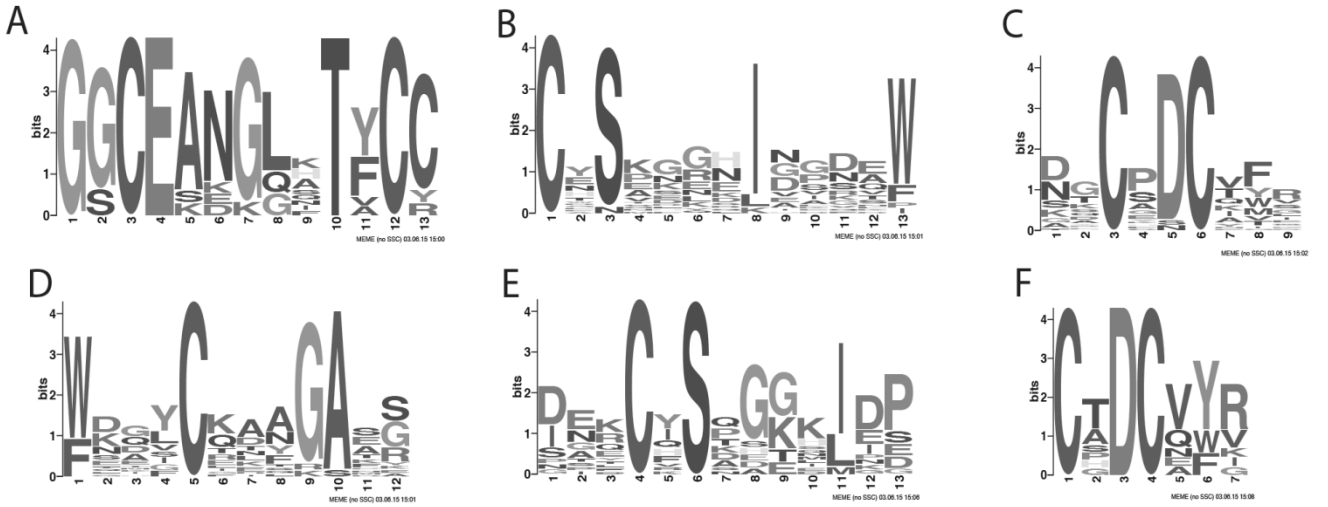

Figure 3. Overrepresented sequence motifs in *H. fraxineus* (A-C) and *H. albidus* (D-F) SSPs. The conserved sequence motifs of *H. fraxineus* and *H. albidus* occur in about 10% of the predicted SSPs (A 23, B 30 and C 33 times in *H. fraxineus.* D 31, E 29, F 27 times in *H. albidus*)

Genes encoding proteins involved in host-parasite interaction are expected to show elevated levels of evolutionary turnover. When amino acid conservation in SSPs between the two *Hymenoscyphus* species was analysed, only 158 ascertained one-to-one orthologous SSP pairs were identified, and the average dN/dS ratio for these genes was 0.6. Only 17 of these SSPs were also conserved in *G. lozoyensis*.

We mined seven available RNAseq libraries of *F. excelsior* infected with *H. fraxineus* (Eshghi Sahraei et al. unpublished), as well as a pure culture RNAseq library (generated in the current study) for expression of the predicted SSPs. Only two *H. fraxineus* SSPs were expressed, HYFRA_T00003917 (protein of unknown function) and HYFRA_T00003226 (similar to SnodProt1). Both genes were expressed *in planta* as well as in pure culture. HYFRA _T00003226 shows similarity to the cerato-platanin family of proteins known from other plant pathogens. No expression of *H. albidus* SSP genes was observed, neither *in planta* in two libraries nor in a single library of pure culture RNASeq data.

Cerato-Platanin (CP) is a non-catalytic phytotoxic hydrophobin-like small secreted protein, which work as virulence factors and/or as elicitors of defense responses and systemic resistance, thus acting as PAMPs (pathogen-associated molecular patterns). Moreover, CP has been defined an expansin-like protein showing the ability to weaken cellulose aggregates, like the canonical plant expansins do (Pazzagli 1999, Luti et al. 2016). The secreted CP-like protein of *H fraxineus* may also protect fungal cell wall polysaccharides from enzymatic degradation by the host in a manner analogous to what recently was suggested for *Fusarium graminearum* (Quarantin et al. 2016).

***Metallopeptidases genes are differentially expressed* in planta**

It has been suggested that fungal metallopeptidases, fungalysins, can target and degrade non-structural defense proteins *in planta* (Jashni et al. 2015, Naumann et al. 2011). Therefore we examined the metallopeptidase and serine protease gene families in *H. fraxineus* and *H. albidus* and found relatively few significant differences in gene family size between the two genomes or in comparison with other Helotialean fungi (Figure 4). However, it is noteworthy that *H. fraxineus* and *H. albidus* each carry two genes of the metallopeptidase families M35 (deuterolysin) and M36 (fungalysin) while the genomes of all other Helotialean fungi, except *G. lozoyensis* and *M. brunnea*, possessed no M36 genes**.** *H. fraxineus*, *H. albidus* and *G. lozoyensis* also contained seven protease genes from family M28A while most other Helotialean fungi only possess two. The expression of M35, M36 and two M28A genes from *H. fraxineus* and *H. albidus* were analysed with qRT-PCR at 7, 14 and 28 dpi on *F. excelsior*.

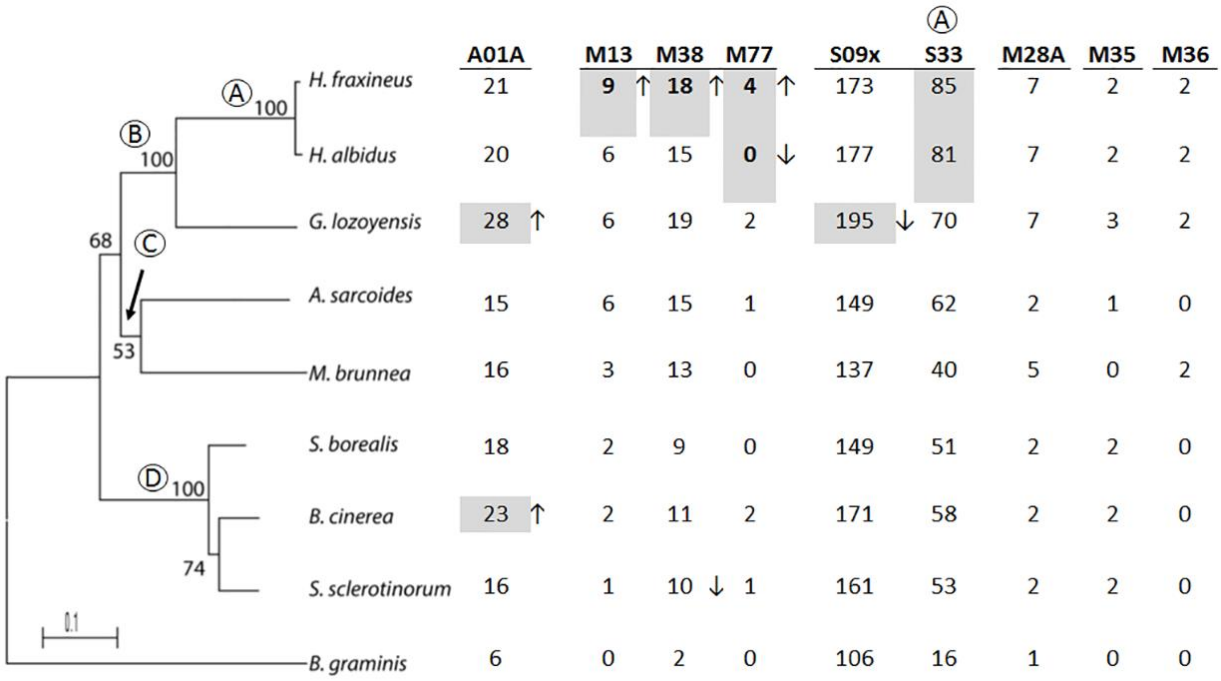

Figure 4. Expansions and contractions among proteolytic gene families in the helotiales: Gene families with significant (Viterbi *P*-value < 0.05) expansions or contractions in specific taxa or ancestral species are shaded in grey. The arrows indicate expansion (↑) or contraction (↓) for individual species along the tree. Expansion or contractions in ancestral species are indicated by encircled A, at the node in question and above the columns of gene families. Numbers in bold font indicate expansions or contractions in the *Hymenoscyphus*

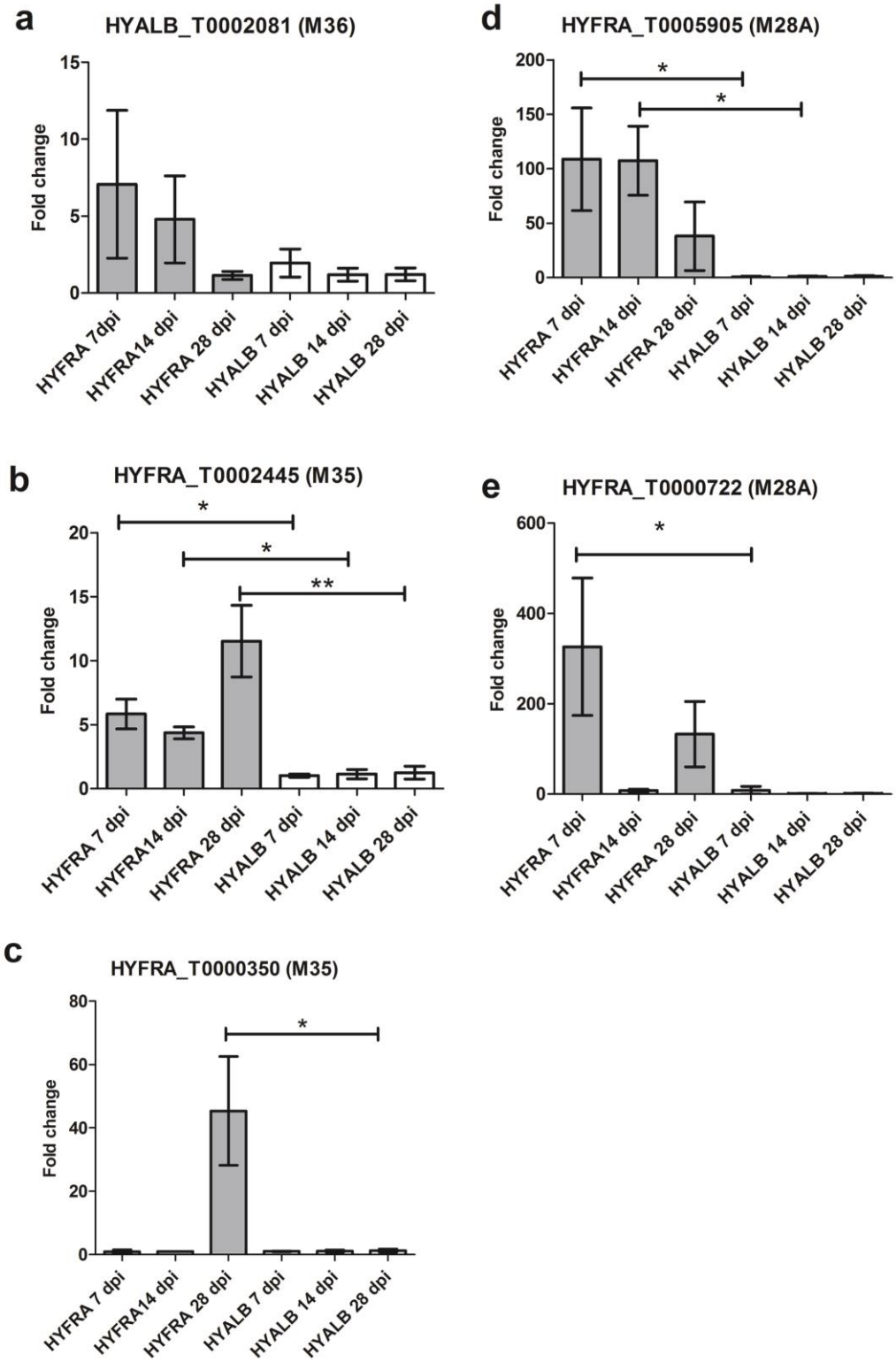

Figure 5. Expression of *Hymenoscyphus* metallopeptidases in interactions with *Fraxinus excelsior*. Relative gene expression levels in *H. fraxineus* (shaded bars) and *H. albidus* (open bars) at 7, 14 and 28 dpi in *F. excelsior* bark: M36_2081 (A); M35_2445 (B); M35_0350 (C); M28A_2445 (D) and M28A_0722 (E). Error bars indicate *SE*. Horizontal bars indicate pair-wise comparisons and asterisks indicate significances (* $p < 0.05$, ** $p < 0.01$, Mann-Whitney U-test)

The two *H. fraxineus* M36 family genes HYFRA_T0002081 and HYFRA_T0004626, and their *H. albidus* orthologs, showed significantly different expression patterns *in planta*. No expression of HYFRA_T0004626 could be detected in either *H. fraxineus* or *H. albidus* interactions with *F. excelsior* (data not shown). On the other hand HYFRA_T0002081 was expressed to similar levels in both *H. fraxineus* and *H. albidus* in interactions with *F. excelsior* at all time-points analysed (Figure 5a).

Both family M35 metallopeptidases (HYFRA_T0002445 and HYFRA_T0000350) showed significantly ($p < 0.05$) higher expression levels in *H. fraxineus* than in *H. albidus* at 28 dpi (Figure 5b and c). However, the expression pattern differentiated at the earlier time-points. HYFRA_T0002445 showed significantly ($p < 0.05$) higher transcript levels in *H. fraxineus* than in *H. albidus* also at 7 and 14 dpi (Figure 5b), while HYFRA_T0000350 showed similar low transcript levels in both species at these time-points (Figure 5c).

The two analysed family M28A metallopeptidases (HYFRA_T0005905 and HYFRA_T0000722) showed very high levels of expression in *H. fraxineus* but not *H. albidus* during their interaction with *F. excelsior* (Figure 5d and e). Interestingly, HYFRA_T0000722 showed the highest degree of induction in both species at 7 dpi, albeit at significantly different levels (Figure 5e).

It is possible that one or several of these metallopeptidases represent an initial step in the arms race between ash and *H. fraxineus*, e.g. by inhibiting chitin-binding domain(CBD)-containing chitinases (Jashni et al. 2015). This may lead to co-evolutionary diversification and adaptation shaping pathogen lifestyle and host defense in the newly formed pathosystem of ash dieback.

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Natural infection of *Fraxinus angustifolia* by *Hymenoscyphus fraxineus* in Slovakia

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Abstract

The fungus *Hymenoscyphus fraxineus* is responsible for dieback of common ash (*Fraxinus excelsior*) and in some parts of Europe also of narrow-leaved ash (*F. angustifolia*). The first symptoms of ash dieback have been recorded on *F. excelsior* in Slovakia since 2004. This study reports about the first natural occurrence of *H. fraxineus* on *F. angustifolia* in Slovakia. The field investigation was carried out in 2014. The segments of diseased shoots and last year's petioles were collected in clonal seed orchard situated in southwest part of the country. The fungus was isolated from infected host tissue and identified using molecular techniques (DNA extraction from pure cultures and apothecia, conventional PCR).

Key words: *Fraxinus angustifolia*, *Hymenoscyphus fraxineus*, molecular techniques, ash dieback.

Introduction

The fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya is the sexual stage of *Chalara fraxinea* T. Kowalski. *H. fraxineus*, the causal agent of ash dieback, attack mainly common ash (*Fraxinus excelsior* L.) and narrow-leaved ash (*F. angustifolia* Vahl). The susceptible hosts have also been recorded black (*F. nigra* Marshall), green (*F. pennsylvanica* Marshall), white (*F. americana* L.), Manchurian (*F. mandshurica* Rupr.) and manna (*F. ornus* L.) ash trees (Drenkhan and Hanso 2010, Kirisits and Schwanda 2015). Symptoms of ash dieback are variously coloured (brownish to orange) bark necroses and cankers without exudates on stems and branches, leading to dieback of trees (Kowalski 2006, Schumacher et al. 2010). Ash dieback was identified for the first time in Poland (Kowalski 2006).

Ash dieback caused by *H. fraxineus* has been reported in many countries in Europe, for example Austria (Cech 2006, Halmschlager and Kirisits 2008), Czech Republic (Jankovský and Holdenrieder 2009), Hungary (Szabo 2009), Latvia, Estonia, Romania and Russia (Kirisits et al. 2009) and others. Since 2004, based on the presence of the symptoms, ash dieback has been recorded also in Slovakia (Kunca 2006). The first report of the *H. fraxineus* occurrence in Slovakia was described only on *F. excelsior* using molecular techniques (Adamčíková et al. 2015).

Development of molecular tools designed in the Internal Transcribed Spacer sequences (ITS) of nuclear ribosomal DNA cistrons for detecting *Chalara fraxinea* was recently performed (Chandelier et al. 2010, Ioos et al. 2009, Johansson et al. 2010). Another molecular technique is identification to species level by conventional PCR according to Johansson et al. (2010), where the targeted gene is the 18S gene and the ITS-2 region of rDNA operon.

The aim of this work is to identify *H. fraxineus* on narrow-leaved ash (*F. angustifolia*) for the first time in Slovakia by molecular tools.

Material and Methods

Sampling

In March 2014, symptomatic twigs of *F. angustifolia* (stem sections 10-20 cm long containing the lesion) were collected. The total number of collected twig or stem samples was 14. Also last year's petioles from leaf litter with apothecia were collected during July 2014. The investigation was carried out in clonal seed orchard of ash species in Trstice, Galanta District, Slovakia (48°24´32"N, 17°47´39"E). The total number of collected apothecia was 8. The samples were placed in paper bags and 1.5-ml Eppendorf sterile tubes and stored at 4 °C before further transferred to laboratory for analysis. Voucher specimens are deposited in the Plant Pathology Herbarium NR (Institute of Forest Ecology of Slovak Academy of Sciences, Nitra, Slovakia).

Sample preparation

Isolation of the pathogen was done from cankers. The outer bark was carefully removed and small sections (0.5 × 0.5 cm) from the canker margin were cut out. After surface sterilization (1 min ethanol 96%, 1 min NaClO 4%, 30 s ethanol 96%), the samples were washed in distilled water, dried on sterile paper and placed in Petri dishes on the surface of 2 % ash leaf malt extract agar (AMEA; 20 g/l malt extract Roth, 15 g/l agar, amended with 50 g fresh *F. excelsior* leaflets removed after autoclaving, Kirisits et al. 2013). AMEA was supplemented with antibiotic (100 mg/l streptomycin sulphate) added before effusing the media in Petri dishes. The Petri dishes were incubated at 20 °C in the dark and subcultures were made on AMEA without antibiotics. The cultures were used for DNA extraction.

DNA extraction

The samples (apothecia, mycelium) were homogenized in the presence of liquid nitrogen using a mortar and pestle. Total DNA of each sample was extracted from 100 mg of homogenized apothecia and mycelium using the EZ-10 Column Plant Genomic DNA Purification Kit (Bio Basic Canada Inc.) following the manufacturer's instructions.

PCR amplification and gel analysis

For analysis of DNA directly from apothecia and mycelium, a conventional PCR approach was used. PCR amplification was performed using the fungal-universal primers ITS1F and ITS4 (White et al. 1990, Gardes and Bruns 1993). DNA sequencing of ITS rDNA operon was done according to Husson et al. (2011). The amplification was carried out in a 20 µl reaction mix containing 2 ng of genomic DNA. The PCR was performed using T1 Thermocycler 96. PCRs were initialized with a denaturation step at 95 °C for 14 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s and extension at 72 °C for 80 s. The thermal cycling ended with a final extension step at 72 °C for 10 min. The PCR products were size separated by gel electrophoresis on 1% agarose gel, stained with an ethidium bromide and visualized under UV light. Prior to sequencing, target fragments were directly purified using a PCR Purification Kit (Macherey-Nagel, Germany). Each amplified product was diluted with 30 µL H_2O and visualized with an ABI3130xl sequencer from Applied Biosystems. The retrieved sequences were compared by BLAST (Basic Local Alignment Search Tool, available at http://www.ncbi.nlm.nih.gov/BLAST/) against DNA sequences deposited in GenBank for *H. fraxineus* (accession number HM193468).

For identification to species level was performed a conventional PCR using specific primers 18S gene and the ITS-2 region of rDNA operon and the expected size of amplicon was 456 bp (Johansson et al. 2010). The amplification of DNA was performed in 20 µL reaction volumes using approximately 2 ng of template DNA. After an initial denaturation step for 15 min at 95 °C, 35 cycles were performed each comprising a denaturation step at 95 °C for 20 s, an annealing step at 62 °C for 30 s and an extension step at 72 °C for 1 min followed by a final extension step for 8 min at 72 °C. Molecular-grade water was used as negative amplification control during preparation of reaction mix.

Results and Discussion

The health state of ash trees in clonal seed orchard situated in Trstice village in Slovakia was investigated and sampled in the period from March to July 2014. The orchard consists of 2 ash species: common ash (*F. excelsior*) and narrow-leaved ash (*F. angustifolia*). The symptoms typical for ash decline and ash dieback were present in the evaluated locality. Ash dieback is characterized by a remarkably wide range of symptoms (Kirisits et al. 2009, Kowalski et al. 2010). Disease symptoms range from necrotic leaf spots to bark cankers associated with xylem necroses and wilting, eventually leading to tree death. The dieback starts with ascospore infection of leaves. Ascospores are produced during summer in apothecia on leaf remnants from the previous year on the ground and dispersed by wind (Kowalski and Holdenrieder 2009, Timmermann et al. 2011, Gross et al. 2012, Kirisits et al. 2012). Leaf infection is followed by necrotic lesions spreading along the rachis into the shoot, and wood discoloration (Kowalski and Holdenrieder 2008, Bakys et al. 2009, Kräutler and Kirisits 2012). The above symptoms were recorded on investigated locality on *F. angustifolia* host tree. Ash dieback was recorded for the first time in the eastern part of Slovakia in 2004 (Kunca 2006). Later, symptoms were observed in other localities (Leontovyč and Kunca 2009) and now ash dieback has spread throughout Slovakia without any natural limits (Kunca et al. 2011). *H. fraxineus* was confirmed in several

localities in different types of vegetation over Slovakia (Adamčíková et al. 2015). All these records originated from Slovakia referred to the ash dieback only on *F. excelsior* host tree.

The fungus isolation from symptomatic *F. angustifolia* host tissues was successful in 3 samples from 14 collected. Colonies were cottony, white, orange-brown or fulvous brown, slow growing as typical for *H. fraxineus*. The obtained pure cultures were used for DNA extraction.

DNA was successfully extracted from 11 samples (3 isolates, 8 apothecia) collected in Trstice. The PCR-based test using species specific primers according to Johansson et al. (2010), forward primer for 18S gene, reverse primer for ITS2 region of the DNA operon produced an amplicon of 456 bp. The 18S ribosomal PCR to test the integrity of the DNA showed positive results for all samples. The confirmation of results, due to possible cross-reaction with *H. albidus* (Gillet) W. Phillips, was done by DNA sequencing of ITS rDNA operon for all samples (DNA extracted from 3 isolates also 8 from apothecia). The ITS sequences obtained with primers ITS1/ITS4 for all samples were identical and showed a high degree of similarity to DNA sequence deposited in GenBank for *H. fraxineus* with accession number HM193468 (Husson et al. 2011). Sequence identity of the rDNA ITS1/ITS4 locus was 100% among the 11 samples from diseased *F. angustifolia*. The ITS sequences obtained in this study were also deposited to GenBank (for GenBank accession numbers see Table 1).

This study reports about the first occurrence of ash dieback and *H. fraxineus* on *F. angustifolia* in Slovakia. Until now it has been confirmed in Slovakia only on *F. excelsior* (Kunca 2006, Adamčíková et al. 2015).

The occurrence of the disease on *F. angustifolia* is not unexpected, but the impact of the disease on this tree species is less documented than that on *F. excelsior* (Kirisits et al. 2010, Hauptman et al. 2012, McKinney et al. 2014). It has also been reported from surrounding countries: Czech Republic (Jankovský and Holdenrieder 2009), Austria and Hungary (Kirisits et al. 2010).

In conclusion, this study demonstrated that the ash dieback pathogen attacks not only *F. excelsior*, but also *F. angustifolia* as in many European countries also in Slovakia.

Table 1. List of samples of *Hymenoscyphus fraxineus* collected from *Fraxinus angustifolia* in clonal seed orchard of ash species in Trstice used for molecular studies

Sample designation	Date of sampling	Fungal tissue* used for DNA extraction	Herbarium specimen no.**	GenBank accession no.
CH_30	26 Mar 2014	M	-	KU736880
CH_31	26 Mar 2014	M	-	KU736881
CH_32	26 Mar 2014	M	-	KU736882
H_61	16 Jul 2014	A	NR 5321	KU736883
H_63	16 Jul 2014	A	NR 5322	KU736884
H_64	16 Jul 2014	A	NR 5320	KU736885
H_71	16 Jul 2014	A	NR 5319	KU736886
H_72	16 Jul 2014	A	-	KU736887
H_73	16 Jul 2014	A	-	KU736888
H_74	16 Jul 2014	A	NR 5317	KU736889
H_75	16 Jul 2014	A	NR 5318	KU736890

*M = mycelium, A = apothecia

** NR = Plant Pathology Herbarium of the Institute of Forest Ecology, Slovak Acad. Sci., Nitra, Slovakia

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First Report of the Invasive Ash Dieback Pathogen *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and *F. angustifolia* in Serbia

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Abstract

In Serbia, unambiguous symptoms of ash dieback disease were for the first time observed in September 2015. Symptoms included dead shoots and occasionally small necrotic lesions in the bark accompanied by characteristic wood discoloration. Isolation of fungal cultures from symptomatic tissues of *F. excelsior* and *F. angustifolia* and their sequencing using the internal transcribed spacer of the rDNA (ITS rDNA) as a marker confirmed the presence of the ash dieback pathogen, *Hymenoscyphus fraxineus.*

Keywords: *Chalara fraxinea*, emerging forest disease, *Fraxinus* spp., new disease report.

Introduction

Ash dieback has been for the first time observed in the early 1990's in north-eastern Poland and has since then spread into large parts of Europe causing decline and mortality in natural stands and plantations of European ash, *Fraxinus excelsior* L. (Timmermann et al. 2011, McKinney et al. 2014). The causal agent of the disease is the ascomycete fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya, which was first found and described in its asexual form as *Chalara fraxinea* T. Kowalski in Poland (Kowalski 2006, Baral and Bemmann 2014, Gross et al. 2014). To date, *H. fraxineus* has been reported from the majority of the distribution range of *F. excelsior* in Europe, excluding, among other areas, Serbia and other parts of the eastern Balkans. *H. fraxineus* is responsible for high mortality rates on *F. excelsior*, but can also cause severe disease on narrow-leaved ash, *F. angustifolia* Vahl (Kirisits et al. 2010, McKinney et al. 2014). In contrast, on flowering or manna ash (*F. ornus* L.) the fungus inflicts only inconspicuous and negligible leaf symptoms, does not progress into woody parts and is therefore not a serious pathogen (Kirisits and Schwanda 2015).

According to the national forest inventory, *F. angustifolia* and *F. excelsior* cover 1.1% and 0.6% of the forest area, respectively, and together constitute 2% of the total standing wood volume in Serbia (Banković et al. 2009). Despite being rare in forest stands, *Fraxinus* spp. are of particular value because they create unique ecological niches in floodplain forests and mixed beech forests and are important as ornamental trees in city parks and greeneries.

In Serbia, the occurrence of *H. fraxineus* has been monitored since ash dieback was reported in neighboring Croatia (Barić and Diminić 2010) and Bosnia and Herzegovina (Treštić and Mujezinović 2013, T. Treštić, pers. comm., the earlier record in this country from 2009 was reported later, in 2014, by Stanivuković et al. 2014). Although symptoms resembling the disease have been observed from 2010 to 2014, *H. fraxineus* was not confirmed, and the observed damages were attributed to other causal agents, such as drought, *Phytophthora* spp. and attacks by the ash weevil (*Stereonychus fraxini* De Geer 1775).

Material and Methods

Sampling was carried out in forests at three localities in September 2015. At each of the localities between 3 and 8 stands or groups of trees were surveyed for the presence of ash dieback symptoms. The focus of the survey was on younger trees (1-3 years old) for the presence of necrotic lesions in the bark, but older trees were also studied for the presence of defoliation and epicormic shoots in the crown. Two localities were in south-western Serbia along the border with Bosnia and Herzegovina, at Tara (N43°56'56'', E19°24'01'', 1000 m a.s.l.) and Debelo Brdo (N44°04'01'', E19°38'16'', 800 m a.s.l.), and one locality was in north-western Serbia at the border with Croatia, at Molovin (N45°11'17'', E19°19'35'', 200 m a.s.l.). The distance between the localities Tara and Debelo Brdo was ca. 40 km, and the distance from these to the locality Molovin was ca. 120 km.

Collected samples included shoots showing symptoms of dieback and necrotic lesions. Disease symptoms on leaves or petioles were not observed during the surveys. In Tara and Debelo Brdo, *F. excelsior* was sampled, while in Molovin shoots were collected from *F. angustifolia*. Fungal isolation followed largely the procedures described by Kirisits et al. (2012). Symptomatic shoots were cut into 5-8 cm long segments containing the zone between necrotic and healthy tissues. Shoot segments were surface sterilized in 96% ethanol for one min, followed by three min sterilization in 4% NaOCl and then for 30 s in 96% ethanol. Subsequently, the outer bark was carefully peeled off and shoot pieces containing phloem and wood were cut off and placed onto 2% malt extract agar (MEA) amended with 100 mg/l of streptomycin sulphate. Petri dishes with shoot pieces were incubated at temperatures between 4 and 10°C in the dark and checked regularly during a period of 6 weeks.

Outgrowing fungal cultures were grouped and preliminary identified based on colony morphology and micromorphological characteristics using microscopy. Molecular identification of fungal cultures was carried out as described by Menkis and Vasaitis (2011). The internal transcribed spacer of the fungal ribosomal DNA (ITS rDNA) was sequenced for representative cultures using primers ITS1F and ITS4 (White et al. 1990). Isolation of DNA was done using the CTAB method, and ITS rDNA was amplified by PCR (Menkis and Vasaitis 2011). Sanger sequencing in both directions was performed by Macrogen Inc. (Seoul, South Korea). Raw sequence data were analyzed using the SeqMan Pro version 12.0 software from the DNASTAR package (DNASTAR, Madison, WI, USA). GenBank database and BLASTn analysis were used to determine the identity of the sequences (Altschul et al. 1997).

Results

The number of trees surveyed during this study was around 1000. At the three study sites, no clearly discernable symptoms of ash dieback were present in the crown of older trees, on which crown thinning and dieback of annual shoots were very similar to the drought driven decline of various other tree species. One- to three-year-old saplings also showed most often only dieback of leading shoots or of smaller lateral shoots, while small lesions in the bark accompanied by wood discoloration, which are typical for ash dieback caused by *H. fraxineus*, occurred sparsely.

During the field survey at three locations, 1-3 symptomatic samples from 45 trees were collected and used for fungal isolation. In total, 10 isolates resembling the ash dieback pathogen were cultured from sampled shoots of *F. excelsior* (7 isolates, 3 from Tara and 4 from Debelo Brdo) and *F. angustifolia* (3 isolates from Molovin). Based on morphological examination, all of them were preliminary identified as *H. fraxineus*. Phialoconidia in short chains were 3.3-4.0 x 2.0 µm in size, while phialides were 15-25 x 3-5 µm in size.

Sequencing of ITS rDNA from four cultures, three from *F. excelsior* and one from *F. angustifolia* resulted in high-quality sequences between 879 and 880 bp in length. Sequence alignment and intraspecific comparisons showed that all of them were identical and therefore belonged to the same fungal species. BLASTn analysis revealed that the closest match of the sequences was to different sequences of *H. fraxineus*, with sequence similarity between 99% and 100% (HM193468, Husson et al. 2011) between them. Sequence similarity to the related and native European species *Hymenoscyphus albidus* (Roberge ex Gillet) W. Phillips was 98% (HM193455, Husson et al. 2011) or lower. The ITS rDNA sequence of one representative *H. fraxineus* isolate from Serbia was deposited at GenBank (accession no. KX255648, isolate NK2-DB 22/3-215 from *F. excelsior*).

Discussion

The results demonstrated that the ash dieback pathogen *H. fraxineus* has spread to Serbia after about 25 years since its first European observation (based on symptoms) in north-eastern Poland. *H. fraxineus* is a wind-dispersed pathogen that spreads with ascospores, which are produced in apothecia on ash leaf rachises and petioles in the leaf litter (Timmermann et al. 2011, Gross et al. 2014). Movement of infected nursery seedlings also played a role in the spread of the fungus across Europe (Timmermann et al. 2011, Kirisits et al. 2012), but this was likely not important in Croatia, Bosnia and Herzegovina, and Serbia, where ash is rarely planted in forests.

On the Balkans and Dinaric Alps, the first report of *H. fraxineus* was from Slovenia in 2007 (Hauptman et al. 2012) and only two years later, in 2009, from Croatia (Zalesina-Gorski kotar, Barić et al. 2012, Županić et al. 2012), from an area which is approximately 400 km from the border with Serbia. Also in 2009, the disease was discovered in the western part of Bosnia and Herzegovina, at Jelašinovci (Stanivuković et al. 2014), and about four years later, in 2013, it was found in a nursery in the central part of Bosnia and Herzegovina (Busovača, Treštić and Mujezinović 2013, T. Treštić, pers. comm.). In 2014, symptoms of ash dieback were observed in eastern Bosnia and Herzegovina, at Vlasenica (N. Keča, unpubl. data), which is located just about 20 km from the border with Serbia. Based on these reports and the results of the present study, it can be estimated that the disease front was moving forward at an average rate of approximately 60 km per year, which is within the range (30-75 km) reported for various parts of Europe, as reviewed by Gross et al. (2014). Natural spread of *H. fraxineus* from Hungary into Serbia is unlikely because there are few ash forests in the northern province of Vojvodina, where the climate is also warm and dry, and thus less suitable for the development of the ash dieback pathogen. According to the most recent reports from Romania (Chira et al. 2016), the pathogen is present in the Carpathian Mountains about 60 km from the Serbian border, but until the end of 2015 symptoms of ash dieback have not been observed on the Serbian side (N. Keča, unpubl. data). Disease incidence at the three localities in Serbia where *H. fraxineus* was confirmed was low, which suggests that *H. fraxineus* was discovered in an early phase of the epidemic.

In the present study, all findings of *H. fraxineus* on *F. excelsior* and *F. angustifolia* were from trees growing in natural forests. Field surveys and sampling in September 2015 showed that the most frequently observed symptom was dead annual shoots, while small necrotic lesions in the bark were encountered only occasionally. Symptoms of decline were observed only on young, 1-3 m high trees in the understory. Fungal culturing and subsequent morphological and molecular identification of isolates confirmed the presence of *H. fraxineus.* Symptoms on *F. angustifolia* were not typical, but the ash dieback pathogen was isolated from totally necrotic 1- to 2-year-old and up to 1 m high understory seedlings which did not show distinct shoot lesions. In 2012 and 2013, such symptoms were connected with extreme drought in the area, but no isolations were attempted. It can therefore not be unambiguously ruled out that trees may already at that time have been damaged by *H. fraxineus*.

It is possible that abundant precipitation in 2014 has stimulated the production of abundant inoculum of *H. fraxineus*, facilitating the pathogen's further spread from Bosnia and Herzegovina through the Dinaric Alps, towards eastern parts of the Balkan Peninsula. Spread towards northern Serbia, i.e. to the region bordering Croatia, was probably along the Sava River where the disease was confirmed in low intensity in 2011 on *F. angustifolia* (Županić et al. 2012).

Further studies are needed in order to evaluate the present distribution and future spread of the ash dieback pathogen into other regions of Serbia and the eastern Balkans as well as its virulence to and impact on different *Fraxinus* species in this part of Europe. It is well documented that the severity of the disease is higher on sites with high relative air humidity, lower temperatures and mesophilic environmental conditions (Keßler et al. 2012, Hauptman et al. 2013, Gross et al. 2014). The coming years will show if warm and dry continental climate will be a limitation for the spread and development of *H. fraxineus* in Serbia and other parts of the eastern Balkans.

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Further Observations on the Association of *Hymenoscyphus fraxineus* with *Fraxinus ornus*

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Abstract

Results of ash dieback symptom observations on 15 seedlings each of *Fraxinus ornus* (flowering ash) and *Fraxinus excelsior* (common ash) which were planted on a forest site in south-eastern Austria where they were exposed to natural inoculum (ascospores) of the ash dieback pathogen *Hymenoscyphus fraxineus* in the growing seasons of 2014 and 2015 are presented. During the observations, until early March 2016 (572-845 days after the seedlings had been planted), necrotic lesions in the bark and wood discoloration, alone or in combination with leaf wilting and/or dieback due to girdling, were cumulatively recorded on all 15 and leaf symptoms associated with *H. fraxineus* on 14 out of the 15 *F. excelsior* seedlings. In contrast, no ash dieback symptoms on woody parts occurred on any of the *F. ornus* seedlings. In autumn 2014 and 2015, a few flowering ash leaves with inconspicuous necrotic lesions on the leaf rachis were observed, and *H. fraxineus* was isolated from 9 out of 20 (45%) symptomatic rachises, and once also from a necrotic leaflet midrib. The observations and fungal isolations confirm and strengthen previous appraisals that *F. ornus* is a host of *H. fraxineus*, that the fungus's occurrence on this ash species is presumably limited to leaves, on which it can complete its life cycle, and that its impact on flowering ash may be low. *F. ornus* is considered to be a highly resistant or tolerant host of *H. fraxineus*, despite it is not known to have undergone co-evolution with this otherwise so damaging invasive alien pathogen.

Key words: *Hymenoscyphus pseudoalbidus*, *Chalara fraxinea*, flowering ash, manna ash, ash dieback

Introduction

Two of the three native European ash species, *Fraxinus excelsior* (common ash) and *Fraxinus angustifolia* (narrow-leaved ash) are highly susceptible to ash dieback (Bakys et al. 2009, Kirisits et al. 2009, 2010, Matlakova 2009, McKinney et al. 2014, Treitler 2014, Hauptman et al. 2016, Havdová et al. 2016), whereas relatively little is known about the association of *Hymenoscyphus fraxineus* with *Fraxinus ornus* (flowering or manna ash). There are no unambiguously confirmed reports that *H. fraxinus* can damage woody parts of flowering ash (e. g. Wallmann and Stingl 2011, Lösing 2013, Kirisits and Schwanda 2015). However, the fungus was recently isolated from leaf rachises with necrotic lesions (Kirisits and Schwanda 2015) and detected with real-time qPCR from asymptomatic leaf blades of *F. ornus* (Schlegel et al. 2016.) Likewise, it was observed to form apothecia on pseudosclerotial leaf rachises and leaflet veins of this ash species in the leaf litter (Gross et al. 2014, O. Holdenrieder, personal communication). The first report of natural infection of *F. ornus* by *H. fraxineus* was based on isolation of the fungus from just four symptomatic leaves (Kirisits and Schwanda 2015). The aim of the present paper is to present further observations on symptoms caused by *H. fraxineus* on flowering ash and on the susceptibility of this ash species to this invasive alien pathogen. A particular purpose is to show photos of symptomatic flowering ash leaves from which *H. fraxineus* was isolated. Based on the present and previous studies, the potential impact of ash dieback on *F. ornus* is appraised and scientific and practical implications are discussed.

Materials and Methods

Observations on the association of *H. fraxineus* with *F. ornus* were made on 15 seedlings of this ash species which had been planted (eight on 16 November 2013, six on 30 March 2014 and one on 16 August 2014) under the canopy of *Picea abies* trees and *Alnus glutinosa* coppice trees at a forest site in Stinatz (Austria, province of Burgenland, 47°12'42.5" N, 16°07'52.9" E, 300 m asl.), outside the natural range of *F. ornus*, where they were exposed to natural inoculum (ascospores) of the ash dieback pathogen. Fifteen seedlings of the highly susceptible *F. excelsior* were planted (six on 16 November 2013, four on 30 March 2014 and five on 16 August 2014) as positive controls along with the *F. ornus* plants. The observations were made on the same seedlings which had been investigated for the first definite report of *H. fraxineus* on *F. ornus* by Kirisits and Schwanda (2015), and further information on the seedlings, the site and the experimental planting can be found there. In summer 2015, two Norway spruce trees on the experimental site were infested and killed by the spruce bark beetle *Ips typographus*. In order to avoid further economic losses, all mature spruce specimens except one were harvested and removed in December 2015; therefore, the seedlings have since then been exposed to more open growing conditions.

The *F. ornus* and *F. excelsior* seedlings were visually inspected for symptoms of ash dieback on the main stem and on side twigs at six dates: 8 November 2014, 24 January, 20 June, 3-4 and 24 October 2015, as well as 10 March 2016. At four dates (8 November 2014, 20 June, 3-4 and 24 October 2015) the plants were also inspected for leaf symptoms. At all inspections symptomatic stem or twig samples (only from *F. excelsior*) and at the inspections on 8 November 2014, 3-4 and 24 October 2015 symptomatic leaf samples (only from *F. ornus*), which were still attached to the seedlings upon collection, were collected and transported to the laboratory, where they were subjected to fungal isolation. In the course of sampling, it was attempted to remove all visible necrotic parts of the stem and symptomatic twigs from affected *F. excelsior* seedlings, in order to free them from the disease. This was done to increase the chance that the plants survive infections, so that they remained available for later inspections.

Fungal isolations were made onto malt extract agar (MEA; 20 g DiaMalt malt extract (Hefe Schweiz AG, Stettfurt, Switzerland), 16 g Becoagar agar (W. Behrens & Co., Hamburg, Germany), 1000 ml tap water, 100 mg streptomycin sulphate (Calbiochem; Merck KGaA, Darmstadt, Germany), added after autoclaving) in 5.2-cm-diameter Petri dishes. From stems and twigs of *F. excelsior* seedlings, 5- to 8-cm-long segments containing, if possible, the transition between necrotic and healthy tissues were cut and subjected to surface sterilization (1 minute in 96% ethanol, 3 minutes in 4% NaClO, 30 seconds in 96% ethanol). The surface sterilized samples were allowed to dry for a few minutes. Thereafter, the outer bark was peeled off from the segments with a sterile scalpel, and 5- to 8-mm-wide discs containing wood and phloem were cut with sterile garden scissors at or near the transition zone between necrotic and healthy phloem or wood (except in the case of entirely necrotic woody parts, where only necrotic samples could be taken). Four discs per stem or twig were placed together in one Petri dish.

For 5- to 8-cm-long leaf rachis sections and leaflets (with necrotic midribs or necrotic dots) of *F. ornus* times of surface sterilization were shortened (30 seconds in 96% ethanol, 2 minutes in 4% NaClO, 30 seconds in 96% ethanol). From leaf rachises the epidermis was peeled off with a sterile scalpel after surface sterilization, but from leaflet midribs the epidermis could only partially be removed or not at all. Care was taken, however, to cut away surrounding parts of the leaf blade as best as possible. Depending on the extension of necrosis, 5- to 10-mm-long fragments from leaf rachises and leaflet midribs were taken at or near the transition between necrotic and healthy tissues or from entirely necrotic tissues. Four to 10 fragments per leaf rachis and four fragments per leaflet midrib were sampled; and four to five fragments from the same rachis or leaflet midrib were placed together in one Petri dish. From leaflets, discs containing necrotic dots were punched out with a sterile 5-mm-diameter cork borer. Three to four leaf discs per leaflet were placed together onto one MEA plate.

The primary isolation plates were incubated either at room temperature and diffuse daylight or at low temperatures (4-6 °C) in the dark, and repeatedly inspected for the growth of microorganisms during a period between six and eight weeks after isolation. *H. fraxineus* was determined based on its colony morphology and morphological characteristics of its asexual stage (Gross et al. 2014). For the first report of *H. fraxineus* on *F. ornus* (Kirisits and Schwanda 2015), the identity of two isolates was verified by ITS rDNA sequencing (GenBank accession numbers: isolate ST/FO/BS/2-1 = CBS 139781, KP994899; isolate ST/FO/BS/3 = CBS 139782, KP994900), following the methods described by Schwanda and Kirisits (2016), but the newly recovered isolates were solely determined morphologically. The isolation of other fungi was recorded, but they were not identified.

Kirisits and Schwanda (2015) reported the symptom observations on and isolation results from the *F. excelsior* and *F. ornus* seedlings at the site in Stinatz up to the inspection on 24 January 2015. These earlier results are incorporated in the present paper, and they are complemented with observations and isolations conducted until March 2016.

Results

During the observation period, until 10 March 2016 (572-845 days (about 82-121 weeks) after the seedlings had been planted), symptoms of ash dieback on woody parts (necrotic lesions in the bark and wood discoloration, alone or in combination with leaf wilting and/or dieback because of stem or twig girdling) were cumulatively recorded on all 15 *F. excelsior* seedlings, of which three (20%) died due to the disease. On 13 plants (87%) symptoms occurred on the stem (on nine of these additionally also on side twigs), and on two (13%) only on side twigs. *H. fraxineus* was isolated from 13 of 14 diseased seedlings (93%) from which isolations were made; from nine it was obtained in pure culture and from four in mixed culture with other fungi (Table 1). In contrast to *F. excelsior*, no ash dieback symptoms on the main stem or on side twigs were observed on any of the 15 *F. ornus* seedlings at any of the six inspection dates.

At the inspections in autumn 2014 and 2015, leaf symptoms (necrotic lesions on rachises and leaflet veins) previously found to be associated with *H. fraxineus* (Bakys et al. 2009, Kräutler and Kirisits 2012, Gross et al. 2014, Steinböck 2014, Schwanda and Kirisits 2016) were frequently observed on *F. excelsior*. In November 2014, 12 of the 15 seedlings (80%) showed such symptoms (the remaining three were already fully defoliated; Kirisits and Schwanda 2015), while in October 2015, symptomatic leaves were recorded on ten of the 12 plants (83%) that were still alive. Considering the autumn assessments in both years, 14 of 15 seedlings (93%) were affected by leaf symptoms in at least one year, while the remaining seedling had shed all its leaves in November 2014 and was already dead at the inspections in October 2015.

At the inspection on 8 November 2014, all *F. ornus* seedlings except the one planted in August 2014 were abundantly affected by leaf symptoms due to various, largely unknown causes, particularly on leaflets, and *H. fraxineus* was (rather unexpectedly) isolated from four leaf rachises (from one in pure culture) with inconspicuous necrotic lesions derived from leaves that were still attached to the plants at the time of collection (see Kirisits and Schwanda (2015) for further details). At the inspection on 24 October 2015, all seedlings except one displayed again various types of leaf symptoms at high frequencies. The most common symptoms were nearly circular or irregularly shaped necrotic lesions or blotches on leaflets presumably caused by the gall midge *Dasineura fraxinea* (Figure 1E-G; Schwerdtfeger 1981). As in the previous year, a few necrotic lesions on leaf rachises (Figure 1A-C) and in one case on a leaflet midrib (Figure1D), which resembled symptoms occurring in connection with ash dieback on *F. excelsior* (Bakys et al. 2009, Kräutler and Kirisits 2012, Gross et al. 2014, Steinböck 2014, Schwanda and Kirisits 2016), were observed. *H. fraxineus* was isolated from 5 of 16 rachises (31%) with necrotic lesions (twice in pure culture; examples of rachises with lesions from which the fungus was isolated are shown in Fig. 1A-C) and in mixed culture with other fungi from the necrotic leaflet midrib just mentioned before (Fig. 1D). Isolations were also done from two other necrotic midribs (one associated with damage by *D. fraxinea*) but *H. fraxineus* was not recovered from them (Table 1). Isolation frequencies from leaf rachises summarized for November 2014 and October 2015 are shown in Table 1. Another symptom observed were small necrotic dots on leaflets (Fig. 1H), which were similar to those caused by *H. fraxineus* on *F. excelsior* (Gross et al. 2014, Steinböck 2014). Attempts to isolate the ash dieback pathogen from leaf blade tissues showing such symptoms were, however, not successful (Table 1).

Table 1. Frequencies of *Hymenoscyphus fraxineus* (Hf) and other fungi (Of) isolated from 2014 to 2016 from stems and twigs of *Fraxinus excelsior* with necrotic lesions in the bark and wood discoloration, and in autumn 2014 and 2015 from different parts of symptomatic *Fraxinus ornus* leaves (leaf rachises and leaflet midribs with necrotic lesions, as well as leaf blades with necrotic dots) at the experimental planting in Stinatz (Austria)

	Sample size (N) and isolation frequency (%)[1]						
Fraxinus species and plant part	N	Hf (total)	Only Hf	Hf and Of	Only Of	Of (total)	Sterile
F. excelsior, stems and twigs	14[2]	93	64	29	7	36	0
F. ornus, leaf rachises	20[3]	45	15	30	55	85	0
F. ornus, leaflet midribs	3[4]	33	0	33	67	100	0
F. ornus, leaf blades	31[4]	0	0	0	39	39	61

[1]For leaf rachises and leaflet midribs percentages refer to samples from which *H. fraxineus* and other fungi were isolated from at least one fragment per rachis or midrib. For leaf blades percentages refer to individual fragments / discs taken from areas of leaflets with necrotic dots (three to four were taken from each of eight leaflets from two trees). Other fungi were not identified. Sterile refers to leaf blade samples yielding no fungal growth.

[2]Isolations were made from samples collected on the following dates (number of seedlings in parentheses): 8 November 2014 (1); 24 January (5), 20 June (3), 3-4 October (3), 24 October 2015 (1); and 10 March 2016 (1).

[3]Isolations were made from four leaf rachises collected on 8 November 2014 (from all of which *H. fraxinus* was isolated; Kirisits and Schwanda 2015) and from 16 leaf rachises collected from seven different trees on 3-4 and 24 October 2015.

[4]Isolations were made from leaves collected on 24 October 2015.

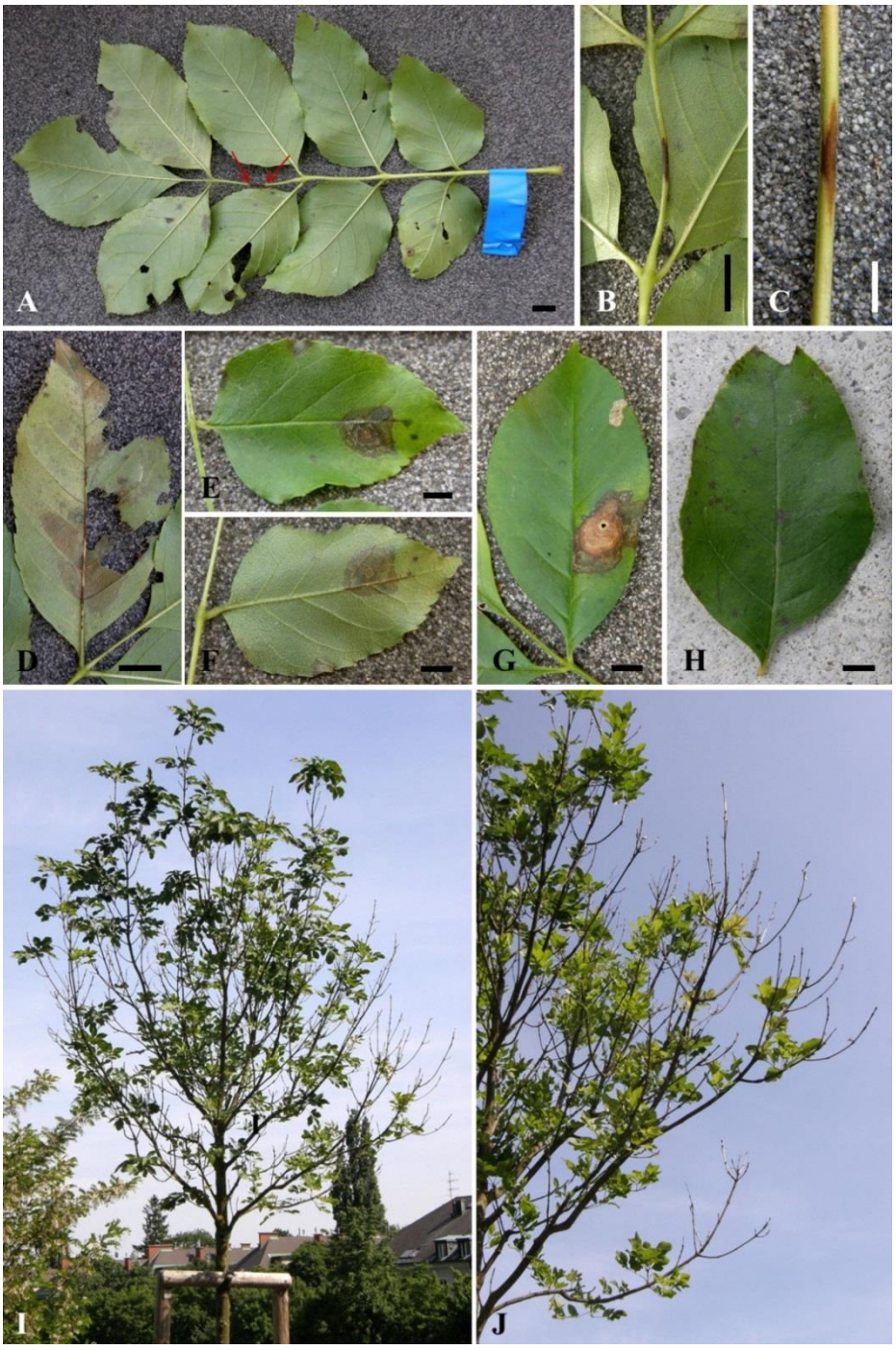

Figure 1. A-H, examples of leaf symptoms on *Fraxinus ornus* at the experimental planting in Stinatz at the inspection on 24 October 2015: A, a leaf with a small, inconspicuous necrotic lesion (indicated with red arrows) on the rachis, shown in (B) at higher magnification, C, another necrotic rachis lesion, D, a leaflet with mechanical damage and necrosis of the midrib, E-G, necrotic lesions and blotches on leaflets presumably caused by the gall midge *Dasineura fraxinea* (E upper side and F lower side of the same leaflet), H, a leaflet with small necrotic dots on the leaf blade; scale bars: 10 mm (A, B, D), 5 mm (C, E, F, G, H); *Hymenoscyphus fraxineus* was isolated in mixed culture with other fungi from the necrotic rachis lesions shown in A and B, and C, as well as from the necrotic leaflet midrib shown in D; I-J, shoot and twig dieback resembling symptoms of ash dieback on a shade tree of *Fraxinus ornus* in an alley in Vienna (Dornbach, 21 May 2009); *Hymenoscyphus fraxineus* was not isolated from any symptomatic shoot or twig of this tree, and the damage was suspected to be due to other causes (probably intensive flowering in the previous year, frost or abiotic stress because of recent planting of the tree)

Discussion

The observations and isolation results reported here confirm and strengthen the appraisals by Kirisits and Schwanda (2015) that *F. ornus* is a host of *H. fraxineus*, that the occurrence of the fungus on this ash species is presumably limited to leaves and that its impact on flowering ash may be low, in contrast to *F. excelsior* and *F. angustifolia* which are severely damaged by ash dieback (Bakys et al. 2009, Kirisits et al. 2009, 2010, Matlakova 2009, McKinney et al. 2014, Treitler 2014, Hauptman et al. 2016, Havdová et al. 2016, Schwanda and Kirisits 2016). The high incidence of ash dieback on the *F. excelsior* seedlings and the mortality of a portion of the plants (despite visually recognizable necrotic parts were removed during inspections) indicate a considerable infection pressure by *H. fraxineus* at the experimental site in Stinatz. Despite this, no ash dieback symptoms on the stem or on side twigs of the *F. ornus* seedlings were recorded.

Careful inspection of just 15 seedlings during about 16 months may be viewed as insufficient to definitely conclude that *H. fraxineus* does not damage woody parts of *F. ornus*. However, this conclusion is supported by the absence of ash dieback on *F. ornus* during inspections in Austria, in forests and on shade trees (Kirisits 2008, Wallmann and Stingl 2011), in a nursery planting experiment (T. Kirisits, C. Gartlehner, C. Lamberg and H. Konrad, unpublished data) as well as on potted plants used for inoculation experiments in the institute's garden (K. Schwanda and T. Kirisits, unpublished observations). In all these situations, at least a few and often many plants of adjacently growing susceptible ash species (*F. excelsior* and sometimes also *F. angustifolia*) showed necrotic lesions in the bark and shoot dieback. Likewise, unambiguous ash dieback symptoms on flowering ash have as yet not been reported from other European countries, including Germany (Lösing 2013), Italy (Luchi et al. 2012, Kirisits and Schwanda 2015, Ghelardini et al. 2017), Slovakia (Longauerová et al. 2012), Slovenia (Hauptman et al. 2012) and Switzerland (Engesser and Meier 2012). Cankers and dieback on *F. ornus* reported by Lehtijärvi et al. (2009) from Turkey and twig dieback by Kopinga and de Vries (2017) from Amsterdam (the Netherlands) were likely caused by other agents; symptoms were not associated with *H. fraxineus* in the former study and pathogen detection was not done in the latter study. At one instance, in May 2009, shoot dieback closely resembling ash dieback was observed on flowering ash trees growing in an alley in Vienna (Figure 1I-J), but *H. fraxineus* was not isolated, which emphasizes that such symptoms can have many causes. In the future, crown dieback symptoms on *F. ornus* should be viewed with caution, and not considered to be caused by *H. fraxineus* unless its involvement is proven by isolation or molecular detection.

In the autumn of both years, foliage of the *F. ornus* seedlings was rarely and negligible affected by necrotic lesions on the rachis and on leaflet veins, the symptoms *H. fraxineus* could be partially linked to. The fungus's low isolation frequency in 2015 may be due to its slow growth, making its detection by isolation onto agar media difficult. It is also possible that other fungi (e. g. some of those mentioned by Schlegel et al. (2016)) can cause necrotic lesions on leaf rachises, particularly on senescent foliage late in the growing season when the inspections were made. Foliage of the *F. excelsior* seedlings at the site in Stinatz was generally more frequently and more severely affected by necrotic lesions on rachises and leaflet veins (shown to be caused by *H. fraxineus* in previous studies; Bakys et al. 2009, Kräutler and Kirisits 2012, Gross et al. 2014, Steinböck 2014, Schwanda and Kirisits 2016). This indicates, in agreement with the total absence of shoot symptoms, that *F. ornus* leaves are highly resistant or tolerant to *H. fraxineus*, respectively that the fungus displays only a low level of virulence to leaves of this ash species. This view is consistent with wound inoculation experiments on leaves, in which *H. fraxineus* was pathogenic to *F. ornus*, but more virulent to *F. excelsior* and *F. angustifolia*, respectively the two latter ash species were more susceptible than the former one (Schwanda and Kirisits 2016). In line with these results, *H. fraxineus* grows significantly slower on agar media amended with leaves of *F. ornus* than on media amended with leaves of *F. excelsior* and *F. angustifolia* (Carrari et al. 2015).

The scarce occurrence of symptoms caused by *H. fraxineus* on *F. ornus* leaves late in the growing season could indicate that the fungus behaves mainly as an endophyte on this ash species, because endophytes often cause symptoms on senescent foliage towards the end of the growing season. Alternatively, the low incidence of symptoms may be because *H. fraxineus* is a relatively rare colonizer of flowering ash leaves. In a study in Switzerland, the ash dieback pathogen was not isolated, but scarcely detected with real-time qPCR from symptomless leaflets (Schlegel et al. 2016). The behaviour and colonization profile of *H. fraxineus* in flowering ash leaves (see Hietala et al. (2013) and Steinböck (2014) for *F. excelsior*) and whether the fungus is associated with other leaf symptoms such as necrotic dots on leaflets observed in this study (Fig. 1H) require further investigation.

In the present study, leaf debris in the litter was not inspected for apothecia of *H. fraxineus*, but fruiting bodies were observed on naturally infected pseudosclerotial leaf rachises and leaflet veins of *F. ornus* in Switzerland (Gross et al. 2014, O. Holdenrieder, personal communication) and Austria (T. Kirisits, unpublished observations), which indicates that the fungus can complete its life cycle on leaves of this ash species. In contrast, apothecia of *Hymenoscyphus albidus* are not known to occur on flowering ash (Baral and

Bemmann 2014); it may therefore not be a host of this long-known native European sibling species of *H. fraxineus*.

All available evidence suggests that *F. ornus* is highly resistant or tolerant to *H. fraxineus* (see Landolt et al. (2016) for a discussion of resistance and tolerance in the context of ash dieback), unless the fungus changes its behaviour. Such a behavioural change could be caused by the introduction of more virulent strains of the pathogen from Asia, as discussed by Gross and Sieber (2016). In wound inoculation trials, *H. fraxineus* incited necrotic lesions and wood discoloration on stems of flowering ash seedlings (Kirisits et al. 2009, Matlakova 2009), which indicates that under natural conditions defence mechanisms on and in leaves or in the leaf scar region prevent the fungus to progress into shoots. Intriguingly, *F. ornus* may be more resistant to ash dieback than *Fraxinus mandshurica* (Manchurian ash), one of the natural hosts of *H. fraxineus* in Asia. On *F. mandshurica*, *H. fraxineus* was originally thought to occur as a harmless leaf endophyte (Gross et al. 2014, Cleary et al. 2016), but Drenkhan et al. (2017) recently reported that the fungus is associated with frequently occurring leaf symptoms on this ash species in its native range in Far East Russia. *H. fraxineus* also causes symptoms on both leaves and shoots of introduced Manchurian ash in Estonia (Drenkhan and Hanso 2010). Moreover, in wound inoculation experiments it incited necrotic lesions in the bark on *F. mandshurica* var. *japonica* seedlings (Gross and Holdenrieder 2015).

Within the genus *Fraxinus*, *F. ornus* belongs to the section *Ornus* (consisting of species occurring in Eurasia), in contrast to *F. excelsior* and *F. angustifolia*, which form part of the section *Fraxinus* (with species distributed in Eurasia and North America) (Wallander 2008). Flowering ash is the first species in the section *Ornus* known to be a host of *H. fraxineus* outside the pathogen's natural range. The high resistance or tolerance of this ash species to ash dieback is despite it is not known to have undergone co-evolution with *H. fraxineus*, which is so damaging to its two other European hosts, *F. excelsior* and *F. angustifolia*. Likewise, the low susceptibility of *F. ornus* to *H. fraxineus* can likely not be explained by co-evolution with *H. albidus*, which has, as mentioned already, so far not been recorded on this ash species. One of the known natural hosts of *H. fraxineus* in Asia, *F. rhynchophylla* or *F. chinensis* subsp. *rhynchophylla* (Korean ash; Han et al. 2014, Gross and Han 2015; treated as a synonym of *F. chinensis* by Wallander (2008)) also forms part of the section *Ornus*, in contrast to *F. mandshurica* which belongs to the section *Fraxinus* (Wallander 2008). This may reflect a shared evolutionary history of *F. ornus* with an Asian host of *H. fraxineus* in the section *Ornus*, which left resistance genes and traits to the fungus in the former ash species. However, in Far East Russia *F. rhynchophylla* was affected by leaf symptoms associated with *H. fraxineus*, suggesting that the fungus is a pathogen on this ash species as well (Drenkhan et al. 2017). Present evidence may suggest that the susceptibility of various ash species to *H. fraxineus* is related to their phylogenetic position within the genus *Fraxinus*, with species in the section *Fraxinus* outside the pathogen's natural range (*F. angustifolia*, *F. excelsior*, *F. nigra*) being highly susceptible, those in the section *Ornus* (*F. ornus*) showing low susceptibility and species in the section *Melioides* (distributed in North and Central America; *F. americana*, *F. pennsylvanica*) displaying intermediate susceptibility (Kirisits et al. 2009, 2010, Matlakova 2009, Drenkhan and Hanso 2010, Lösing 2013, Gross et al. 2014, McKinney et al. 2014, Treitler 2014, Gross and Sieber 2016, Kowalski et al. 2015, Hauptman et al. 2016, Havdová et al. 2016, Schwanda and Kirisits 2016). However, knowledge on more ash species is necessary to definitely infer on the relationship between the phylogeny of various *Fraxinus* spp. and their susceptibility to *H. fraxineus*.

Based on the present state of knowledge, *H. fraxineus* will likely not have a serious impact on *F. ornus* in natural and managed ecosystems and not impair its future use as forest and ornamental tree. In fact, the further invasion of the fungus into the natural distribution range of flowering ash (from which it is presently largely absent) may occur inconspicuously (if it does not co-occur with one or both of the other, susceptible, European ash species) because symptoms of infections are not striking and difficult to detect. In the search for the genetic background of resistance or susceptibility to *H. fraxineus* within the genus *Fraxinus*, flowering ash can be viewed as a "model" species of a highly resistant host (Harper et al. 2016). The findings presented here have also quarantine implications because *H. fraxineus* may be moved to new areas on plants with leaves or with pseudosclerotial leaf parts of *F. ornus*, a risk which can be greatly reduced if only bare-rooted material from which any accompanying leaf debris is removed is traded. Finally, the example of *F. ornus* emphasizes that exposing plants to high loads of ascospores under field, semi-field or laboratory conditions is presumably the best way to study the susceptibility of ash species to *H. fraxineus*, the symptomatology of ash dieback on various hosts and the infection biology of the pathogen (Cleary et al. 2013, Kirisits and Schwanda 2015).

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Fungal Community in Symptomatic Ash Leaves in Spain

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Abstract

Mycobiota inhabiting symptomatic leaves of *Fraxinus excelsior* from two sites in Asturias, northern Spain, was analyzed to investigate the occurrence of pathogenic fungal species on European ash such as *Hymenoscyphus fraxineus*. Leaves were collected in fall 2014 and isolations were made from petioles showing discolorations. The morphological characterization of 173 isolates resulted in eight morphotypes, whereas the phylogenetic analysis resulted in seven genera, *Alternaria*, *Phomopsis* and *Phoma* being the most frequently isolated. Neither *H. fraxineus*, nor other *Hymenoscyphus* species were detected. The absence of *H. fraxineus* is consistant with field observations where no typical ash dieback symptoms were recorded. Most of the fungi isolated are known plant pathogens and some of them have occasionally produced disease symptoms on ash after artificial inoculations. Nevertheless, their natural behaviour as pathogens on *F. excelsior* remains unclear, and could be significantly influenced by different factors as environmental conditions or endophyte presence.

Keywords: *Fraxinus excelsior*, northern Spain, symptomatic leaves, pathogenic fungi, ITS-sequencing.

Introduction

Fraxinus is a genus of flowering plants belonging to the Oleaceae family. Species in this genus are usually medium to large trees and widespread across much of Europe, Asia and North America. The European ash or common ash (*Fraxinus excelsior*) is an ecologically important tree in Asturias, a region in northern Spain, where it has played an important role throughout history presenting a wide range of uses, such as wood resource, human and cattle nourishment, or as a medicinal plant. At present, *F. excelsior* in Europe is threatened by the Ascomycete fungus *Hymenoscyphus fraxineus* (synonym: *Hymenoscyphus pseudoalbidus*, basionym: *Chalara fraxinea*), the causal agent of ash dieback. *H. fraxineus* is an alien invasive fungus originated in East Asia (Zhao et al. 2012) which has been spreading across Europe since the mid-1990s, causing massive damage and mortality to susceptible ash species, mainly *F. excelsior* but also *F. angustifolia* (Gross et al. 2014). At present, this fungus has not been reported in Spain, therefore it would be interesting to study whether this pathogen has arrived before clear symptoms are visible. The aim of the present work was to study the fungal biota inhabiting common ash leaves with disease symptoms in Asturias. For this purpose, a morphological and molecular characterization of fungal cultures isolated from symptomatic ash petioles was conducted.

Material and methods

Study site description and sampling design

The study area is located in Asturias, officially the Principality of Asturias, a region in north-western Spain. Plant material was collected in autumn 2014 in two central areas, Aller (site 1, coordinates: 43.0621, 5.3450), and Llanera (site 2, coordinates: 43.2716, 5.5108). There were 5 sampling points within each area, which were at least 5 km apart from each other. At each sampling point (approx. 50 ha), 10 leaves from 10 trees were collected. Totally, a hundred ash trees were sampled. Even though trees showed a healthy common appearance, all collected leave samples showed disease symptoms such as leaf spots and petiole discoloration.

Analysis of samples, fungal isolation and morphological characterization

Isolation was conducted as follows, two leaf petioles per sample were washed with distilled water and blotted

dry. Next, they were disinfected by submerging them into 100% ethanol for 30 s, air-dried, peeled and cut off in three segments with a sterile scalpel. A small part of each segment was placed onto ash-agar medium (15 g/l of Agar, 20 g/l of Bacto Malt Extract, 50 g/l of ash leaf solution and 0.4 ml/l of Streptomycin) plates, and next incubated in the dark at room temperature during 4-6 weeks. The plates were inspected daily, and one outgrowing culture per leaf petiole was sub-cultured onto potato dextrose agar (PDA, Difco) plates. The plates were incubated at room temperature on a laboratory bench at daylight conditions for 15 days. Isolates were morphologically assessed and classified into morphological similarity groups.

DNA extraction, PCR and ITS sequencing

A representative number of isolates for each morphological group was chosen for DNA analysis. DNA extraction was performed according to Schoebel et al. 2014. The species identity was verified by ITS sequencing using ITS1 and ITS4 primers (White et al. 1990). Thermal cycling conditions were: 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55°C, 2 min at 72 °C, and a final extension of 72 °C for 10 min. PCR products were fractionated by electrophoresis and visualized under UV. For PCR purification the IllustraExoStar 1-Step kit (Fisher scientific) was used. Cycling conditions were: 1 min at 96 °C, followed by 25 cycles of 10 s at 95 °C, 5 s at 50°C and 1 min at 60 °C. Sequencing was performed in both directions, using an ABI PRISM 3130 (Applied Biosystems).

Phylogenetic analysis

Sequences were manually checked and edited to obtain a consensus sequence, and compared with reference sequences deposited in the BLAST database (http://www.ncbi..nlm.nih.gov/BLAST/). Assembled sequences were aligned and used to reconstruct a phylogenetic tree using the UPGMA method with kimura 80 as nucleotide distance measure, using CLC Genomics Workbench (version 8.0 Beta 4). All ITS sequences obtained in this work were deposited at GenBank.

Estimation of frequencies

Isolation frequencies of each genera or species were estimated. Isolation frequency was calculated as the percentage of isolates of individual genera or species in relation to the total number of isolates obtained from each site.

Results

Morphological characterization of fungal isolates

In total, 173 fungal cultures were isolated from the 200 leaf petioles analysed. Most of the cultures shared common morphological characteristics, therefore they were classified in groups of similar morphology. Totally, there were eight morphotypes (table 1), which included 163 isolates. In addition, 10 isolates were morphologically different from all other isolates so they were considered as single morphotypes. Only few isolates showed a morphology similar to *H. fraxineus.*

Genetic characterization of morphotypes

A selection of 31 representative isolates of morphological groups and single isolates, were identified by ITS sequencing. The results of the identification are presented in table 1 and the phylogenetic relationship is depicted in figure 1. Even though a few isolates showed morphological characteristics similar to *H. fraxineus*, the subsequent ITS sequencing showed that all of them did not belong to this species. As it can be seen in the phylogenetic tree (figure 1) there were two main clusters and one isolate (C9b: *Harzia acremonioides*) very dissimilar to both clusters. One cluster included 15 isolates of the morphological groups III, IV, VI, VII and a group of single isolates. The second cluster comprised 15 isolates of the morphotypes I, II, V, VIII and four single isolates.

Estimation of frequencies

From the 200 petioles analyzed (two petioles per sample), 173 (86.5%) gave fungal growth. There were seven fungal genera included in the eight morphological groups, and three genera, one family and three species belonging to single morphotypes. Data are shown in table 2.

Discussion and conclusions

This study reveals a high prevalence of plant pathogenic fungi in European ash in Asturias. Based on morphological culture characteristics of the isolates, eight morphotypes were established. ITS sequencing and phylogenetic analysis revealed seven genera among these morphotypes. These genera included *Phomopsis* spp, *Phoma* spp, *Fusarium* spp, *Epicoccum* spp,. and *Rossellinia* spp, in descending order in respect to isolation frequency. In addition, there were two closely related genera included in morphotype II, belonging to the Pleosporales, *Stemphylium* spp and *Alternaria* spp, this last being more frequently isolated (3:1). Each genera group corresponded to a different morphological group, with the exception of *Phoma* spp and *Phomopsis* spp, which include two morphotypes each (I and VIII, VI and VII, respectively). There were no large morphological differences observed between these pairs of groups.

Totally, 173 fungal isolates were obtained from both sampling sites, 89 isolates from site 1 and 84 from site 2. The fungal community was very similar at both sites with *Alternaria* spp, *Phomopsis* spp, *Phoma* spp and *Fusarium* spp being the most frequent followed by *Rosellinia* spp and

Table 1. ITS-based identification of fungal cultures isolated from leaves of *Fraxinus excelsior* in Asturias

Isolates						Most closely related database sequence				
Code	Site	MG	GB accession no.	SL (bp)	Identification	GB accession no.	QC (%)	Id (%)	Identification	Reference
C2b	1	VII	KT948355	552	*Phomopsis rudis*	KC343230.1	100	99	*Diaporthe rudis (Phomopsis rudis)*	Gomes et al. 2013
						FJ228188.1			*Diaporthe viticola (P. rudis)*	Bakys et al. 2009
C3b	1	*	KT948356	492	Fungal sp.	FJ228206.1	96	99	Fungal sp.	Bakys et al. 2009
						HQ414588.1				Scholtysik et al. 2013
C7b	1	VII	KT948357	545	*Phomopsis sp.*	KC843328.1	100	100	*Diaporthe cotoneastri* (*P. cotoneastri*)	Gomes et al. 2013
						KC343073.1			*Diaporthe eres* (*P. oblonga*)	
						HE774484.1			*Phomopsis sp.*	Hauptman et al. 2013
C9b	1	*	KT948358	569	*Harzia acremonioides*	HQ698593.1	97	99	*Harzia acremonioides*	nd
CAO1b	1	VI	KT948359	536	*Phomopsis sp.*	FN386273.1	99	99	*Phomopsis sp.*	nd
CAO4a	1	I	KT948360	502	*Phoma sp.*	EU852354.1	100	100	Uncultured *Phoma*	Bakys et al. 2009
CAO5	1	IV	KT948361	522	*Fusarium sp.*	KM189440.1	100	100	*Fusarium avenaceum*	nd
						GQ922561.1			*Fusarium tricinctum*	
CAO7b	1	I	KT948362	506	*Phoma sp.*	EU852354.1	100	99	Uncultured *Phoma*	Bakys et al. 2009
						AJ608976.1			*Phoma exigua*	nd
						EU343118.1			*Phoma exigua* var. *exigua*	
						JQ804843.1			*Phoma exigua* var. *foveata*	
R6b	1	II	KT948371	531	*Alternaria sp.*	KM215624.1	100	100	*Alternaria sp.*	nd
R7b	1	V	KT948372	505	*Epicoccum nigrum*	AF455395.1	100	100	*Epicoccum nigrum*	nd
R10b	1	III	KT948373	558	*Rosellinia mammiformis*	KF719200.1	100	100	*Rosellinia mammiformis*	nd
ST1b	1	I	KT948380	502	*Phoma sp.*	EU852354.1	100	100	Uncultured *Phoma*	Bakys et al. 2009
ST6a	1	*	KT948381	506	*Phoma sp.*	JX160059.1	100	99	*Phoma sp.*	nd
T4a	1	*	KT948382	514	*Phacidium mollerianum*	KR873247.1	100	100	*Phacidium mollerianum*	nd
T5b	1	II	KT948383	531	*Alternaria sp.*	KJ541477.1	100	100	*Alternaria sp.*	nd
T6b	1	*	KT948384	520	Fungal sp.	GU174285.1	100	99	Uncultured fungus	nd
T7	1	*	KT948385	520	Fungal sp.	EF040841.1	100	99	Uncultured fungus	nd
F1b	2	IV	KT948363	522	*Fusarium lateritium*	JQ693397.1	100	100	Fusarium lateritium	nd
F5a	2	VIII	KT948364	501	*Phoma* sp.	JX160059.1	100	100	*Phoma* sp.	nd
F9b	2	II	KT948365	543	*Stemphylium* sp.	AB979903.1	100	98	Stemphylium sp.	nd
L2b	2	*	KT948366	503	*Mycosphaerellaceae* sp.	EU167596.1	100	99	*Micosphaerella coacervata*	nd
						EU167590.1			*Micosphaerella linorum*	
						AB435070.1			*Micosphaerella delegatensis*	
						EF394864.1			*Septoria* sp	
L9	2	VII	KT948367	539	*Phomopsis theicola*	HE774477.1	100	100	*Diaporthe foeniculina* (*P. theicola*)	Hauptman et al. 2013

MG: morphological group, GB accession no: GeneBank accession number, SL: sequence length, QC: query cover, Id: identity, *: single isolate, nd: no data

Table 1. Continued

Isolates						Most closely related database sequence to isolates of this work				
Code	Site	MG	GB accession no.	SL (bp)	Identification	GB accession no.	QC (%)	Id (%)	Genus or species	Reference
M5a	2	IV	KT948368	524	*Fusarium sp.*	JX114790.1	100	100	*Fusarium acuminatum*	nd
						AY188923.1			*Fusarium tricinctum*	
M6b	2	VI	KT948369	545	*Phomopsis sp.*	NR_119726.1	100	100	*Diaporthe cotoneastri* (*P. cotoneastri*)	nd
						KC343073.1			*Diaporthe eres (P. oblonga*)	Gomes et al. 2013
						HE774484.1			*Phomopsis sp*	Hauptman et al. 2013
M10b	2	III	KT948370	558	*Rosellinia mammiformis*	KF719200.1	100	100	*Rosellinia mammiformis*	nd
S1b	2	II	KT948374	503	*Alternaria sp.*	KM215624.1	100	100	*Alternaria sp.*	nd
S4	2	*	KT948375	506	*Epicoccum nigrum*	KP132016.1	100	100	*Epicoccum nigrum*	nd
S10	2	*	KT948376	541	*Periconia byssoides*	KC954157.1	100	100	*Periconia byssoides*	nd
SC5b	2	V	KT948377	508	*Epicoccum nigrum*	KM036093.1	100	99	*Epicoccum nigrum*	nd
SC7b	2	*	KT948378	518	*Cladosporium sp.*	KF367491.1	100	98	*Cladosporium sp.*	nd
SC8b	2	I	KT948379	502	*Phoma sp.*	EU852354.1	100	100	Uncultured Phoma	Bakys et al. 2009

MG: morphological group, GB accession no: GeneBank accession number, SL: sequence length, QC: query cover, Id: identity, *: single isolate, nd: no data

Epicoccum spp. Three cultures could not be identified at site 1 because they had database hits as fungal sp or uncultured fungus. Interestingly, one of them, C3b isolate (table 1) is similar to fungal isolates previously recovered from ash (Bakys et al. 2009, Scholtysik et al. 2013). Several isolates from this work showed high genetic identity with isolates previously reported from ash. This is the case of *Phomopsis rudis* (C2b) or *Phoma* sp (CAO4a, CAO7b, ST1b, SC8b) (Bakys et al. 2009, Gomes et al. 2013), *Phomopsis* sp (C7b, M6b) (Gomes et al. 2013, Hauptman et al. 2013), and *Rossellinia mammiformis* (R10b, M10b) (unpublished data, sequences submitted in GenBank by Hamelin et al. 2013) (table 1). Results from our work are in line with previous studies (Przybyl 2002, Pukacki and Przybyl 2005, Bakys et al. 2009, Scholtysik et al. 2013) where *Alternaria* spp, *Cladosporium* spp, *Epicoccum* spp, *Fusarium* spp, *Phoma* spp and *Phomopsis* spp were also the most frequent fungi isolated from symptomatic ash tissues. In our study area, *Cladosporium* spp seems to be rare with only one isolate recovered from site 2 (table 2).

The isolates in the genera *Alternaria* were most frequently isolated, a result that was also obtained by Scholtysik et al. 2013. These authors suggested that high infec-

Table 2. Fungal genera and species isolated from *Fraxinus excelsior* leaves from Asturias

MG	Fungal genera/species identified	No. of isolates and frequency of isolation (%)	
		Site 1	Site 2
I, VIII, *	*Phoma spp*	15 (16.85%)	10 (11.90%)
II	*Alternaria spp-Stemphylium spp (Pleosporales)*	37 (41.57%)	32 (38.10%)
III	*Rosellinia spp*	2 (2.25%)	6 (7.14%)
IV	*Fusarium spp*	9 (10.11%)	8 (9.52%)
V, *	*Epicoccum spp*	4 (4.49%)	4 (4.76%)
VI, VII	*Phomopsis spp*	17 (19.10%)	21 (25%)
*	*Periconia byssoides*	0 (0%)	1 (1.19%)
*	*Phacidium mollerianum*	1 (1.12%)	0 (0%)
*	*Mycosphaerellaceae spp*	0 (0%)	1 (1.19%)
*	*Cladosporium spp*	0 (0%)	1 (1.19%)
*	*Harzia acremonoides*	1 (1.12%)	0 (0%)
*	Not identified	3 (3.37%)	0 (0%)
		89	84

MG: morphological group, *: single isolate

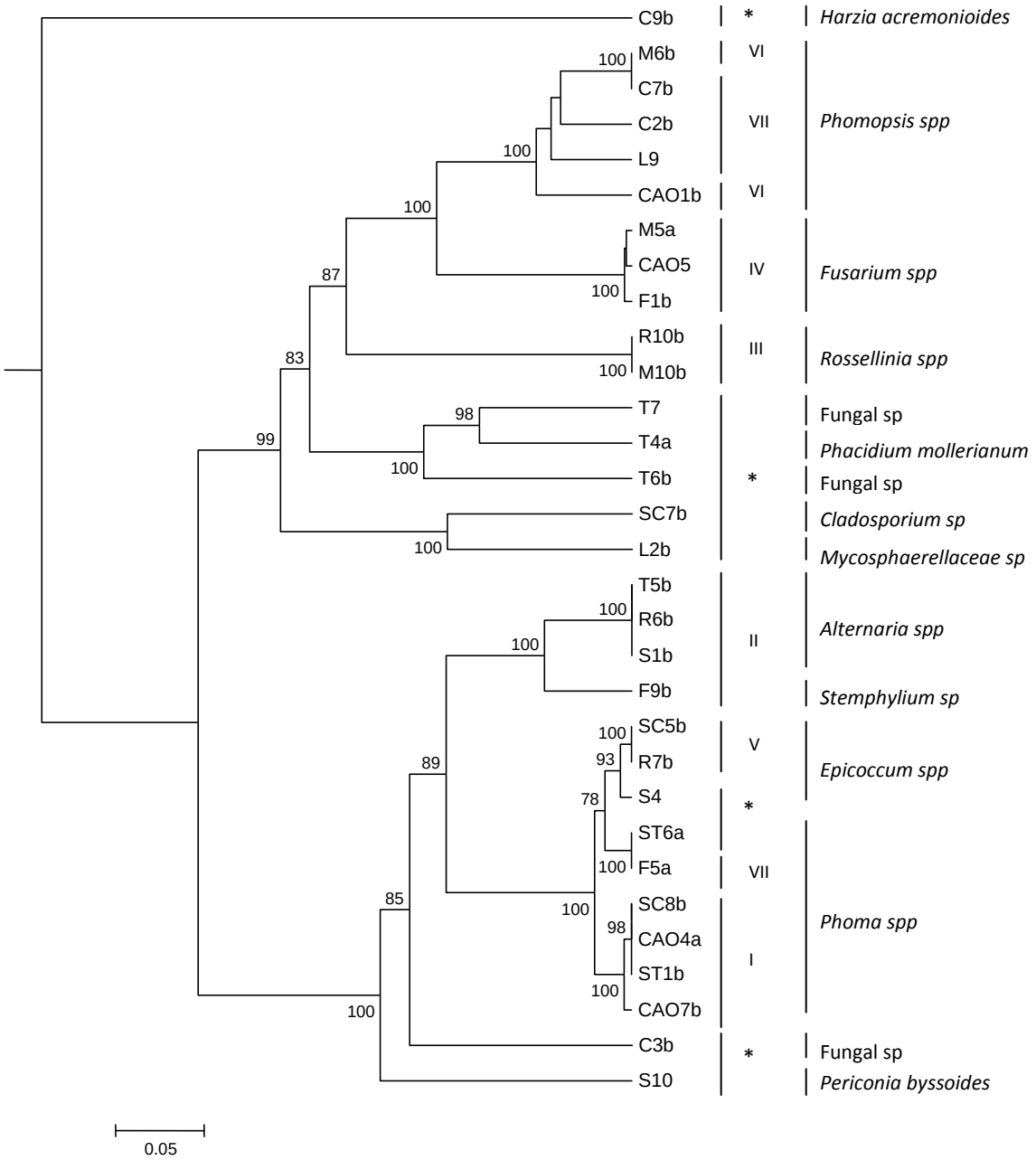

Figure 1. UPGMA tree depicting the phylogenetic relationship between the fungal isolates. Bootstrap support resulting from 1000 replicates is shown on the internodes. The morphological groups and the related fungal genera or species are shown

tion rates of *Alternaria* spp could lead to a premature leaf abscission. Other shared taxa was *Gibberella avenacea* isolated from ash shoots by Bakys et al. 2009, and also obtained in the present work (synonym: *Fusarium avenaceum*, CAO5) (table 1). Therefore, we can observe a similar fungal community isolated from common ash trees, independently of the study area. Concerning pathogenicity, *Phoma exigua* was supposed to cause brown spotting on shoots of European ash seedlings, being responsible for mass dying of seedlings at nurseries in Normandy, France (Boudier 1994). According to Bakys et al. 2009, four species (*A. alternata*, *E. nigrum*, *Phomopsis* sp., and *H. frax-*

ineus) isolated from ash were able to cause necrotic lesions on bark and cambium after artificial inoculation of ash seedlings, however the lesions were relatively small and most of the inoculated trees remained visually healthy. In that study, severe disease symptoms were only observed on seedlings inoculated with *H. fraxineus*. These results are consistent with our field observations in Asturias, where all collected leaf samples showed disease symptoms, but most of the trees remain apparently healthy, and *H. fraxineus* was not isolated. In addition, there are different observations about the susceptibility of *F. excelsior* to ash dieback, partially influenced by climatic stressors such as drought and frost (Pukacki and Przybyl 2005), and possibly the synergistic action of several fungal species (Bakys et al. 2009). Therefore, environmental factors and fungal interactions in addition to genetic resistance should be considered as possible modifiers of the pathogenicity of ash mycobiota in nature. In conclusion, even though some of fungal species isolated in this work are known plant pathogens and they were likely responsible for the disease symptoms observed on *F. excelsior* leaves, their behaviour as pathogens of ash remains still unclear and further research about their role in ash would be highly interesting.

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European *Fraxinus* species Introduced into New Zealand Retain Many of their Native Endophytic Fungi

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Abstract

Fraxinus species were introduced in to New Zealand as amenity trees as early as the mid-1850s. As a likely consequence of this early introduction and of their geographic isolation, *Fraxinus* species in New Zealand have not yet been subjected to the devastating impacts of ash decline caused by *Hymenoscyphus fraxinus*. This study used isolations, PCR and cloning methods to examine the endophytic fungi associated with *Fraxinus excelsior* and *F. angustifolia* on the north island of New Zealand.

Keywords: *Fraxinus excelsior*, *Fraxinus angustifolia*, endophytic communities

Introduction

Endophytic fungi, that is those species growing within plants for all or at least a part of their life cycle without causing any obvious symptoms, are a critical component of the plant ecosystem. Endophytes are known to confer benefits to their host, for example, by increasing host tolerance to stress (Rodriguez and Redman 2007), reducing herbivory through the production of toxic alkaloids (Wilkinson et al. 2000), and via antagonistic effects that reduce infection of plant tissues by pathogens (Arnold et al. 2003). International trade and transportation of tree species for use in forestry, horticulture and as amenity trees in parks and landscapes has led to the potential redistribution of endophytes as cryptic hitchhikers within their inhabited host which may lead to new possibilities for endophyte-host interactions. In theory, a plant introduced into a new environment will either remain colonised by its associated fungi from within its native range (the cointroduction hypothesis) or become colonized by fungi from its new environment

(the host-jumping hypothesis) (Shipunov et al. 2008), or both. Although the ecological implications of either of these events are not well known, the plasticity of endophytic interactions could promote parasitism if hosts are physiologically stressed and/or newly acquired fungi could increase host competitiveness (Brown and MacAskill 2005). In extreme cases, fungal assemblages associated with introduced species have caused extensive damage to native tree species following successful host jumps, e.g. *Neonectria fuckeliana* introduced to New Zealand affecting *Pinus radiata* (Dick and Crane 2009) and *Hymenoscyphus fraxineus* introduced to Europe affecting native *Fraxinus* species (Kowalski 2006; Gross et al. 2014). Thus, the endophyte communities of introduced forest species are of fundamental importance to forest ecosystem health.

Since the early-mid 1990s European ash (*Fraxinus excelsior*) has experienced widespread population decline across Europe (Juodvalkis and Vasiliauskas 2002, Timmerman 2011). The causal agent of this decline has been identified as *Hymenoscyphus fraxineus* (Baral et al. 2014); syn. *H. pseudoalbidus*; anamorph *Chalara fraxinea* T. Kowalski (Kowalski 2006, Queloz *et al*. 2010). Typical symptoms include small necrotic spots on leaves, necrotic lesions on rachises and bark, discoloration of wood, and eventual dieback of twigs, branches and crown (Cleary et al. 2013; Bakys et al. 2009a,b). Trees of all age classes are affected. *Hymenoscyphus fraxineus* in Europe causes symptoms on native *Fraxinus*, namely *F. excelsior*, *F. angustifolia*, and *F. ornus* (Kirisits and Schwanda 2015, Kirisits et al. 2010), but also North American *Fraxinus* species have been affected to some degree (Drenkhan and Hanso 2010; McKinney et al. 2014). Asian *Fraxinus* species planted in Europe and in their native origin of East Asia exhibit only minor or no dieback damage to the crown of trees (Drenkhan and Hanso 2010; McKinney et al. 2014; Cleary et al. 2016).

Fraxinus excelsior and *Fraxinus angustifolia* were introduced to New Zealand by European colonists in the mid-1800s. Since then, both species have established naturally, are commonly found throughout the country, and frequently planted as amenity trees (Allan Herbarium, 2000). No ash species are native to New Zealand. *Hymenoscyphus fraxineus* has not been identified on either *F. excelsior* or *F. angustifolia* in New Zealand, and neither has its non-pathogenic European relative *H. albidus*. The geographic separation of *F. excelsior* and *F. angustifolia* from its native range in Europe provides a unique opportunity to examine endophytic fungal communities in an introduced environment and compare with those documented within its native range to decipher the degree at which communities are influenced by their native or novel associates following establishment. The objective of this study was to: 1) describe the fungal communities associated with *F. excelsior* and *F. angustifolia* in New Zealand and 2) compare community structure to that documented on the same host species in other European countries.

Methods

Study sites and sampling

Field samples were collected from *F. excelsior* and *F. angustifolia* trees at two urban locations in Rotorua, New Zealand (-38.160S, 176.263E; -38.085S, 176.216E). *Fraxinus excelsior* samples were taken from three trees approximately 60-70-years-old that appeared to be healthy. From each tree, three branches were sampled from the lower part of the crown. Samples were collected in August 2009 (during winter) from three different tissue types: bud, bark and wood. *Fraxinus angustifolia* samples were collected in November 2009 (during spring) from rachises of three healthy 20-year-old trees. Samples from all three trees were pooled.

Isolation of fungi in pure culture

Fungal isolations from *F. excelsior* samples were made from all three tissue types (buds, bark and wood). Tissue was surface sterilised with 70% EtOH for 1 min., 3% NaClO for 5 min, repeated 70% EtOH for 1 min, followed by ddH_2O for 1 min, and small sections of tissue (3 x 3 mm) were plated onto 1% malt-extract and incubated at 20°C. Fungal outgrowth was observed for up to 8 weeks thereafter and subcultures of all filamentous fungi was performed to obtain clean isolates. Mycelial tissue was harvested from a select number of representative plates and stored at -20℃ in preparation for DNA extraction. No isolations were performed on *F. angustifolia* samples.

Isolation of DNA, amplification and sequencing

DNA was extracted from both the mycelia of pure cultures and directly from tissue of *F. excelsior* samples. For *F. angustifolia* samples, DNA was extracted directly from tissues. All samples were freeze dried and ground to powder with a Precellys® 24 tissue homogenizer (Bertin Technologies). DNA was extracted using 3% CTAB method described in Cleary et al. (2013), and then purified using the JET quick kit (GENOMED GmbH). All DNA samples were quantified using the NanoDrop (NanoDrop Technologies) and diluted to 1 ng/μL.

PCR amplification of all DNA mycelial samples obtained from pure cultures was performed using primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). The thermal cycling condition was initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30s, annealing at 55°C for 30s, and 72°C for 30s, followed by a final extension at 72°C for 7 min.

Amplifications were performed using the Veriti Thermal Cycler (Applied Biosystems) in 40 μL reactions

containing the following final concentrations: 1 ng/µL template DNA, 0.03 U Dream Taq Polymerase (Fermentas), 200 µM of dNTPs, 2.75 mM of $MgCl_2$, and 0.2 µM of each primer in 1x Buffer. PCR products were visualised on 1% Agarose gels using Gel Green dye (Biotium).

For tissue samples, DNA extraction and PCR was performed similarly to that described above. PCR products were then cloned using the TOPO TA Cloning kit with pCR®2.1-TOPO vector and One Shot TOP10 chemically competent *E. coli* (Invitrogen). Only those samples exhibiting clear bands on the gel were selected for cloning. Bacterial colonies were added directly to a PCR cocktail as described above, but with primers M13 Forward (GTAAAACGACGGCCAG) and M13 Reverse (CAGGAAACAGCTATGAC) (Griffin and Griffin 1993).

All PCR products were purified using AMPure (Agencourt) and sent to Macrogen (Seoul, Korea) for Sanger sequencing on the ABI3730XL in both forward and reverse directions. Sequences were manually aligned and edited using Seqman (DNASTAR Lasergene 8) and Geneious version 5.4.6 (Biomatters Ltd.), respectively. Fungal taxa were determined by comparing sequences with reference databases using BLASTN at GenBank (NCBI), also considering geographic origin of the closest BLAST-sequence match. The ITS sequence homology for delimiting fungal taxa was set at between 98 and 100% for species level, and between 94 – 97% for genus level (Glen et al. 2001; Bakys et al. 2009). Principal component analysis (PCA) was performed on all samples using a presence/absence matrix with Canoco version 4.5 (Plant Research International).

Results

Fungal isolates from F. excelsior:

A total of 36 twig samples were collected from branches of *F. excelsior* trees which yielded 30 bud samples, 34 bark samples and 35 wood samples. Sixty-eight isolates were obtained from these samples, but only 36 isolates were used for sequence identification (the remaining 32 were omitted from further analysis due to unforeseen contamination). Of those 36 samples, 19 originated from bud samples, 15 from bark and two from wood. In total, 23 fungal taxa were detected; ten of which were identified to the species level based on sequence similarities with GenBank entries, 12 were identified to the genus level and one remained unidentified. The fungal taxa belonged to eight orders of Ascomycetes: Eurotiales, Capnodiales, Glomerellales, Hypocreales, Pleosporales, Botryosphaeriales, Diaporthales, Xylariales and an unknown Ascomycete, and to four orders of Basidiomycetes: Ustilaginales, Sporidiales, Cantharellales, Tremellales, and an unknown Microbotryomycetes (Table 1). The most frequently detected fungal taxa were *Phoma* sp. (18%), but this only occurred in the bud tissue.

Tissue samples from F. excelsior

PCR products from 15 of 30 bud samples, 15 of 34 bark samples, and 16 of 35 wood samples were selected for cloning, and was successful on all with the exception of two bark samples. Seventy-eight fungal taxa were identified of which 21 were identified to species level, 23 were identified to genus level, and 34 remained unidentified (Table 2). These fungal taxa represented by 11 orders of Ascomycetes: Helotiales, Dothideales, Hypocreales, Botryosphaeriales, Capnodiales, Glomerellales, Pleosporales, Eurotiales, Chaetothyriales, Diaporthales, Saccharomycetales, and two unknown ascomycetes, and seven orders of Basidiomycetes: Agaricostilbales, Tremellales, Exobasidiales, Malasseziales, Polyporales, Sporidales, Boletales, and one unknown basidiomycete.

Tissue samples of F. angustifolia

Twenty fungal taxa were identified from rachises of *F. angustifolia*, of which eight were identified to species level, eight to a genus level and four remained unidentified. The fungal taxa represented three orders of Ascomycetes: Dothideales, Diaporthales, Pleosporales, and three orders of Basidiomycetes: Tremellales, Erythrobasidiales, Sporidales, and four unknowns.

Comparison of community composition

To look for similarities in *Fraxinus* fungal communities between New Zealand and Europe, the identified fungal taxa on *F. excelsior* and *F. angustifolia* were compared to the published literature of Przybyl (2002a,b) Lygis et al. (2006), Bakys et al (2009a,b), Davydenko et al. (2013), Scholtysik et al. (2013), Hauptman et al. (2013), and Kowalski and Czekaj (2010), all which report associated fungal taxa to either diseased or healthy *F. excelsior* trees. Of the fungal taxa detected in New Zealand *Fraxinus* samples, several taxa were similar to that reported in *F. excelsior* in Europe (Tables 1-3). At the species level, familiar fungi included *Aureobasidium pullulans*, *Colletotrichum acutatum*, *Epicoccum nigrum*, *Fusarium lateritium*, *F. oxysporum*, *Neofabraea alba*, *Penicillium canescens*, *Phoma exigua*, *P. exigua* var. *exigua*, and *Venturia fraxini*. Of all the fungal taxa detected in New Zealand *Fraxinus* samples, only one species was uniquely associated to *F. angustifolia*: *Erythrobasidium hasegawianum*. It is not known how these communities may compare to those located on native New Zealand trees, though some evidence suggest that *Epicoccum nigrum*, *Phoma* sp., and *Pleosporaceae* sp. may be more cosmopolitan in nature (Ganley 2008; Johnston et al. 2012).

Table 1. Identification of fungal isolates from *F. excelsior* samples in New Zealand

Putative fungal taxon	Closest BLAST match	Number of base-pairs matched	% Match	Origin of closest match	Tissue type	Presence on *Fraxinus* in Europe[a]
Aspergillus versicolor	*Aspergillus versicolor*	567/570	99%	Lithuania	Bud	
Ascomycota sp.	*Ascomycota sp.*	505/511	98%	China	Bark	
Ceratobasidium sp.	*Ceratobasidium sp.*	539/541	99%	Sweden	Bark	
Cladosporium phaenocomae	*Cladosporium phaenocomae*	566/566	100%	Netherlands	Bud	
Colletotrichum acutatum	*Colletotrichum acutatum*	583/583	100%	Germany	Bud	+
Davidiella sp.	*Davidiella sp.*	559/559	100%	Czech Republic	Bark	
Epicoccum nigrum	*Epicoccum nigrum*	549/549	100%	Czech Republic	Bud	+
Fusarium lateritium	*Fusarium lateritium*	567/567	100%	Germany	Bud	+
Lophiostoma corticola	*Lophiostoma corticola*	553/553	100%	New Zealand	Bark , Bud	+
Microbotryomycetes sp.	*Microbotryomycetes sp.*	655/692	94%	USA	Bark	
Neofusicoccum parvum	*Neofusicoccum parvum*	589/589	100%	Sweden	Wood	
Penicillium sp.	*Penicillium sp.*	604/604	100%	China	Bark, Bud, Wood	+
Phaeosphaeria sp.	*Phaeosphaeria sp.*	432/439	98%	New Zealand	Bud	
Phoma exigua	*Phoma exigua*	542/542	100%	Germany	Bud	+
Phoma sp.	*Phoma sp.*	540/542	99%	USA	Bud	+
Phomopsis sp.	*Phomopsis sp.*	585/589	99%	USA	Bark	+
Pleosporales sp.	*Pleosporales sp.*	497/519	95%	USA	Bark, Bud	
Rhodosporidium babjevae	*Rhodosporidium babjevae*	594/594	100%	USA	Bark	
Rhodotorula bacarum	*Rhodotorula bacarum*	656/667	98%	Japan	Bark	
Rhodotorula sp.	*Rhodotorula sp.*	575/581	98%	Finland	Bark, Bud	
Tremellales sp.	*Tremellales sp.*	511/515	99%	USA	Bark	
Xylariaceae sp.	*Xylariaceae sp.*	525/545	96%	USA	Bud	+

[a] compared to that reported by Przybyl (2002a,b), Lygis et al. (2006), Bakys et al. 2009a,b; Davydenko et al. 2013; Scholtysik et al. 2013; Hauptman et al. 2013; Kowalski and Czekaj 2010. '**+**' indicates similar presence of the fungal organism on both *Fraxinus* species in New Zealand and Europe.

Principal Component Analysis of fungal community for trees and different tissue types

The fungal species community composition from *F. excelsior* samples was analysed by principal component analysis (PCA) and grouped by tissue type (bark, wood and bud). There was no clear delineation of fungal community among sampled trees and no differentiation in fungal community structure between the different tissue types sampled (Figure 1).

Discussion

Ninety fungal taxa were detected in this study; an extremely high richness of fungi given the small number of samples taken from just six ash trees in New Zealand. Of these, it was possible to give species or generic identities to more than half. Species able to be identified included those previously known to be endophytes (e.g. *Bionectria ochroleuca*; Promputtha et al. 2005), ubiquitous filamentous fungi such as *Penicillium* spp. as well as yeasts such as *Cryptococcus favescens*, *Rhodotorula bacarum* and *Saccharomyces cerevisiae* already known to inhabit plants.

The well-known pathogen of ash in Europe, *Hymenoscyphus fraxinus* and its non-pathogenic relative *Hymenoscyphus albidus* were not found in this study. *Hymenoscyphus fraxinus* has spread very fast since it was first recorded in Poland and Lithuania (Juodvalkis and Vasiliauskas, 2002, Przybył 2002a), but it has not yet been recorded as an introduced, invasive species outside of Europe. Though other *Hymenoscyphus* species can be found naturally associated with native *Nothofagus* species in New Zealand (Johnston et al. 2012), this study provides no evidence of any *Hymenoscyphus* species associated with exotic *Fraxinus* in New Zealand although the use of species-specific primers (e.g. Johansson et al. 2010) and more extensive sampling of ash from a wider geographic distribution would be more conclusive. The only pathogen of ash previously recorded in New Zealand, *Hysterographium fraxini*, was not found (Cannon 1999). This is not unexpected as *H. fraxini* is primarily found on the south island of New Zealand (http://www.nzffa.org.nz/farm-forestry-model/the-essentials/forest-health-pests-and-diseases/diseases/Hysterographium-fraxini), not the north where the samples were taken for the present study.

Several of the fungi found in healthy ash tissue in this study are known and are recognised as pathogens of ash in Europe and North America. These include *Neofabraea alba*, *Fusarium lateritium*, *Phoma exigua* and

Table 2. Identification of fungi directly from *F. excelsior* tissue samples in New Zealand via direct DNA extraction and PCR cloning

Putative fungal taxon	Closest BLAST match	Number of base-pairs matched	% Match	Origin of closest match	Tissue type	Presence on *Fraxinus* in Europe[a]
Articulospora sp.	*Articulospora proliferate*	512/542	94%	Canada	Bud	
Ascomycete sp.	*Ascomycete sp.*	606/608	99%	Brazil	Wood	
Ascomycota sp.	*Ascomycota sp.*	530/545	98%	China	Bark, Bud, Wood	
Aureobasidium pullulans	*Aureobasidium pullulans*	616/616	100%	Spain	Bud	
Basidiomycota sp.	*Basidiomycota sp.*	617/639	97%	Czech Republic	Bark, Bud, Wood	
Bensingtonia yuccicola	*Bensingtonia yuccicola*	679/683	99%	USA	Wood	
Bionectria ochroleuca	*Bionectria ochroleuca*	608/608	100%	Czech Republic	Wood	
Botryosphaeria parva	*Botryosphaeria parva*	594/597	99%	Brazil	Wood	
Cladosporium sp.	*Cladosporium sp.*	591/591	100%	China	Wood	
Colletotrichum acutatum	*Colletotrichum acutatum*	618/620	99%	Germany	Bud	+
Cryptococcus flavescens	*Cryptococcus flavescens*	568/568	100%	Austria	Bud, Wood	
Cryptococcus sp.	*Cryptococcus sp.*	523/524	98%	USA	Bud	+
Dioszegia sp.	*Dioszegia sp.*	467/479	97%	USA	Bud	
Epicoccum sp.	*Epicoccum sp.*	566/583	97%	USA	Bark, Bud, Wood	+
Exobasidium arescens	*Exobasidium arescens*	605/609	99%	Germany	Wood	
Exobasidium sp.	*Exobasidium rhododendri*	591/615	96%	England	Bud	
Fusarium lateritium	*Fusarium lateritium*	595/600	99%	Germany	Bud, Wood	+
Fusarium oxysporum	*Fusarium oxysporum*	580/582	99%	Czech Republic	Bark, Bud, Wood	+
Fusarium sp.	*Fusarium sp.*	590/595	99%	Czech Republic	Bark, Bud, Wood	
Herpotrichia parasitica	*Herpotrichia parasitica*	515/532	98%	USA	Wood	
Herpotrichia sp.	*Herpotrichia parasitica*	515/532	96%	USA	Bark	
Kabatina sp.	*Kabatina thujae*	595/631	94%	USA	Bud	
Malassezia restricta	*Malassezia restricta*	767/773	99%	Belgium	Wood	
Malassezia sp.	*Malassezia globosa*	767/789	97%	Germany	Wood	
Neofabraea alba	*Neofabraea alba*	578/582	99%	Netherlands	Bark	+
Neofusicoccum parvum	*Neofusicoccum parvum*	596/597	99%	Sweden	Wood	
Penicillium brevicompactum	*Penicillium brevicompactum*	619/619	100%	Japan	Wood	
Penicillium canescens	*Penicillium canescens*	622/622	100%	Brazil	Bud	
Penicillium sp.	*Penicillium sp.*	620/621	99%	China	Bud, Wood	+
Penicillium spinulosum	*Penicillium spinulosum*	616/616	100%	Czech Republic	Wood	
Phaeomoniella sp.	*Phaeomoniella sp.*	513/520	98%	Korea	Bud	
Phialophora sp.	*Phialophora europaea*	580/613	95%	Switzerland	Wood	+
Phoma exigua	*Phoma exigua*	576/579	99%	Germany	Bud	+
Phoma sp.	*Phoma sp.*	577/579	99%	USA	Bud	+
Phomopsis sp.	*Phomopsis sp.*	592/599	98%	USA	Bark, Wood	+
Pilidium concavum	*Pilidium concavum*	485/486	99%	USA	Bark	
Pleosporales sp.	*Pleosporales sp.*	682/689	98%	China	Bark, Bud, Wood	
Polyporus tuberaster	*Polyporus tuberaster*	647/647	100%	USA	Bark, Bud, Wood	
Rhodotorula sp.	*Rhodotorula sp.*	637/641	99%	Germany	Bud	
Saccharomyces cerevisiae	*Saccharomyces cerevisiae*	877/891	98%	France	Bark	
Suillus granulatus	*Suillus granulatus*	706/715	98%	China	Wood	
Suillus sp.	*Suillus tomentosus*	715/732	97%	Canada	Bud, Wood	
Tremellales sp.	*Tremellales sp.*	561/565	99%	USA	Bud, Wood	
Venturia fraxini	*Venturia fraxini*	585/586	99%	Netherlands	Wood	+

[a] compared to that reported by Przybyl (2002a,b), Lygis et al. (2006), Bakys et al. 2009a,b; Davydenko et al. 2013; Scholtysik et al. 2013; Hauptman et al. 2013; Kowalski and Czekaj 2010. '**+**' indicates similar presence of the fungal organism on both *Fraxinus* species in New Zealand and Europe

Table 3. Identification of fungi from *F. angustifolia* tissue samples in New Zealand via direct DNA extraction and PCR cloning, and comparative presence of similar type taxa to *F. excelsior* in Europe

Fungal taxon	Closest BLAST match	% Match	Number of base-pairs matched	Origin of closest match	Presence on *Fraxinus* in Europe[a]
Ascomycota sp.	Ascomycota sp.	97	525/539	USA	
Aureobasidium pullulans	*Aureobasidium pullulans*	100	619/619	Spain	+
Basidiomycete	Uncultured basidiomycete yeast	98	641/653	Germany	
Cryptococcus sp.	Uncultured Cryptococcus clone	99	554/557	Sweden	+
Cryptococcus sp.	Uncultured Cryptococcus clone	99	526/527	Sweden	+
Cryptococcus sp.	*Cryptococcus sp.*	99	521/524	USA	+
Diaporthe eres	*Diaporthe eres*	98	596/608	Lithuania	
Dioszegia sp.	*Dioszegia sp.*	98	470/479	USA	
Dioszegia takashimae	*Dioszegia takashimae*	99	534/542	Germany	
Epicoccum nigrum	*Epicoccum nigrum*	99	578/580	Sweden	+
Erythrobasidium hasegawianum	*Erythrobasidium hasegawianum*	98	605/617	Germany	
Lewia infectoria	*Lewia infectoria*	99	633/637	United Kingdom	
Phaeomoniella sp.	*Phaeomoniella capensis*	89	512/574	New Zealand	
Phaeosphaeria sp.	*Phaeosphaeria sp.*	97	539/639	Spain	+
Phaeosphaeria sp.	*Phaeosphaeria sp.*	96	556/582	USA	+
Phoma exigua var. exigua	*Phoma exigua var. exigua*	99	573/576	Germany	+
Phoma sp.	*Phoma sp.*	99	573/575	Brazil	
Rhodotorula sp.	*Rhodotorula aurantiaca*	100	415/425	USA	
Rhodotorula sp.	Uncultured Rhodotorula clone	98	409/418	Austria	
Tremellomycetes sp.	Uncultured Tremellomycetes	99	558/561	Germany	
Trichosporon sp.	*Trichosporon laibachii*	99	530/531	China	
Unidentified	Uncultured soil fungus clone	95	605/638	USA	
Unidentified	Uncultured fungus clone	99	586/589	USA	
Unidentified	*Sporobolomyces syzygii*	89	571/641	Japan	

[a] compared to that reported by Przybyl (2002a,b), Lygis et al. (2006), Bakys et al. 2009a,b; Davydenko et al. 2013; Scholtysik et al. 2013; Hauptman et al. 2013; Kowalski and Czekaj 2010. '**+**' indicates similar presence of the fungal organism on both *Fraxinus* species in New Zealand and Europe

Venturia fraxini. *Neofabraea alba* was detected in healthy bark samples in the present study and is known to cause Coin Canker of Ash in Northeastern North America (Angeles et al. 2006). *Fusarium lateritium* is one of the most common fungi on diseased ash (Kowalski and Czekaj 2010), often isolated from dead buds and necrotic stems in European ash (Pukacki and Przybyl 2005). *Fusarium lateritium* was obtained from bud samples in the present study. *Phoma exigua* is the causal agent of ash seedling canker, a disease reported from nurseries in Belgium where it caused severe losses (Schmitz et al. 2006). This fungus has also been reported from ash seedlings in France and Great Britain causing little or no symptoms (Schmitz et al. 2006). *Venturia fraxini*, the causal agent of Fraxinus leaf blotch (Anselmi 2001) was detect from wood samples in the present study. Several other, more generalist pathogens, previously identified as causing disease in other woody plants or shrubs were also found in the present study. These include *Colletotriochum actuatum*, which causes disease on a range of crops and fruit trees (Freeman 2008), The presence of all of these pathogens in healthy plant tissue is not unexpected; pathogens, especially those causing disease in woody tissue, often exhibit a latent endophytic phase which reverts to a pathogenic state when environmental conditions become suitable (e.g. Brown and MacAskill 2005; Crane et al. 2009).

Of the 90 species detected in this study, almost one third had been previously reported from ash species in Europe. Many of these are fungi with a worldwide distribution such as *Fusarium lateritium*, *Fusarium oxysporum* and *Phoma exigua*, however several are known predominantly from Europe and have not previously been recorded in New Zealand. It is not possible to determine from this study whether the specific strains observed arrived in New Zealand with the introduction of ash or colonised ash once it was established in New Zealand. This is also the case for the majority of the other species observed only on ash in this study (and not previously known from European studies), as these species are generally widely distributed throughout the world.

The presence of several common European taxa in European *Fraxinus* established in New Zealand lends support to the cointroduction hypothesis, that is, that these species were cryptic hitchhikers on ash plants when they

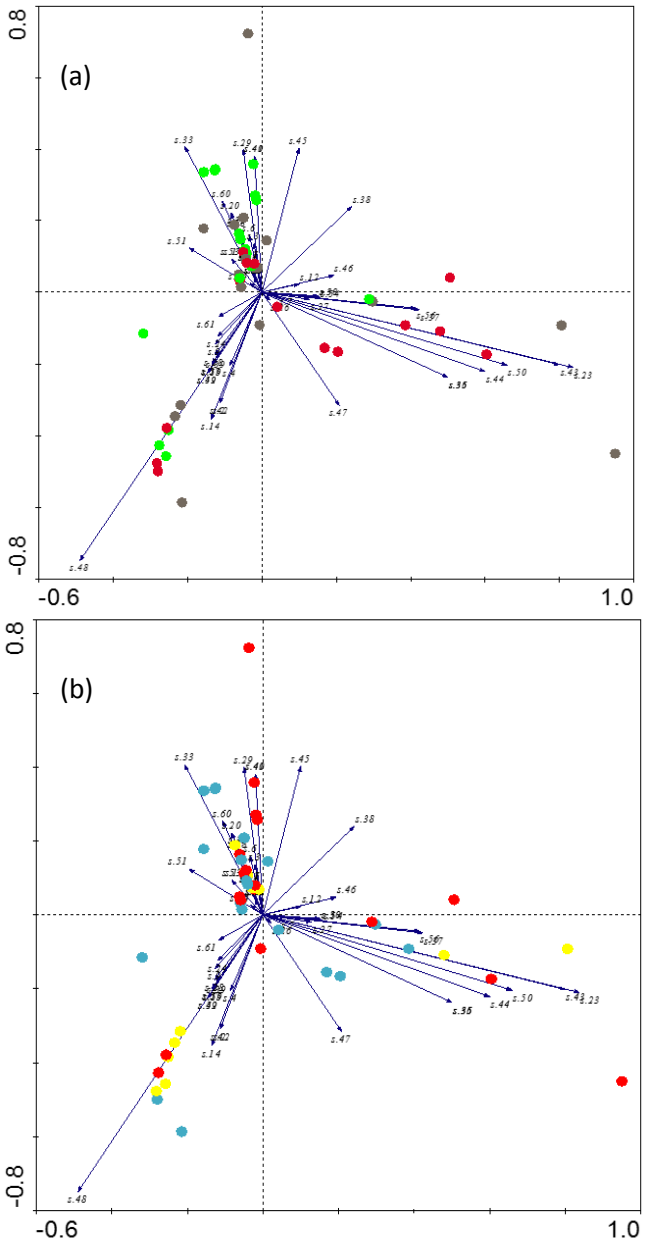

Figure 1. PCA ordination of *Fraxinus excelsior* fungal community. Arrows denote fungal species driving the interactions. Coloured dots denote (a) samples from buds (green), bark (red) and wood (grey), and (b) different trees

were first introduced. In contrast, few fungal species were identified that could be definitively described as being exclusively from New Zealand (and thus also in support of the host-jumping hypothesis) (Shipunov et al. 2008). The high number of known pathogens associated with ash in this study, many of which could have been cointroduced, highlights the importance of the live plant pathway for the movement of potential pathogens around the world.

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Acceleration of Ash Petiole Decomposition to Reduce *Hymenoscyphus fraxineus* Apothecia Growth – a Feasible Method for the Deprivation of Fungal Substrate

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Abstract

A fungal pathogen, the ascomycete *Hymenoscyphus fraxineus*, is causing serious damages in the European common ash (*Fraxinus excelsior*) populations. Despite intensive investigations, there is currently no method to cure or slow down the ash dieback disease. A possible control strategy could be the treatment of the ash leaf litter to accelerate its decomposition and subsequently deprive the fungus of its substrate, thus preventing apothecia growth and its sporulation the next year. The aim of this study is to investigate the decomposition of ash petioles when covered with the leaf litter of different broad-leaved tree species (*Tilia cordata*, *Carpinus betulus* and *Juglans nigra*). Mass loss, physical fragmentation and lignin content of ash petioles were followed over a decomposition time of one year. Litter quality was determined based on their C, N and lignin contents and C/N and lignin/N ratios. Ash petioles covered with *Tilia* leaf litter had significantly higher fragmentation and decomposition rates after a one year decomposition time compared to petioles without covering. Since all treatment plots had the same microclimatic conditions (temperature and relative humidity), the effect of higher fragmentation and decomposition was induced as a result of the chemical properties and quality of the covering litter type.

Keywords: *Hymenoscyphus fraxineus*, ash petiole, leaf litter decomposition, litter quality, disease control

Introduction

The ascomycete *Hymenoscyphus fraxineus*, an invasive and highly pathogenic fungus with East Asian origin (Zhao et al. 2012), is causing serious damages in the European common ash (*Fraxinus excelsior*) populations. Ash dieback was first observed in Poland in the 1990s and then described as the causal agent *Chalara fraxinea* (Kowalski 2006). It is now found in more than twenty countries (Timmerman et al. 2011). Initially, the sexual stage of the fungus was incorrectly identified as *H. albidus*, a litter decomposing fungus already known in Europe (Kowalski and Holdenrieder 2009). First in 2011, after genetic analysis, it became clear that it was a new species and was identified as *H. pseudoalbidus* (Queloz et al. 2011). Since 2014, the new correct scientific name of the pathogen for both sexual and asexual stages is *H. fraxineus* (Baral et al. 2014). The infection begins with the fruiting body, or apothecium. During the summer they are growing on the ash petioles fallen in the previous year. The ascospores spread very effectively over long distances due to the wind. After adhering to the leaf surface, the ascospores penetrate the ash leaves and start to colonize the leaf tissue (Kirisits and Cech 2009, Timmermann et al. 2011, Cleary et al. 2013). After leaf fall in autumn, the fungus starts to produce pseudosclerotial plates and overwinters on the petioles. In the next year, new apothecia will develop from the pseudosclerotia thus closing the infection cycle (Gross and Holdenrieder 2013). Even two years after of the leaf fall, new apothecia can develop on the remaining petioles (Gross and Holdenrieder 2013). The leaf litter of ash has been described as one of the most degradable compared to other broad-leaved trees in temperate forests (Mitchell et al. 2014). Different studies showed faster litter decomposition

compared to *Coryllus avellana*, *Quercus robur*, *Q. rubra*, *Fagus sylvatica*, *Tilia ssp.*, *Carpinus betulus* or *Acer pseudoplatanus* (Hagen-Thorn et al. 2004, Bjornlund and Christensen 2005, Jacob et al. 2009). It is explained due to its lower C/N ratio and lower lignin content (Cotrufo et al. 1998, Bjornlund and Christensen 2005, Jacob et al. 2010). However, in these studies there was no distinction between leaf and petiole. The results were referring mainly to the foliar litter mass. While the leaf decomposition itself is really a fast procedure, the petiole could remain longer, often up to two years and more after leaf fall.

Our objective was to investigate specifically the decomposition of the ash petioles because of its major role in the fungal growth and in the infection cycle. We used different covering treatments with the leaf litter of different broad-leaved tree species to influence the decay in litterbag experiments. According to our hypothesis, if we were able to increase the petiole break down process, it could have a negative effect on the next year apothecia growth – and so finally on the sporulation.

Materials and methods

Study site description

The study site was located in an ash – maple mixed forest, situated in Ismaning, Germany (48°12´43.0" N, 11°40´52.7" E). The soil is moderate fresh lime-rich clay. Mean annual temperature of the side during the experiment was 9.6 °C and mean relative humidity was 86.54 %, measured with HOBO Logger (HOBO Pendant temperature/light data logger UA-002-64, Onset Computer Corporation, USA) in the study site at 1.3 m height. Precipitation during the experiment (October 2013 – October 2014) was 897.9 mm, recorded at the meteorological station Eichenried.

Litterbag experiment design

Freshly fallen leaves of ash trees (*Fraxinus excelsior*) with signs of *H. fraxineus* infection were collected from two pure ash forest stands in Oberhummel and in Freising (30 and 20 km from Ismaning). The ash petioles were removed from the leaves and oven-dried at 60 °C for 48 h. Ten grams of the petioles (approx. 40 – 50 intact petioles) were placed in litter bags, 50 cm x 50 cm, made of PVC coated fiberglass net with a mesh size of 2 mm. A total of 27 bags per treatment were placed in an ash – maple mixed forest located in Ismaning (Munich).

A 40 m x 40 m area was separated in the forest, and divided into 10 m x 10 m plots for the different cover treatment types and for control plots. Twelve 10 m x 10 m parcels were random selected for three different cover treatment types and for control (three replicates for each). Nine litter bags were placed in the middle of each plot. The litter bags were covered with the leaf litter of three different broad-leaved tree species: small-leaved lime (*Tilia cordata*), hornbeam (*Carpinus betulus*) and black walnut (*Juglans nigra*). For control, litter bags with petioles were placed in three plots on the forest ground without any leaf cover. The experiment was started in October 2013. One litter bag from each plot was collected in January, April, July and October 2014. The remaining ash petioles were carefully removed from the bags and oven-dried at 60 °C for 48 h for weight determination and further chemical analysis.

At all plots, the air temperature was continuously measured with temperature sensors (HOBO Pendant temperature/light data logger UA-002-64, Onset Computer Corporation, USA). Relative humidity was recorded with HOBO Pro v2 U23-001 data logger.

Chemical analyses

Carbon (C) and nitrogen (N) content of the petioles were measured with the elementary analysis using Vario EL III CN elemental analyzer (Elementar Analysensysteme, Germany).

For lignin content determination, the Klason procedure was used (Hatfield and Fukushima, 2005). Briefly, 500 mg of dried and pulverized material per sample was suspended in 10 mL of 75% (v/v) H_2SO_4 and incubated for 2 h at room temperature. The total contents were transferred into 500 mL Duran glasses and diluted with distilled water to a final volume of 300 mL and boiled for 4 h. After cooling, the solution was filtered and the lignin was collected in a pre-weighed Millipore membrane filter. The acid residues were washed out with deionized water. The filters were dried overnight in the oven at 80°C. Lignin content was calculated as percentage by weight of the dried sample.

Calculations and Statistical Analysis

Total N and C were expressed as a percentage of the initial value, based on ash-free dry mass. The mass loss was expressed in percentage of the remaining dry mass compared to the initial dry weight. Mean yearly decomposition rates (k) were calculated using negative exponential decay model (Olson, 1963) for each treatment: $k = \ln(X_0/X_t)/t_t\text{-}t_0$. Differences among the litter and lignin decomposition were tested with one-way analysis of variance (ANOVA), followed by pairwise *t*-tests using Bonferroni correction (α=0.05, $P < 0.05$, n= three samples per treatments). Differences among the ash petiole fragmentation were tested with ANOVA and Tukey test was applied for post-hoc comparison with significance set at $P < 0.05$. All analyses were performed with SigmaPlot data analysis package.

Results

Decomposition of the petioles

After one year, the remaining petiole biomass was approximately 35-40% for control plots, followed by the cover treatments: 30% under *Carpinus*, 18% under *Juglans*

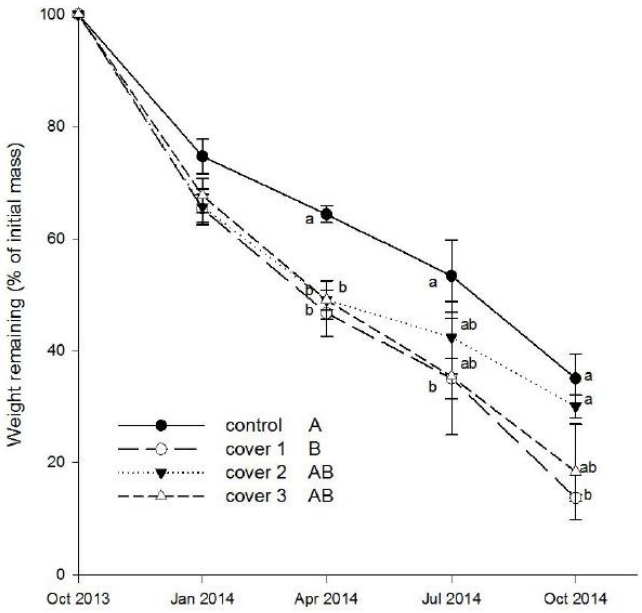

Figure 1. Decomposition of ash petioles under different treatment conditions as percent of their initial mass (mean ± SE). Covering treatments with *Tilia cordata* (cover 1), *Carpinus betulus* (cover 2) and *Juglans nigra* leaf litter (cover 3). Different capital letters indicate significant differences ($P < 0.05$) among the treatments for the entire time course; different lower case letters indicate significant differences ($P < 0.05$) on a given date

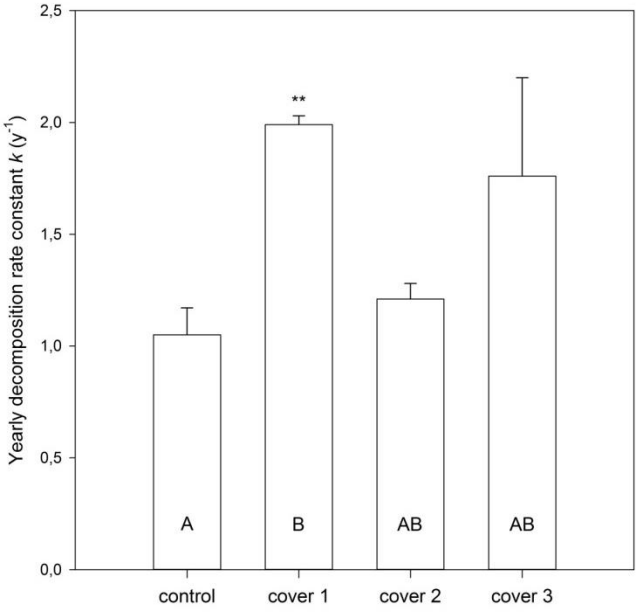

Figure 2. Yearly decomposition rate constants (*k*) of ash petioles under the different treatment conditions (mean ± SE). Different capital letters indicate significant differences among the treatments ($P < 0.01$)

and 13% under *Tilia* leaf cover (Figure 1). There was a significant effect in all treatments compared to the control plots after 6 months (October – April). However, only the mass remaining under *Tilia* cover was significantly lower than the control samples over the entire time course (October 2013 – October 2014). Figure 2 shows the decomposition rates *(k)* of petiole litter. Generally litter cover treatments have higher *k* values, but only *Tilia* leaf cover induced a significant change ($P < 0.01$).

In contrast to previously published data, increasing lignin contents were measured as percentage by weight over the whole experimental time in all petiole samples (Figure 3). Interestingly the opposite effect could be observed by the weight remaining. Ash petioles under leaf litter cover have in general, higher lignin contents in percent of their initial mass than petioles without covering treatments. However, there were no significant differences over the entire time course or on given sampling dates.

The length of the petioles and the length of the remaining petiole fragments were recorded at the beginning of the experiment and after one year of decomposition (Figure 4) to additionally detect signs of degradation besides the chemical decomposition. At the beginning, most of the intact petioles had a length about 17-18 cm. After one year, the petioles from the control bags were still 8-10 cm long with mainly intact parts. The petioles from the covering treatments were more fragmented (main length 3-7 cm), but only below the lime leaf (cover 1) was significant the length reduction.

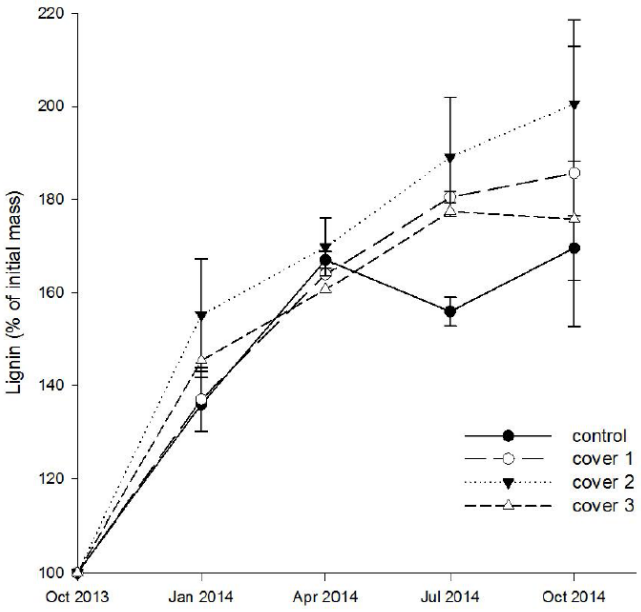

Figure 3. Petiole lignin contents in percent of their initial mass (mean ± SE). Covering treatments with *Tilia cordata* (cover 1), *Carpinus betulus* (cover 2) and *Juglans nigra* leaf litter (cover 3)

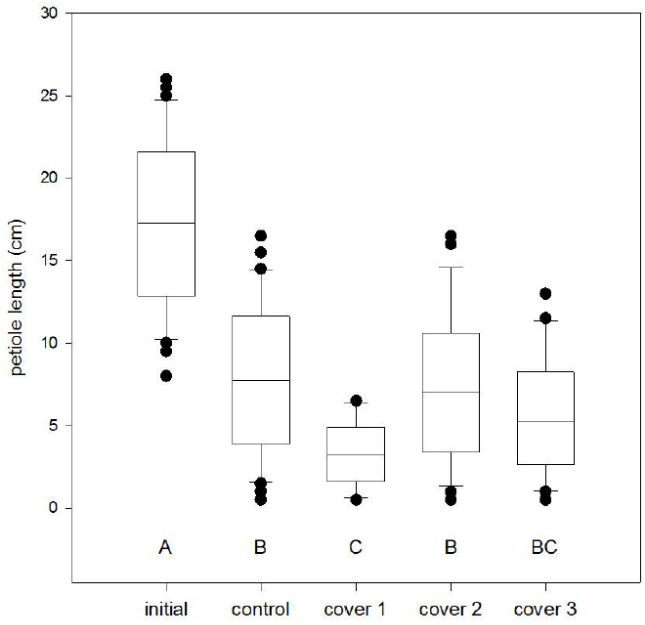

Figure 4. Initial length of petioles at the beginning of the experiment, and the remaining fragments length after one year decomposition time. Cover treatments with *Tilia cordata* (cover 1), *Carpinus betulus* (cover 2) and *Juglans nigra* leaf litter (cover 3)

Impact of the cover effect

We assumed that the leaf litter covers of the petioles may induce changes in the local microclimate (temperature and relative humidity) which can be the promoting effect during the rotting process. But this was not the case; there were neither significant differences between the treatment plots in temperature or in relative humidity, nor in night and in day data (Table 1).

Table 1. Mean annual night and day temperature and relative humidity in the study site and in the plots

	Temperature night (°C)	Temperature day (°C)	Relative humidity night (%)	Relative humidity day (%)
Study site	7.4	13.8	93.2	72.7
control	7.7 ± 0.2	14.5 ± 0.5	97.1	85.4
cover 1	7.3 ± 0.3	14.5 ± 0.5	98.2	84.2
cover 2	8.2 ± 1.6	14.2 ± 0.8	98.1	82.5
cover 3	7.5 ± 0.2	14.5 ± 0.8	98.1	90.4

Temperature of the study site was recorded at 1.3 m above the forest floor. Temperatures of the treatment plots were recorded directly under the leaf litter cover. Covering treatments were performed with *Tilia cordata* (cover 1), *Carpinus betulus* (cover 2) and *Juglans nigra* leaf litter (cover 3).

Initial nutrient concentrations and quality parameters of ash petioles and leaf litter, which determine the speed of the decomposition, are listed in Table 2. Ash petioles have a high lignin concentration (186.1 mg g^{-1} DW) as well as high lignin/N (on average 26.27 g g^{-1}) and C/N ratios (on average 64.4 g g^{-1}) compared to the other leaf litter species (Table 2). Although the litter of *Tilia* had approximately equal lignin concentration (188.4 mg g^{-1} DW), it displayed the lowest C/N ratio (on average 20.86 g g^{-1}). The litter of *Juglans* had the lowest lignin concentration (on average 70 mg g^{-1}) but simultaneously a high C/N ratio (59.8 g g^{-1}).

Table 2. Litter quality properties for the ash petioles and the three leaf litter species

	N (%)	Lignin (%)	C:N (g/g)	Lignin:N (g/g)
Ash petioles	0.71	18.61	64.4	26.27
	± 0.04	± 0.97	± 3.58	± 2.6
cover litter 1	2.31	18.84	20.86	8.24
	± 0.17	± 2.14	± 1.79	± 1.5
cover litter 2	1.34	15.49	33.95	11.68
	± 0.11	± 6.88	± 3.68	± 4.16
cover litter 3	0.76	2.14	59.8	2.76
	± 0.11	± 0.32	± 8.13	± 0.43

The percentage of Nitrogen (N), lignin and the ratio for Carbon (C) and N and lignin and N is given for the ash petioles and the cover litters (*Tilia cordata* - cover litter 1, *Carpinus betulus* - cover litter 2 and *Juglans nigra* - cover litter 3) at the beginning of the experiment.

Lime leaf litter had a significant positive effect on the petiole decomposition ($P < 0.01$), whereas a moderate effect was recognized in case of *Juglans* and *Carpinus* leaf litter cover. Rates of ash petiole decomposition (k) plotted against the initial nutrient and lignin concentrations or ratios of the covering leaf species showed no correlation.

Discussion

Effect of microclimate and microbial activity

However only *Tilia* leaf litter had a significant effect on the ash petiole decomposition, a moderate positive effect was recognized for the petioles covered with *Carpinus* and *Juglans* leaf litter. The promoting effect could be explained by different mechanisms. The determining factors in the leaf litter decomposition are temperature, humidity, microbial activity and leaf litter chemistry (Couteaux et al. 1995).

The macroclimate is determining the decomposition process on a large scale; but within a climatic zone the substrate quality plays a more important role. The microclimate could influence the local decomposer community and lead to different decomposition efficiencies (Couteaux et al. 1995, Zhou et al. 2008). We assumed that under the leaf litter cover, the temperature and humidity will increase, and could increase the petiole decomposition due to elevated decomposer activity of microbes. Actually, no significant differences in temperature and relative humidity were found between the sampling plots (Table 1). Nevertheless,

the covering can influence the decomposer community in other ways: it provides a better habitat for invertebrates than poorly covered forest topsoil. Higher fragmentation of the petioles may be due to the higher micro-fauna activity, and the greater fragmentation allows more effectively microbial decomposition (Couteaux et al. 1995). On the other hand, the leaf cover could have a direct effect on the microbial abundance. They have their own endophytic and epiphytic fungal inhabitants that could also affect the decomposition process (Unterseher et al. 2013).

Relationship between litter quality and decomposition

The quality of the litter is an additional and often more important factor in the decomposition than climate. The chemical properties like the initial lignin and N content, C/N ratio and lignin/N ratio basically determine the decomposition process (Cotrufo et al. 1998, Zhou et al. 2008, Jacob et al. 2010, Rahman et al. 2013). According to literature data, ash leaf litter is one of the most quickly degradable among the temperate zone broad-leaved tree species. It is rich in calcium and nitrogen with low lignin content, C/N and lignin/N ratios (Mitchell et al. 2014). However, these data refer to the foliar litter quality while the ash petiole itself has, according to our chemical analysis, rather different chemical compositions with significantly higher lignin content, C/N and lignin/N ratios and low N content (Table 2). These chemical properties make the ash petioles more persistent and allow them to remain longer on the forest ground. Furthermore, the pseudosclerotial plates formed by *H. fraxineus* on infected ash petioles protect them additionally from other decomposition processes. There are also some data in the literature that ascomycetes could decelerate the decomposition via exclusion of secondary saprophytic basidiomycetes (Purahong and Hyde 2011). We found that covering the ash petioles with leaf litter of other species promotes the decomposition, nevertheless, according to our results this effect correlated with the enhanced litter quality and not to the microclimatic changes, as we first assumed. Positive effects of tree diversity on leaf decomposition rate were also reported (Kominoski et al. 2007; Purahong et al. 2014). It must be noted here, that the relationship between leaf litter diversity and decomposition is more complex. Many research groups published partly different, even contrary results, and thus we cannot conclude a general rule (Gessner et al. 2010).

Disease control

From a vast international research effort in the last years, we have increased knowledge about the ash dieback disease and the causative fungal pathogen. Disease symptoms and host range, life cycle and reproductive mode, as well as genetic information are well described (Kowalski and Holdenrieder 2009, Bakys et al. 2009, Drenkhan and Hanso 2010, Schumacher et al. 2010, Kräutler and Kirisits 2012, Cleary et al. 2013). Also research in host resistance to the pathogen is occurring in many countries (McKinney et al. 2011, Kjær et al. 2012, Stener 2012). Nevertheless, we still cannot mitigate the serious damages caused by *H. fraxineus*. There is currently no method to stop or to cure the disease. Hauptman and coworkers (Hauptmann et al. 2014) published a study about possible chemical control. They tested eight different fungicides on mycelia growth and apothecia development *in vitro*. They found that the fungicide carbendazim effectively inhibited the growth of the pathogen *in vitro*, but there is no further evidence of how it works under field conditions. In the same study, they also tested the efficacy of urea against the mycelia and apothecia development with positive results. The use of urea could be more favorable than fungicide application. Besides its direct suppressing effect on the pathogen, it can accelerate the leaf litter decomposition and therefore provides a cost-effective and environmental friendly alternative. However, the promotion of mixed forest stands in forestry practice for enhancing the litter quality and promoting the ash petiole decomposition could be more practicable, near to nature and could be used as a long term disease control method (Scherer-Lorenzen et al. 2007, Jactel et al. 2009, Wagner et al. 2014). This hypothesis is confirmed by a previous study carried out by the Bavarian State Institute of Forestry in mixed and pure ash forest stands (data not published). The vitality of ash trees was evaluated in a total of 16 forest stands in four locations (two pure ash forest stands and two ash mixed forest stands in each case) according to a five-step scoring scale (Lenz et al., 2012). On average, ash trees in all mixed forest stands had better vitality compared to pure ash stands.

Conclusions

Leaf litter decomposition is a complex process controlled by a range of factors like leaf litter diversity, litter chemistry, microclimatic conditions and microbial decomposer activity. Foresighted forest system management planning in reforestation decisions - like using specific selected alternative tree species - can influence the decomposition rates of persistent leaf debris like the ash petioles and moderate the infection spread. Surely, this cannot stop the disease, but slowing down the infection spread may provide enough time for natural resistance development against the pathogen and time for the research community to develop new disease control methods.

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Fungal Endophytes in Ash Shoots – Diversity and Inhibition of *Hymenoscyphus fraxineus*

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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: *Fraxinus excelsior*, 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. 2014). 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 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, Faculty of Science, Prague, 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 was 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).

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.

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 to 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% 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 (one positive out of five) 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% inhibition and reisolation of the pathogen was successful (three positive out of five). The inhibition rates of endophytes (for each isolate of *H. fraxineus*), the results of reisolation and identification of endophyte species are available in the Supplementary Data (Suppl. Table 1 and Suppl. Table 2).

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 (identical to Fungal sp. 104 in Bakys et al. 2009a 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. 2009b). *Dendrothyrium* sp., *Bjerkandera adusta* and *Coprinellus disseminatus* were recorded in ash shoots with dead tops (Bakys et al. 2009a).

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 multiforme	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)	–	(-|-)
Coniochaetales	*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 ($P < 0.05$)

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

Ecological group of fungi	Summer		Winter	
	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

Pyrenochaeta corni was noted as an endophyte of living ash leaves (Scholtysik et al. 2013) and current year seeds (Cleary et al. 2013). 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.

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,

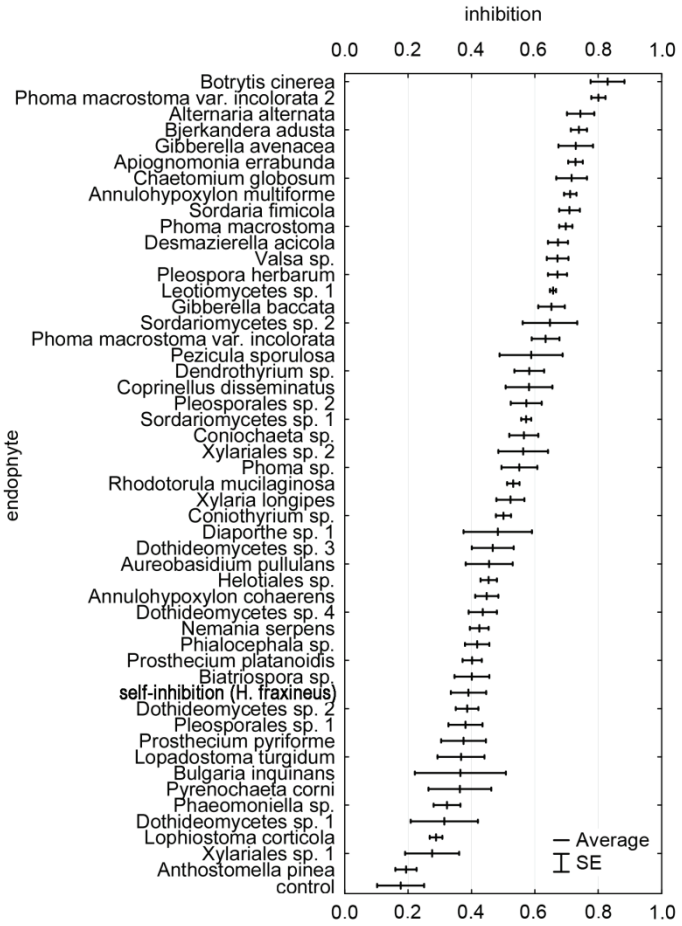

Figure 1. Average growth inhibition of *H. fraxineus* caused by endophytes isolated from healthy ash shoots (including *Biatriospora* sp. from CCF). Horizontal bars indicate standard errors. Identical isolates of *H. fraxineus* were used to test the self-inhibition and a pairing with an agar plug was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

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

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* *Epicocum nigrum* *Giberella avenacea*	Ascomycota sp. *Fusarium* sp. unidentified sp. 119	*Alternaria arborescens* *Aureobasidium pullulans* *Cladosporium cladosporioides* *Cladosporium tenuissimum*	*Alternaria alternata* *Pezicula cinnamomea* *Phomopsis* spp.	*Aureobasidium pullulans* *Phoma macrostoma* var. *incolorata* unidentified yeast	*Diaporthe* sp. 2 Dothideomycetes sp. 1 *Lophiostoma* sp.
Species congruently recorded in present study	*Alternaria alternata* *Aureobasidium pullulans* *Diaporthe* sp. 1 *Gibberella avenacea* *Valsa* sp.	*Gibberella baccata* *Lophiostoma corticola*	*Aureobasidium pullulans*	*Aureobasidium pullulans* *Alternaria alternata* *Prosthecium pyriforme* *Nemania serpens*		

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

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*). Similarly 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 fungal 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 synthesize 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) and *G. baccata* are weak parasites (De Gruyter et al. 2002, 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.

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Supplementary material

Suppl. Table 1. Inhibition rates of endophytes and the results of *H. fraxineus* reisolation after interaction

Suppl. Table 1a. Average inhibition rate of the endophytes againts two isolates of *H. fraxineus*. Endophytes connected with the same vertical line belong to the same homogeneous group and are not significantly different ($P > 0.05$) according to Tukey multiple comparisons of means. 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

Endophyte	Inhibition rate	Homogeneous groups
Botrytis cinerea	0.83	|
Phoma macrostoma var. *incolorata 2*	0.8	| |
Bjerkandera adusta	0.74	| | | | |
Alternaria alternata	0.74	| | |
Apiognomonia errabunda	0.73	| | | | |
Gibberella avenacea	0.73	| | | |
Chaetomium globosum	0.72	| | | | |
Sordaria fimicola	0.71	| | | | |
Annulohypoxylon multiforme	0.71	| | | | |
Phoma macrostoma	0.7	| | | | | |
Pleospora herbarum	0.67	| | | | | | |
Valsa sp.	0.67	| | | | | | |
Desmazierella acicola	0.67	| | | | | | |
Leotiomycetes sp. 1	0.66	| | | | | | | |
Sordariomycetes sp. 2	0.65	| | | | | | | | |
Gibberella baccata	0.65	| | | | | | | |
Phoma macrostoma var. *incolorata*	0.63	| | | | | | | | | |
Pezicula sporulosa	0.59	| | | | | | | | | |
Coprinellus disseminatus	0.58	| | | | | | | | | |
Dendrothyrium sp.	0.58	| | | | | | | | | |
Coniochaeta sp.	0.57	| | | | | | | | | | |
Sordariomycetes sp. 1	0.57	| | | | | | | | | |
Pleosporales sp. 2	0.57	| | | | | | | | | |
Xylariales sp. 2	0.56	| | | | | | | | |
Phoma sp.	0.55	| | | | | | | | | |
Rhodotorula mucilaginosa	0.53	| | | | | | | | | | |

Suppl. Table 1.a (Continued)

Endophyte	Inhibition rate	Homogeneous groups
Xylaria longipes	0.52	| | | | | | | | | | |
Coniothyrium sp.	0.5	| | | | | | | | | | | |
Diaporthe sp. 1	0.48	| | | | | | | | | | |
Dothideomycetes sp. 3	0.47	| | | | | | | | | |
Aureobasidium pullulans	0.46	| | | | | | | | |
Annulohypoxylon cohaerens	0.45	| | | | | | | | |
Helotiales sp.	0.45	| | | | | | | | |
Dothideomycetes sp. 4	0.44	| | | | | | | | |
Phialocephala sp.	0.42	| | | | | | | | |
Nemania serpens	0.42	| | | | | | | | |
Biatriospora sp.	0.4	| | | | | | | |
Prosthecium platanoidis	0.4	| | | | | | | |
Dothideomycetes sp. 2	0.39	| | | | | | |
self-inhibition *(H. fraxineus)*	0.39	| | | | | | |
Prosthecium pyriforme	0.38	| | | | | | |
Pleosporales sp. 1	0.38	| | | | | | |
Bulgaria inquinans	0.37	| | | | | | |
Lopadostoma turgidum	0.37	| | | | | | |
Pyrenochaeta corni	0.36	| | | | | | |
Phaeomoniella sp.	0.32	| | | | | |
Dothideomycetes sp. 1	0.31	| | | | |
Lophiostoma corticola	0.29	| | | |
Xylariales sp. 1	0.28	| | | |
Anthostomella pinea	0.19	| | |
control	0.18	|

Suppl. Table 1b. Average inhibition rate of the endophytes againts *H. fraxineus* isolate LUZ. Endophytes connected with the same vertical line belong to the same homogeneous group and are not significantly different ($P > 0.05$) according to Tukey multiple comparisons of means. 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.

Endophyte	Inhibition rate	Homogeneous groups
Botrytis cinerea	0.92	|
Phoma macrostoma var. *incolorata* 2	0.84	| |
Alternaria alternata	0.84	| |
Chaetomium globosum	0.78	| | |
Pezicula sporulosa	0.76	| | |
Desmazierella acicola	0.73	| | | |
Apiognomonia errabunda	0.72	| | | | | |
Phoma macrostoma	0.72	| | | | | |
Coprinellus disseminatus	0.72	| | | | |
Annulohypoxylon multiforme	0.71	| | | | | |
Gibberella avenacea	0.7	| | | | | | |
Bjerkandera adusta	0.68	| | | | | | | | | | | |
Leotiomycetes sp. 1	0.67	| | | | | | | |
Pleospora herbarum	0.66	| | | | | | | |
Sordaria fimicola	0.65	| | | | | | | | |
Valsa sp.	0.63	| | | | | | | | | |
Gibberella baccata	0.63	| | | | | | | | | |
Phoma macrostoma var. *incolorata*	0.61	| | | | | | | | | | |

Suppl. Table 1.b (Continued)

Endophyte	Inhibition rate	Homogeneous groups
Sordariomycetes sp. 1	0.59	| | | | | | | | | | | |
Pleosporales sp. 2	0.59	| | | | | | | | | | | |
Dendrothyrium sp.	0.57	| | | | | | | | | | | | |
Dothideomycetes sp. 3	0.57	| | | | | | | | | | | | |
Coniothyrium sp.	0.54	| | | | | | | | | | | | |
Rhodotorula mucilaginosa	0.53	| | | | | | | | | | | | |
Phoma sp.	0.5	| | | | | | | | | | | | | |
self-inhibition (*H. fraxineus*)	0.5	| | | | | | | | | | | | | |
Coniochaeta sp.	0.49	| | | | | | | | | | | | | |
Aureobasidium pullulans	0.49	| | | | | | | | | | | | | |
Sordariomycetes sp. 2	0.49	| | | | | | | | | | | | | |
Xylaria longipes	0.48	| | | | | | | | | | | | | |
Pleosporales sp. 1	0.46	| | | | | | | | | | | | | | |
Helotiales sp.	0.45	| | | | | | | | | | | | | | |
Phialocephala sp.	0.41	| | | | | | | | | | | | | | |
Xylariales sp. 2	0.4	| | | | | | | | | | | | | | |
Nemania serpens	0.39	| | | | | | | | | | | | | | |
Prosthecium platanoidis	0.39	| | | | | | | | | | | | | | |
Annulohypoxylon cohaerens	0.38	| | | | | | | | | | | | | | |
Xylariales sp. 1	0.37	| | | | | | | | | | | | | | |
Dothideomycetes sp. 4	0.35	| | | | | | | | | | | | | | |
Dothideomycetes sp. 2	0.34	| | | | | | | | | | | | | | |
control	0.34	| | | | | | | | | | | | | | |
Phaeomoniella sp.	0.34	| | | | | | | | | | | | | | |
Lophiostoma corticola	0.32	| | | | | | | | | | | | | | |
Biatriospora sp.	0.3	| | | | | | | | | | | | | | |
Lopadostoma turgidum	0.28	| | | | | | | | | | | | | | |
Anthostomella pinea	0.25	| | | | | | | | | | | | | | |
Diaporthe sp. 1	0.24	| | | | | | | | | | | | | | |
Prosthecium pyriforme	0.23	| | | | | | | | | | | | | | |
Pyrenochaeta corni	0.17	| | | | | | | | | | | | | | |
Bulgaria inquinans	0.13	| | | | | | | | | | | | | | |
Dothideomycetes sp. 1	0.09	| | | | | | | | | | | | | | |

Suppl. Table 1c. Average inhibition rate of the endophytes againts *H. fraxineus* isolate HER. Endophytes connected with the same vertical line belong to the same homogeneous group and are not significantly different ($P > 0.05$) according to Tukey multiple comparisons of means. 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

Endophyte	Inhibition rate	Homogeneous groups
Sordariomycetes sp. 2	0.8	|
Phoma macrostoma var. *incolorata* 2	0.77	| |
Sordaria fimicola	0.77	| |
Gibberella avenacea	0.76	| | |
Bjerkandera adusta	0.76	| | |
Apiognomonia errabunda	0.74	| | | |
Botrytis cinerea	0.74	| | | |
Xylariales sp. 2	0.73	| | | |
Bulgaria inquinans	0.71	| | | | |
Valsa sp.	0.71	| | | |

Suppl. Table 1.c (Continued)

Endophyte	Inhibition rate	Homogeneous groups
Annulohypoxylon multiforme	0.71	| | | |
Gibberella baccata	0.68	| | | | |
Coniochaeta sp.	0.68	| | | | | |
Phoma macrostoma	0.68	| | | | |
Pleospora herbarum	0.68	| | | | |
Chaetomium globosum	0.66	| | | | |
Phoma macrostoma var. *incolorata*	0.66	| | | | |
Leotiomycetes sp. 1	0.65	| | | | |
Alternaria alternata	0.65	| | | | |
Diaporthe sp. 1	0.64	| | | | |
Desmazierella acicola	0.61	| | | | | | |
Dendrothyrium sp.	0.6	| | | | | | | |
Phoma sp.	0.6	| | | | | | | |
Sordariomycetes sp. 1	0.56	| | | | | | | | |
Pyrenochaeta corni	0.56	| | | | | | | | |
Xylaria longipes	0.56	| | | | | | | | |
Pleosporales sp. 2	0.55	| | | | | | | | |
Dothideomycetes sp. 1	0.54	| | | | | | | | |
Rhodotorula mucilaginosa	0.53	| | | | | | | | |
Annulohypoxylon cohaerens	0.52	| | | | | | | | |
Dothideomycetes sp. 4	0.52	| | | | | | | | |
Prosthecium pyriforme	0.52	| | | | | | | | |
Biatriospora sp.	0.51	| | | | | | | | | |
Nemania serpens	0.46	| | | | | | | | | | |
Coniothyrium sp.	0.46	| | | | | | | | | | |
Helotiales sp.	0.46	| | | | | | | | | | |
Lopadostoma turgidum	0.45	| | | | | | | | | | |
Dothideomycetes sp. 2	0.44	| | | | | | | | | | |
Coprinellus disseminatus	0.44	| | | | | | | | | | |
Phialocephala sp.	0.43	| | | | | | | | | | |
Aureobasidium pullulans	0.42	| | | | | | | | | | |
Pezicula sporulosa	0.42	| | | | | | | | | | |
Prosthecium platanoidis	0.41	| | | | | | | | | | |
Dothideomycetes sp. 3	0.37	| | | | | | | | | | |
Pleosporales sp. 1	0.31	| | | | | | | | | | | |
Phaeomoniella sp.	0.3	| | | | | | | | | | | |
self-inhibition (*H. fraxineus*)	0.28	| | | | | | | | | | | |
Lophiostoma corticola	0.26	| | | | | | | | | | | |
Xylariales sp. 1	0.18	| | | | | | | | | | | |
Anthostomella pinea	0.13	| | | | | | | | | | | |
control	0.01	| | | | | | | | | | | |

Suppl. Table 1d. Average reisolation of both isolates of *H. fraxineus* from five discs cut from the interaction zone with the given endophyte species. The endophytes are ordered alphabetically

Endophyte species	Average reisolation		Endophyte species	Average reisolation	
	Isolate HER	Isolate LUZ		Isolate HER	Isolate LUZ
Alternaria alternata	2.0	0.0	*Lopadostoma turgidum*	0.3	0.0
Annulohypoxylon cohaerens	5.0	5.0	*Lophiostoma corticola*	4.3	0.0
Annulohypoxylon multiforme	5.0	5.0	*Nemania serpens*	0.0	0.0
Anthostomella pinea	5.0	5.0	*Pezicula sporulosa*	3.7	0.0
Apiognomonia errabunda	4.7	5.0	*Phaeomoniella* sp.	5.0	5.0
Aureobasidium pullulans	1.0	2.7	*Phialocephala* sp.	2.0	3.7
Biatriospora sp.	3.0	3.7	*Phoma macrostoma*	5.0	4.3
Bjerkandera adusta	3.0	0.0	*Phoma macrostoma* var. *incolorata*	4.7	0.0
Botrytis cinerea	1.3	0.0	*Phoma macrostoma* var. *incolorata* 2	5.0	0.0
Bulgaria inquinans	5.0	5.0	*Phoma* sp.	0.3	1.0
Coniochaeta sp.	5.0	3.7	*Pleospora herbarum*	3.7	1.3
Coniothyrium sp.	5.0	0.0	Pleosporales sp. 1	3.3	5.0
Coprinellus disseminatus	3.7	2.7	Pleosporales sp. 2	4.7	3.3
Dendrothyrium sp.	3.7	3.0	*Prosthecium platanoidis*	5.0	3.3
Desmazierella acicola	1.7	2.7	*Prosthecium pyriforme*	5.0	5.0
Diaporthe sp. 1	1.3	2.3	*Pyrenochaeta corni*	5.0	1.7
Dothideomycetes sp. 1	4.3	5.0	*Rhodotorula mucilaginosa*	4.7	5.0
Dothideomycetes sp. 2	3.0	1.7	*Sordaria fimicola*	5.0	5.0
Dothideomycetes sp. 3	1.7	0.0	Sordariomycetes sp. 1	1.0	5.0
Dothideomycetes sp. 4	5.0	5.0	Sordariomycetes sp. 2	4.0	0.0
Gibberella avenacea	3.3	0.0	*Valsa* sp.	5.0	3.7
Gibberella baccata	0.0	0.0	*Xylaria longipes*	0.3	2.7
Helotiales sp.	4.3	4.7	Xylariales sp. 1	5.0	5.0
Chaetomium globosum	0.0	5.0	Xylariales sp. 2	0.7	2.7
Leotiomycetes sp. 1	3.0	1.7			

Suppl. Table 2. Identification of isolates obtained from *Fraxinus excelsior* shoots based on molecular data (ITS rDNA) and morphology

Strain ID	Identification	Phenotype	Closest BLAST match	Accession Nr.	Identities (base pairs)
F1	*Valsa* sp.	*Cytospora* sp.	*Valsa* sp.	FJ228166	489/490
F2	*Sordaria fimicola*	*Sordaria fimicola*	*Sordaria fimicola*	AY681188	470/470
F3	*Phoma macrostoma*	*Phoma macrostoma* var. *macrostoma*	*Phoma macrostoma*	DQ474069	493/493
F4	*Aureobasidium pullulans*	*Aureobasidium pullulans*	*Aureobasidium pullulans*	JN886798	534/534
F5	*Phoma* sp.	*Phoma* sp.	*Phoma caloplacae*	JQ238635	526/547
F6	unidentified yeast	white yeast	DNA of poor quality		
F7	*Phoma macrostoma* var. *incolorata*	*Phoma macrostoma* var. *incolorata*	*Phoma macrostoma* var. *incolorata*	DQ474071	492/942
F8	*Gibberella baccata*	*Fusarium lateritium*	*Fusarium lateritium*	AF310980	518/518
F9	*Diaporthe* sp.	*Phomopsis* sp.	*Phomopsis quercina*	JX262803	534/534
F10	*Gibberella avenacea*	*Fusarium avenaceum*	*Fusarium avenaceum*	JX534353	512/512
F11	*Pyrenochaeta corni*	*Pyrenochaeta corni*	*Pyrenochaeta corni*	GQ387608	502/502
F12	Pleosporales sp. 1	yellowish to light olive coelomycete	Pleosporales sp.	JF449873	551/560

Suppl. Table 2. (Continued)

Strain ID	Identification	Phenotype	Closest BLAST match	Accession Nr.	Identities (base pairs)
F13	Pleosporales sp. 2	grey coelomycete, *Pyrenochaeta*-like	Pleosporales sp.	HQ207056	593/593
F15	*Desmazierella acicola*	*Desmazierella acicola*	*Desmazierella acicola*	LN589957	574/574
F16	*Penicillium citrinum*	*Penicillium* sp.	*Penicillium steckii*	HM469415	547/547
F17	*Lophiostoma corticola*	grey coelomycete	*Lophiostoma corticola*	AF383957	468/468
F18	*Alternaria alternata*	*Alternaria alternata*	*Alternaria alternata*	GQ328849	760/760
F20	*Prosthecium pyriforme*	*Stegonsporium pyriforme*	*Prosthecium pyriforme*	EU039975	522/526
F22	*Nemania serpens*	*Geniculosporium serpens*	*Hypoxylon serpens*	HM036598	538/538
F23	*Anthostomella pinea*	white mycelium	*Anthostomella pinea*	HQ599578	544/547
F24	*Cladosporium* sp.	*Cladosporium* sp.	*Cladosporium* sp.	KT270233	502/502
F25	*Botrytis cinerea*	*Botrytis cinerea*	*Botryotinia fuckeliana*	JQ693407	493/493
F27	*Bulgaria inquinans*	orange mycelium	*Bulgaria inquinans*	AY789345	472/473
F28	Dothideomycetes sp. 1	black-brown meristematic mycelium	*Endosporium aviarium*	EU304353	480/500
F29	*Aspergillus versicolor*	*Aspergillus versicolor*	*Aspergillus versicolor*	EF652478	663/663
F30	*Pleospora herbarum*	pale brown mycelium	*Pleospora herbarum*	GU584954	503/503
F31	*Aspergillus psudoglaucus*	*Aspergillus pseudoglaucus*	*Aspergillus pseudoglaucus*	FR839678	776/776
F32	*Rhodotorula mucilaginosa*	pink yeast	*Rhodotorula mucilaginosa*	AF444541	551/552
F34	*Chaetomium globosum*	*Chaetomium globosum*	*Chaetomium globosum*	JF773585	539/539
F38	Sordariomycetes sp. 1	ochre mycelium	Sordariomycetes sp.	JQ759839	443/458
F41	*Annulohypoxylon multiforme*	grey-brown mycelium	*Annulohypoxylon multiforme*	GU062284	500/500
F45	*Coniochaeta* sp.	yellow-orange mycelium	*Coniochaeta* sp.	JQ904605	543/543
F46	Dothideomycetes sp.2	pale grey coelomycete	Dothideomycetes sp.	JQ759636	504/516
F47	*Xylaria longipes*	white mycelium with brown margin	*Xylaria longipes*	AY909017	501/501
F52	*Annulohypoxylon cohaerens*	*Nodulisporium* sp.	*Annulohypoxylon cohaerens*	EF026140	566/566
F53	*Diaporthe* sp. 2	*Phomopsis* sp.	*Diaporthe cynaroidis*	EU552122	892/900
F57	*Lophiostoma* sp.	grey mycelium	*Lophiostoma* sp.	HE998729	414/414
F58	*Pezicula sporulosa*	*Cryptosporiopsis quercina*	*Pezicula sporulosa*	AF141166	521/523
F68	Sordariomycetes sp. 2	dark brown hyphomycete	Sordariomycetes sp.	KM519327	807/810
F69	Pleosporales sp. 3	olive mycelium with red pigment	*Phoma aliena*	KC311486	910/913
F71	Sordariomycetes sp. 3	dark brown hyphomycete	Sordariomycetes sp.	JQ760660	846/848
F75	*Prosthecium platanoidis*	brown-white coelomycete	*Prosthecium innesii*	JF681964	471/471
F81	*Coprinellus disseminatus*	pale yellow mycelium	*Coprinellus disseminatus*	JN159560	686/686
F89	Leotiomycetes sp. 1	white-orange coelomycete	Leotiomycetes sp.	JQ758675	690/720
F90	*Apiognomonia errabunda*	*Disculla umberinella*	*Apiognomonia errabunda*	AJ888477	550/551
F93	Dothideomycetes sp. 3	grey mycelium with olive-brown margine	Dothideomycetes sp.	HQ433040	794/823
F97	*Coniothyrium* sp.	*Coniothyrium* sp.	*Coniothyrium* sp.	EU852367	361/364
F98	Helotiales sp.	orange to ochre coelomycete	Helotiales sp.	HQ207037	585/618
F99	*Phaeomoniella* sp.	*Phaeomoniella* sp.	*Phaeomoniella* sp.	JN225891	341/343
F103	*Dendrothyrium* sp.	*Dendrothyrium* sp.	*Dendrothyrium variisporum*	JX496053	525/536
F106	Leotiomycetes sp. 2	white-orange hyphomycete	Leotiomycetes sp.	JQ758675	658/685
F108	Xylariales sp	white mycelium	*Anthostomella pinea*	HQ599578	449/474

Suppl. Table 2. (Continued)

Strain ID	Identification	Phenotype	Closest BLAST match	Accession Nr.	Identities (base pairs)
F109	Dothideomycetes sp. 4	white-brown coelomycete	Dothideomycetes sp.	JQ759636	538/585
F110	*Lopadostoma turgidum*	white mycelium	*Lopadostoma turgidum*	KC774617	627/630
F112	*Cryptodiaporthe hystrix*	*Diplodina acerina*	*Cryptodiaporthe hystrix*	EU255021	492/493
F114	Xylariales sp.2	white-brown mycelium	*Xylaria polymorpha*	AB512310	835/837
F115	*Bjerkandera adusta*	white mycelium	*Bjerkandera adusta*	FJ608590	736/736
F116	*Phialocephala* sp.	*Phialocephala* sp.	*Phialocephala* sp.	EU434850	341/347

HfMV1 and Another Putative Mycovirus in Central European Populations of *Hymenoscyphus fraxineus*, the Causal Agent of Ash Dieback in Europe

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Summary

The occurrence of putative, doubled-stranded (ds) viral RNA particles was investigated in the decaying ascomycetous fungal pathogen *Hymenoscyphus fraxineus*. In total, 134 isolates obtained from 134 common European ashes (*Fraxinus excelsior* and *Fraxinus angustifolia*) were obtained from three European countries (Austria, Czech Republic and Slovakia). Three different dsRNA bands of *ca* 2.2, 2.5 and 4.5 kb were confirmed in 28.4 % of the examined *H. fraxineus* samples. The dsRNA band of *ca* 2.2 kb was the most frequent, with 21.6 % occurrence, while the *ca* 4.5 kb band was the least abundant, with 15.7 % occurrence. Complementary, Hymenoscyphus fraxineus mitovirus 1 (HfMV1) was recorded through high-throughput sequencing of dsRNA in one Czech isolate and confirmed to occur in all isolates presenting bands of *ca* 2.5 and/or 2.2 kb in size using direct specific retro-transcriptase (RT) PCR. The Czech mitovirus strain contained a single ORF of 2154 nt, encoding an RNA-dependent RNA polymerase (RdRp). These results confirm the presence of HfMV1 in Central Europe and provide evidence of a potential novel mycovirus in *H. fraxineus*.

Keywords: *Chalara fraxinea*, Illumina sequencing, mycoviruses, mitovirus.

Introduction

The ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya, comb. nov. (Baral *et al.* 2014) (syn. *Hymenoscyphus pseudoalbidus* Queloz; anamorph *Chalara fraxinea* Kowalski) causes a lethal disease known as ash dieback in Europe. This disease was first identified in Poland (Kowalski, 2006), but it rapidly spread throughout eastern, central and northern Europe. Because no genetic variability has been observed between European *H. fraxineus* and Asian saprophytic fungus *Lambertella albida*, it has been suggested that it likely originated in East Asia (Zhao et al. 2012; Gross et al. 2014a).

Mycoviruses are primarily RNA viruses that infect all major groups of plant pathogenic fungi (Ghabrial and Suzuki, 2009) and are transferred intracellularly through hyphal anastomosis (horizontal transmission) and spores (vertical transmission) (Ghabrial and Suzuki, 2009). Most

mycoviruses produce cryptic infections. However, some mycoviruses identified within fungal genera, i.e., *Botrytis*, *Diaporthe*, *Fusarium*, *Helicobasidium*, *Helminthosporium*, *Ophiostoma*, *Rhizoctonia*, *Rosellinia* and *Sclerotinia*, have harmful consequences on the host organisms (Wu et al. 2007; Preisig et al. 2000; Chu et al. 2002; Ikeda et al. 2004; Ghabrial and Suzuki, 2009; Doherty et al. 2006; Lakshman et al. 1998; Kanematsu et al. 2014; Chiba et al. 2009; Deng et al. 2003; Yu et al. 2010). The best example of a mycovirus with negative effects on the fungal host is *Cryphonectria hypovirus 1* (CHV1), which is currently used as an effective biocontrol agent against *C. parasitica*, the main driver of the chestnut blight in Europe (Anagnostakis and Day, 1979; MacDonald and Fulbright, 1991).

Mycoviruses have been traditionally detected through the isolation of their dsRNA. The size and the number of dsRNA fragments suggest that these molecules might represent mycovirus genomes. In particular, *Mitovirus* (family *Narnaviridae*), the genus of the recently reported Hymenoscyphus fraxineus mitovirus 1 (HfMV1) (Schoebel et al. 2014), only occurs in fungi and has a linear genome of approximately 2.5 kb with no capsid structure. The genome includes one major open reading frame (ORF), which encodes the RNA-dependent RNA polymerase (RdRp) and has a low GC content (approximately 30 %). Mitoviruses are located and translated in the mitochondria, where these organisms typically exist as dsRNA replicative forms (Ghabrial and Suzuki, 2009).

Although mycoviruses have commonly been detected based on dsRNA isolation, the current sequencing and next generation sequencing (NGS) methods, such as Illumina, Roche or Life Technologies platforms, are proving a useful technique for the direct identification of mycoviruses (Al Rwahnih et al. 2011; Vainio et al. 2015; Ezawa et al. 2015; Feldman et al. 2013). Thus, based on previous studies concerning mycoviruses and the need to identify sustainable control methods that might inhibit the development of ash dieback, we conducted a basic investigation complemented with new technologies to describe the occurrence of fungal viruses in *H. fraxineus*. Specifically, the main objectives of the present study was (1) to investigate the occurrence of viral dsRNA particles among the Central European population of *H. fraxineus* and (2) describe potential new viruses.

Materials and Methods

Sampling

In total, 134 isolates of *H. fraxineus* from Austria, Czech Republic and Slovakia were analysed in the present study (Table 1). Forty-three samples were isolated in the laboratory of Forest Pathology in Mendel University in Brno. The other isolates were obtained thanks to the cooperation within COST Action FP1103 (FRAXBACK).

Fungal isolation

The fungus samples were isolated from ash twigs collected in the field and transported to the laboratory. The sterilisation protocol involved incubation in 70 % ethanol (30 s), followed by sodium hypochlorite solution (30 s), 10 % ethanol (30 s) and sterilised distilled water (2 min). Subsequently, the samples were dried on filter paper and transferred to a Petri dish with Malt Extract Agar (MEA) medium (HiMedia, Mumbai, India). When the mycelium was visible, the sample was immediately transferred to AMEA medium (MEA amended with 50 g fresh, frozen or dried *Fraxinus excelsior* leaves, which were removed after autoclaving) (Kirisits et al. 2013).

DsRNA analyses, cDNA synthesis and HfMV1 screening

DsRNA molecules were extracted following a modified version (Botella *et al*. 2015) of the protocol of Morris and Dodds (1979). Although CF-11 cellulose specifically binds to dsRNA, the 38 positive isolations were repeated twice to confirm the results. Furthermore, S1 Nuclease (Thermo Fisher Scientific, Inc.) treatment was conducted to degrade single-stranded RNA (ssRNA); as suggested by the manufacturer. The dsRNA-banding patterns were assessed through electrophoresis (Botella et al. 2015). The sizes of the dsRNA bands were determined through visual comparison with a Gene Ruler 1 kb Plus DNA Ladder (New England Biolabs).

Sequencing of the 2.5 and 2.2 kb bands and GenBank deposition

To confirm the presence of HfMV1 in the 35 isolates with the bands of *ca* 2.2 and/or 2.5 kb, the corresponding total dsRNA was used for the synthesis of first-strand cDNA by direct specific reverse transcription (RT) PCR as performed in Schoebel *et al.* (2014). The amplified region (*ca* 500-bp RT-PCR products) was separated through electrophoresis on 1 % agarose gels (SERVA).

The isolate MeU_1721 was randomly selected among the isolates presenting both 2.5 and 2.2 kb bands to confirm that both bands belong to HfMV1. The total dsRNA was extracted. Each dsRNA band was cut from the gel and purified to subsequently synthesise the corresponding cDNA and perform direct RT-PCR as described above. The corresponding sequences for MeU_1721-band 1 (2.5 kb) and MeU_1721-band 2 (2.2 kb) were deposited in GenBank/NCBI (accession numbers KT809401 and KT809402).

Description of the full-length sequence of HfMV1 within the isolate MEU_1739 using high-throughput sequencing

Among the positive isolates, MeU_1739 (Table 1), which contained a mixture of 3 dsRNA bands of 2.2, 2.5, and 4.5 kb in size (Figure 1), was selected for the determination and sequencing of potential viruses. A 100-200-ng/µl sample of the extracted dsRNA of MeU_1739 was sent to Macrogen Korea for high-throughput sequencing. Illumina HiSeq 2500 sequencing was performed. The subsequent data processing pipeline included the following steps. (1) Sequence quality was checked using fastQC-0.10.1. (2) The adaptors were trimmed the FASTX Toolkit (FASTX-clipper). (3) To obtain unique reads, the FASTX Toolkit (FASTX-collapser) was used. (4) The contigs were created using the Velvet-1.2.10 assembler (Zerbino and Birney, 2008). (5) Subsequently, the libraries were blasted against GenBank/NCBI. (6) The Short Oligonucleotide Alignment Program (SOAP aligner 2.21) (Li et al. 2009) was used for the efficient gapped and ungapped alignment of short oligonucleotides onto reference sequences selected in the Blastn analysis. (6) Sequences were retrieved using the map format in SAMtools (Li et al. 2009). In addition, complete packages, such as the CLC Genomic Workbench 6.5 (CLC Bio), were used for the visualisation of partial evaluation steps.

The NCBI Protein Blast (Blastx) was used to search for similar sequences and conserved domains. The NCBI OrFinder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and Geneious Pro 5.1.6 software was used to identify ORFs. The final sequence was deposited in GenBank/NCBI (accession number KT809403).

Results

Occurrence of dsRNA banding patterns and HfMV1

Three dsRNA bands of *ca* 2.2, 2.5 and 4.5 kb were observed in 28.4 % (38 out of 134) of the observed isolates (Table 1). The three different dsRNA bands infected and co-infected different isolates. Thus, the 2.2-kb band was the most abundant, occurring in nearly 21.6 % of the isolates. The other two dsRNA bands were represented in similar amounts: 17.9 % (2.5 kb) and 15.7 % (4.5 kb). All three bands were identified in 40 % of the positive samples, and more than one band was identified in more than 55 % of cases.

A comparison of the dsRNA presence in these three Central European countries revealed that isolates from the Czech Republic (N= 69) more commonly (36.2 %) hosted dsRNA segments compared with Austrian isolates (N= 26) (23.1 %) and Slovak isolates (N= 39) (17.9 %).

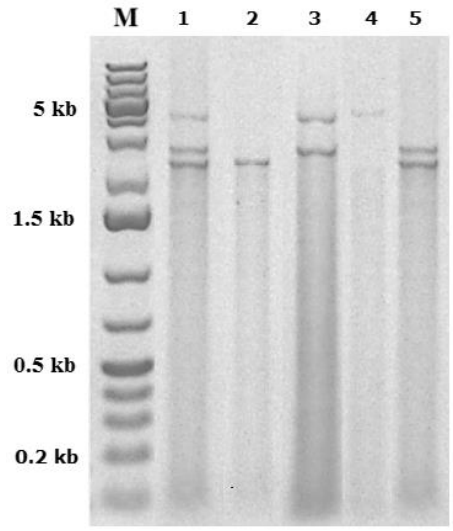

Figure 1. Gel electrophoretic profiles of the dsRNAs (*ca* 2.2 kb, 2.5 kb and 4.5 kb) observed in Central European populations of *H. fraxineus*. M, DNA marker (GeneRuler 1 kb Plus DNA Ladder, 75-20000bp, Thermo Scientific), (1) isolates MeU_1939, (2) MeU_1654, (3) KAR 4, (4) C50/12B, (5) TU 3/2/1/1

Based on direct specific RT-PCR, HfMV1 was confirmed in all the isolates harbouring *ca* 2.2 and/or 2.5 kb-bands. The PCR product was *ca* 500 bp in length, confirming the presence of HfMV1 in Central Europe. Additionally, the 2.2 and 2.5 kb bands belonging to isolates MeU_1721 and MeU_1739, respectively, were processed, and first strand cDNA was synthesised. The direct RT-PCR of the corresponding cDNA using primers specific for HfMV1 confirmed that both bands might belong to the same virus.

Description of the full-length HfMV1 sequence of isolate 1739 using high-throughput sequencing

Among the positive isolates, MeU_1739 (Table 1), which contained a mixture of three dsRNA bands of 2.2, 2.5, and 4.5 kb in size (Figure 1), was selected for the determination and sequencing of potential viruses. Two readings were obtained using Illumina HiSeq 2000 and 100-bp paired-end chemistry. The readings were generated from the end of one read from the sample (after shearing) of either direction. In total, 3,931,685 nucleotides (nt) in raw data were obtained. After quality trimming and sequence unification were completed, 1,738,530 nt were obtained from paired-end reads. The Blastn results showed hits of *H. fraxineus* mitovirus 1 in 22× and hits of *Sclerotinia sclerotiorum* mitovirus 5 in 3×. Based on these results, unique reads were aligned with reference sequences of mitoviruses. The analysis provided a total read count of 27,104 nt and high coverage totalling 1,686 nt of reference sequence (KJ667051) (Schoebel et al. 2014). The resulting consensual sequence was 2350 nt long against 2387 nt

Table 1. Data collection of the *Hymenoscyphus fraxineus* isolates analysed in this study

Code of samples	Year of collection	Locality	Country	dsRNA bands
MeU_1693	2012	Brno - Vojtova street	Czech Republic	(-)
MeU_1694	2012	Brno - Vojtova street	Czech Republic	(-)
MeU_1735	2013	Podolí	Czech Republic	(-)
MeU_1716	2013	Highlands - Třemošnice - Počátky	Czech Republic	(-)
MeU_1723	2013	Seč - Javorka	Czech Republic	(-)
MeU_1705	2013	Highlands - Běštvina - Hlubošský rybník	Czech Republic	(-)
MeU_1724	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1654	2013	Brno - Ivanovice	Czech Republic	2.2kb
MeU_1721	2013	Highlands Seč Javorka	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1737	2013	Podolí	Czech Republic	2.2kb
MeU_1704	2013	Vranovice	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1701	2013	Vranovice	Czech Republic	2.2kb, 4.5kb
MeU_1653	2013	Brno - Havelková street	Czech Republic	(-)
MeU_1730	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1732	2013	Lužické hory	Czech Republic	(-)
MeU_1738	2013	Podolí	Czech Republic	(-)
MeU_1657	2012	Brno - Ústřední hřbitov	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1665	2012	Brno - Ústřední hřbitov	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1720	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1656	2012	Brno - Ústřední hřbitov	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1702	2013	Highlands - Běštvina - Hlubošský rybník	Czech Republic	(-)
MeU_1714	2013	Highlands - Třemošnice - Počátky	Czech Republic	(-)
MeU_1734	2013	Podolí	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1731	2013	Lužické hory	Czech Republic	(-)
MeU_1699	2013	Vranovice	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1657	2012	Brno - Ústřední hřbitov	Czech Republic	(-)
MeU_1700	2013	Vranovice	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1698	2013	Vranovice	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1718	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1739	2013	Podolí	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1712	2013	Highlands - Třemošnice - Počátky	Czech Republic	2.2kb, 4.5kb
MeU_1713	2013	Highlands - Třemošnice - Počátky	Czech Republic	2.2kb
MeU_1711	2013	Highlands - Třemošnice - Počátky	Czech Republic	2.5kb
MeU_1696	2013	Highlands - Běštvina - Hlubošský rybník	Czech Republic	(-)
MeU_1726	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1703	2013	Highlands - Běštvina - Hlubošský rybník	Czech Republic	(-)
MeU_1733	2013	Lužické hory	Czech Republic	(-)
MeU_1743	2013	Lužické hory	Czech Republic	2.2kb
MeU_1725	2013	Highlands - Seč Javorka	Czech Republic	2.2kb
MeU_1742	2013	Lužické hory	Czech Republic	2.2kb
MeU_1652	2012	Brno - Bohunice, ul Havelková	Czech Republic	(-)
MeU_1729	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_13	2013	Vranovice	Czech Republic	(-)
C54/12B	2012	Lovečkovice -Ústecký kraj	Czech Republic	(-)
C52/12A	2012	Lovečkovice -Ústecký kraj	Czech Republic	(-)
C46/12B	2012	Lovečkovice -Ústecký kraj	Czech Republic	(-)
C75/12B	2012	Krásné Pole - Ústecký kraj	Czech Republic	(-)
C8/12B	2012	Výrava - Královéhradecký kraj	Czech Republic	2.2 kb
C106/13A	2013	Dolní Světlá - Liberecký kraj	Czech Republic	(-)
C102/13C	2013	Mnichovo Hradiště - Středočeský kraj	Czech Republic	(-)
C5/12C	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C6/12B	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C11/12B	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C19/12A	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)

Table 1. (Continued)

Code of samples	Year of collection	Locality	Country	dsRNA bands
C09/12A	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C99/13B	2013	Vranovice	Czech Republic	(-)
C28/12C	2012	Ploužnice - Liberecký kraj	Czech Republic	(-)
C60/12C	2012	Krásné Pole - Ústecký kraj	Czech Republic	(-)
C66/12A	2012	Krásné Pole - Ústecký kraj	Czech Republic	2.2kb, 2.5kb, 4.5kb
C40/12B	2012	Ploužnice´- Liberecký kraj	Czech Republic	2,5kb
C13/12B	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C50/12B	2012	Lovečkovice -Ústecký kraj	Czech Republic	4.5kb
C74/12C	2012	Krásné Pole - Ústecký kraj	Czech Republic	(-)
C107/13B	2013	Teplice - Ústecký kraj	Czech Republic	(-)
C48/12B	2012	Krásné Pole - Ústecký kraj	Czech Republic	(-)
C103/13B	2012	Krásné Pole - Ústecký kraj	Czech Republic	2.2kb, 2.5kb, 4.5kb
C5/12C	2012	Ploužnice - Liberecký kraj	Czech Republic	(-)
C61/12C	2012	Ploužnice - Liberecký kraj	Czech Republic	(-)
C34/12B	2012	Ploužnice - Liberecký kraj	Czech Republic	2.2kb, 2.5kb, 4.5kb
GRO 2	2010	Gröbming, Styria	Austria	(-)
KAR 4	2009	Karlsbach, Upper Austria	Austria	2.5kb, 4.5kb
HAS/FP/4/2	2011	Hasenauerstrasse, Vienna	Austria	(-)
GB 14	2011	Gansbach, Lower Austria	Austria	(-)
HO/II/6/1	2008	Hohenau an der March, Lower Austria	Austria	2.2kb, 2.5kb, 4.5kb
KAB 9	2009	Kapuzinerberg, Salzburg	Austria	(-)
BI/2/2	2009	Bad Ischl, Upper Austria	Austria	(-)
BB/1/19	2009	Bisamberg, Lower Austria	Austria	(-)
VER/2	2009	Verditz, Carinthia	Austria	(-)
N5/4/A	2007	Altaussee, Styria	Austria	2.2kb, 2.5kb
LOF 5	2009	Lofer, Salzburg	Austria	(-)
SAB 2/1	2008	Saberda, Carinthia	Austria	(-)
KOR/FP/17/2	2011	Langenzersdorf, Lower Austria	Austria	2.2kb, 2.5kb, 4.5kb
STL 1/6	2010	Lorenzen ob Murau, Styria	Austria	(-)
KOR/FP/7	2011	Langenzersdorf, Lower Austria	Austria	(-)
KOR/FP/3	2011	Langenzersdorf, Lower Austria	Austria	2.5 kb
BIZ/1	2010	Bizau, Vorarlberg	Austria	(-)
GOT 1/1/2	2009	Götzis, Vorarlberg	Austria	(-)
T 4/2	2013	Kolsass, Tyrol	Austria	(-)
WER 2/17	2009	Werfen, Salzburg	Austria	(-)
TU 3/2/1/1	2008	Tulln, Lower Austria	Austria	2.2kb, 2.5kb
KOR/FP/7	2011	Langenzersdorf, Lower Austria	Austria	(-)
SFB/II/7/2	2008	Vienna-Schafberg, Vienna	Austria	(-)
ST1	2010	Stinaz, Burgenland	Austria	(-)
NWE/1/2/H1	2008	Vienna-Neuwaldegg, Vienna	Austria	(-)
N 1/3/Holz	2008	Vienna-Neuwaldegg, Vienna	Austria	(-)
Z E7/48	2014	Zbojská	Slovakia	(-)
Z E17/5 1	2014	Zbojská	Slovakia	4.5kb
Z E7/48 1	2014	Zbojská	Slovakia	(-)
Z O 13/10	2014	Zbojská	Slovakia	(-)
Z G13/10 2	2014	Zbojská	Slovakia	(-)
Z B17/44	2014	Zbojská	Slovakia	(-)
Z J15/55 2	2014	Zbojská	Slovakia	(-)
Z E13/42 4	2014	Zbojská	Slovakia	(-)
Ú 3o	2014	Úľany nad Žitavou	Slovakia	(-)
Ú 1T	2014	Úľany nad Žitavou	Slovakia	2.2 kb
Ú 1g	2014	Úľany nad Žitavou	Slovakia	(-)
Ú 1B	2014	Úľany nad Žitavou	Slovakia	(-)
Ú 3i	2014	Úľany nad Žitavou	Slovakia	(-)

Table 1. (Continued)

Code of samples	Year of collection	Locality	Country	dsRNA bands
Ú1k	2014	Úľany nad Žitavou	Slovakia	(-)
Č 2J	2014	Černík	Slovakia	2.5 kb
Č 1c	2014	Černík	Slovakia	(-)
Č 2H	2014	Černík	Slovakia	2.2kb, 2.5kb
Č 2S	2014	Černík	Slovakia	(-)
Č 2Q	2014	Černík	Slovakia	(-)
Č 2G	2014	Černík	Slovakia	(-)
Č 2x	2014	Černík	Slovakia	2.5 kb
TR 19	2014	Trstice	Slovakia	(-)
Hladomer 4	2013	Lovce	Slovakia	(-)
Hladomer 1	2013	Lovce	Slovakia	(-)
Príbelce 3	2013	Príbelce	Slovakia	(-)
Štitáre 6E	2013	Štitáre	Slovakia	(-)
Štitáre 5B	2013	Štitáre	Slovakia	(-)
Jarok 2	2013	Jarok	Slovakia	(-)
Jarok 1a4	2013	Jarok	Slovakia	4.5kb
L9	2014	Ladzany	Slovakia	(-)
SA PC 2 E	2013	Svätý Anton	Slovakia	(-)
SA NI 7B	2013	Svätý Anton	Slovakia	(-)
SA 8 A	2013	Svätý Anton	Slovakia	(-)
SA 2/B	2013	Svätý Anton	Slovakia	(-)
SA NC 2A	2013	Svätý Anton	Slovakia	(-)
SA PC 2A	2013	Svätý Anton	Slovakia	2.2 kb
SA NC 1C	2013	Svätý Anton	Slovakia	(-)
SA NI 6B	2013	Svätý Anton	Slovakia	(-)
SA NI 10C	2013	Svätý Anton	Slovakia	(-)

(KJ667051). Unexpectedly, in contrast with the Blastn result of *Sclerotinia sclerotiorum* mitovirus 5, mapping/aligning was not successful and no coverage was determined (KJ62509) (Khalifa and Pearson, 2014).

Genome organisation of HfMV1-strain 1739

The total length of this dsRNA segment was 2350 nt with a 44.5 % GC content. Using the mitochondrial translation table, a single large ORF of 2154 nt was determined. The putative start codon AUG, occurring in an AU-rich context, was located at position 197, and the stop codon UAG was located at position 2348, potentially yielding a protein of 783 aa in length with a predicted molecular weight of 87.308 KDa, an isoelectric point of 10.13 and an extinction coefficient of 137,905.

A comparison of the full-length genome sequence in GenBank (Blastx) revealed the highest similarity with HfMV1 (88 %), followed by *Cryphonectria cubensis* mitovirus 1a (51 %) and *Sclerotinia sclerotiorum* mitovirus 15 (50 %). In addition, the alignment of the identified sequences with the Polish and German strains of HfMV1 (Schoebel et al. 2014) revealed 2,202 (93.7%) identical sites, and an overall pairwise identity of 95.4 %. According to the nomenclature practices of Schoebel *et al.* (2014), this strain was designated H. fraxineus mitovirus 1-strain 1739.

Discussion

Sequencing is the most reliable and adequate method for identifying fungal viruses, but the molecular weight of the dsRNA banding patterns might indicate the type of putative viruses involved and facilitate further analyses (Herrero et al. 2009; Botella et al. 2011). In the present study, we screened potential virus molecules in 134 isolates of *H. fraxineus* obtained from three different countries in Central Europe. Three different dsRNA bands of *ca* 2.2, 2.5 kb and 4.5 kb were confirmed to occur at a moderate frequency (28.4 % of the total contained the three bands). Different factors might influence the dsRNA frequency within fungi, i.e., the type of spore (sexual vs. asexual) (Pearson et al. 2009) that the fungus uses as its primary reproduction system. Conidiospores are more efficient for transferring mycoviruses, while virus transmission through sexual spores is a less efficient particularly in ascomycetes (Polashock et al. 1997; Buck, 1998; Pearson et al. 2009). Ascospores are the primary dispersion and infectious mechanism of *H. fraxineus* in nature (Gross et al. 2014b), and recently it was also shown that HfMV1 is vertically transmitted into sexual ascospores at a high frequency (Schoebel et al. 2017). However, in the present study, only mycelia derived from lesions were analysed. As lesions are consid-

ered a dead-end for the fungus, this might potentially decrease the capacity of mycovirus transfer. On the other hand no significant differences in prevalence, genotype diversity and virulence could be detected for HfMV1 (Burokiene et al. 2015, Lygis et al. 2016, Schoebel et al. 2017). The observed rates of virus transmission greatly differ for diverse fungus/virus combinations (Pearson et al. 2009). The transmission of mycoviruses is also controlled through a genetic self/non self-recognition system designated heterokaryon or vegetative incompatibility (vic). Although little is known about the vegetative compatibility groups (VCGs) of *H. fraxineus* in Europe, Brasier and Webber (2013) showed that in the UK, most genetic *H. fraxineus* individuals are likely vegetatively incompatible, suggesting that vegetative incompatibility indirectly influences the flow of viruses in *H. fraxineus*.

The first two bands observed were approximately 2.2 and 2.5 kb in size, which could be preliminarily classified as members of the family *Narnaviridae*, such as the genera *Mitovirus* or *Narnavirus*. Based to Schoebel *et al.* (2014), which previously described the full-length sequence of Hymenoscyphus fraxineus mitovirus 1 (HfMV1) in one Polish and one German *H. fraxineus* isolate, we used RT-PCR and specific-primers to determine whether HfMV1 corresponds to the *ca* 2.2 and/or 2.5 kb bands. Moreover, HfMV1 was identified in positive *H. fraxineus* isolates examined. In parallel, the direct high-throughput sequencing of one Czech isolate hosting the three determined bands (Figure 1) corroborated the presence of HfMV1 and also confirmed the presence of this virus in Austria, Czechia and Slovakia, as it has been recently published by Schoebel *et al.* (2017).

The novel defined Czech strain (sequence) was called HfMV1-strain 1739 and according to its size (2350 nt), this virus could correspond to a band of approximately 2.5 kb. This sequence was slightly shorter than the Polish sequence C402 (2439 nt) and slightly longer than the German sequence C9444 (2281 nt) (Schoebel et al. 2014). HfMV1-1739 is the only mitovirus species detected in the pool of data, and the fact that we detected HfMV1 in isolates harbouring both or only one band suggested that both bands might correspond to HfMV1. We confirmed this supposition through the direct RT-PCR of the cDNA from each band. However, whether the smaller segment (2.2 nt) has a shorter ORF or whether part of the 5' and/or 3' untranslated regions (UTRs) is lost should be further addressed. Mitoviruses are widespread in pathogenic fungi, such as *Ophiostoma novo-ulmi* (Cole et al. 1998), *Gremmeniella abietina* (Tuomivirta and Hantula, 2003; Botella et al. 2012), *Rhizoctonia cerealis* (Zhang et al. 2015), *Fusarium coeruleum*, *Fusarium globosum* (Osaki et al. 2015), *Fusarium circinatum* (Martinez-Alvarez et al. 2014), *Thielaviopsis basicola* (Park et al. 2006), *Helicobasidium mompa* (Osaki et al. 2005) and *Heterobasidion annosum* (4.4 kb) (Vainio et al. 2015). However, no evidence of the potential loss of part of the genome has been ever reported in mitoviruses.

The third band was 4.5 kb in size and remains unidentified; thus, further analyses are required. Nevertheless, we hypothesize this band does not belong to HfMV1, as the direct specific RT-PCR of the corresponding cDNA was negative in the isolate MeU_1739. According to the molecular weight of the detected segment (4.5 kb), this virus could be included in the family *Totiviridae*, which normally contains a monosegmented dsRNA of 4.6-7.0 kb. Totiviruses have been reported in both *Diplodia pinea* and *D. Scrobiculata* (Wet et al. 1998) and *Gremmeniella abietina* (Tuomivirta and Hantula 2005). However, we did not detect any putative member of *Totiviridae* among the pool of identified sequences. Therefore, although we cannot fully discard this idea, this virus might belong to another viral family or even a novel species belonging to an as yet undescribed virus family, not included in the reference libraries. Moreover, the intensity of this dsRNA segment is markedly faint (Fig. 1), suggesting that 4.5 kb dsRNA might correspond to a virus with ssRNA genome and low copy numbers of replicative dsRNA (Vainio et al. 2015).

In conclusion, the results of the present study confirm the presence of three dsRNA bands within *H. fraxineus* in Austria, Czech Republic and Slovakia. Two of them (2.2 and 2.5 kb) belong to HfMV1 and the third (4.5 kb) suggests the occurrence of another putative novel virus, which remains unidentified and suggests that there is another mycovirus present in *H. fraxineus*. Furthermore, a novel full-length strain of HfMV1 is described. This work contributes to an ongoing attempt to understand the role of mycoviruses as potential biological control agents (BCAs) for *H. fraxineus*.

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Challenges in Assessing the Ecological Impacts of Tree Diseases and Mitigation Measures: the Case of *Hymenoscyphus fraxineus* and *Fraxinus excelsior*

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Abstract

Forests worldwide are currently threatened by a number of non-native tree diseases. Widespread death of a tree species will have ecological impacts on species that in some way depend on that tree species to complete their life-cycle. One measure to mitigate these impacts is to establish alternative tree species to replace the threatened tree species. These alternative tree species should be as similar as possible to the threatened tree species in terms of species supported, tree traits and the environmental conditions under which the tree will grow. This study assesses the availability and quality of data to assess the ecological impact of *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and the suitability of 48 alternative trees to replace *F. excelsior* in the UK. To make this assessment data were collected on 1) species use (whether the 955 ash-associated species will use the alternative tree species), 2) traits (bark pH, deciduous, floral reward, fruit type, height, leaf dry matter content, leaf shape, length of flowering time, mycorrhizal association, pollen vector and specific leaf area) and 3) site requirements (occurrence within northern/southern and upland/lowland Britain, detailed climatic and soil nutrient requirements). For all three assessment methods there was lower confidence in the suitability of non-native tree species to replace *F. excelsior* due to lack of data. Different alternative tree species were ranked most suitable depending on the methods used. We conclude that no one species is suited to all the site types associated with *F. excelsior*, nor will any one tree species support a high percentage of the ash-associated species while also matching many of the traits of *F. excelsior*. Our work provides broad guidance on the suitability of the 48 alternatives but site specific information is required to refine this selection at each site. The study highlights a lack of information to make a full assessment of the suitability of many species, particularly non-native species and calls for the collation of biological records so that rapid assessments of the potential ecological impacts of the loss of any given tree species and the suitability of their alternative tree species can be made.

Keywords: adaptive forest management, ash dieback, Chalara, *Fraxinus excelsior*, diseases, *Hymenoscyphus fraxineus*, mitigation, pests

Introduction

The rate of spread of tree diseases and the number of different diseases causing serve impacts appears to have been increasing in recent years due, in part, to climate change and global trade (Woodward and Boa 2013). In North America, chestnut blight has caused near complete loss of *Castanea dentata* chestnuts (Jacobs 2007), Dutch elm disease has caused a similar loss of *Ulmus* spp. elms in Europe and North America (Potter et al. 2011) and several species of *Pinus* pine around the world are now threatened with the fungus *Gibberella circinata* which causes pine pitch canker (Wingfield et al. 2008). *Fraxinus excelsior* ash trees are currently dying in Russia and North America due to the emerald ash borer (*Agrilus planipennis*), a beetle from the Buprestid beetle family (commonly known as jewel beetles or metallic wood-boring beetles) native to China (Cappaert et al. 2005; Poland and McCullough 2006) and in Europe due to the ascomycete *Hymenoscyphus fraxineus* (Kjær et al. 2012; Baral et al. 2014) (previously called *Chalara fraxinea* and *H. pseudoalbidus*). Following common convention we call the disease caused by *Hymenoscyphus fraxineus* ash dieback throughout. The disease was first recorded in the UK in February 2012 and has since spread throughout the UK (see Clark and Webber 2017).

Tree diseases can cause severe economic and cultural impacts through loss of trade and cultural/ionic trees and/or forests (Boyd et al. 2013). However, widespread death of one tree species may also have huge environmental and ecological implications, in particular for other species that in some way depend on that species of tree to complete their life-cycle (associated-species). For species that predominately only use one species of tree, the loss of that tree species could lead to declines in populations or even extinctions (Ellis et al. 2012; Pautasso et al. 2013; Lohmus and Runnel 2014; Mitchell et al., 2014a). Wide spread loss of a tree species may also impact on ecosystem function, e.g. nutrient cycling and carbon storage (Mitchell et al. 2016) if tree species composition within a forest changes radically following the arrival of a particular disease.

One way of mitigating ecological impacts of tree diseases is to establish alternative tree species (Meason and Mason 2014, Wilson 2014), here defined as a tree species other than the one which is threatened by the disease. From an ecological view point it is critical that these alternative tree species are as similar as possible to the threatened tree species in terms of the associated species supported and the tree traits if the alternative tree is to provide successful mitigation. The ecological traits of a tree, such as height, bark pH, leaf shape, deciduous/evergreen, floral reward, pollinator and seed type will alter the environmental conditions or resources created by the tree and will influence which associated species will use the tree for feeding/breeding or as a habitat such as epiphytic species. In addition the site requirements (climate, soil type) of any alternative tree species must be similar to that of the threatened tree species to enable it to establish and grow at the site.

When identifying the most suitable alternative tree species as mitigation for production purposes there are a limited number of criteria on which to focus such as site requirements, growth rates, volume of timber produced and timber quality. However, identifying the most suitable alternative tree species as mitigation for ecological impacts is more complicated, as there are a larger number of factors to be considered, many of which interact, and this requires extensive data to be collated; specifically information on the species assemblages associated with candidate alternative species and on the traits of the trees. Our aim here is to collate information on a) species use, b) tree traits and c) site requirements for 48 tree species that are currently being considered as alternatives to replace *F. excelsior* in the UK. We aim to 1) identify the most suitable ecological alternative tree species as assessed using each of these three types of data and 2) collate information on the availability and quality of these data, as good quality data for all tree species under assessment is essential if comparisons between tree species are to be made accurately.

While this study focuses on the challenges of obtaining data to make an ecological assessment of the suitability of alternative tree species as mitigation for *Hymenoscyphus fraxineus* we aim to use this as a case study to illustrate the types of data required and generic problems that may occur with this type of assessment for any tree species. The UK is acknowledged as having excellent biological recording systems (Pocock et al. 2015) and *F. excelsior* is a common tree in the UK, occurring in 88% of 10 km grid squares (Preston et al. 2002). Thus one would expect data collation to be easier and more complete than for many other tree species or countries. With the increase in tree diseases, the need to identify the availability of this type of ecological data is critical if suitable ecological assessments of alternative tree species are to be made.

Method

Species use

Information on which species use *F. excelsior* was gathered for 6 taxon groups: birds, bryophytes, fungi, invertebrates, lichens and mammals. For each taxon group a "taxon expert" who knew the taxon group well and the available data sources (Table S1) was identified to carry out the assessment. Each taxon expert was asked to identify ash-associated species and their level of association with *F. excelsior*. The only *Fraxinus* species native and common in the UK is *F. excelsior* – thus the term ash-associated species refer to species that use *F. excelsior* in the UK. The level of association of the species with *F. excelsior* was

defined as: obligate - unknown from other tree species; highly associated - rarely uses other tree species; partially associated - uses *F. excelsior* more frequently than its availability; cosmopolitan - uses *F. excelsior* as frequently as or less than its availability; uses - uses *F. excelsior* but the importance of *F. excelsior* for this species is unknown. Taxon experts were asked to note specific difficulties in identifying which species were ash-associated species.

Forty-eight alternative tree species were assessed as to whether or not the ash-associated species would also use these alternative tree species. The 48 tree species assessed included all native tree and shrub species (27 species) likely to occur on sites where *F. excelsior* is currently present in the UK and 21 non-native tree species which have been proposed as possible alternatives to *F. excelsior* where commercial production of *F. excelsior* is currently the primary objective of woodland management. The taxon experts used the same data sources as for identification of the ash-associated species but recorded the use made of the alternative tree species into one of 5 categories: yes – known to use the tree, rare – only occasional records of the species using the tree, likely – no specific information on the use of the tree species by the ash-associated species but the taxon expert suggested that the ash-associated species was likely to use that tree species, for example when the ash-associated species was known to use other tree species in the same genera and known to use a wide range of deciduous tree species; No – ash-associated species thought not to use the alternative tree species, unknown – no information on whether or not the ash-associated species will use the alternative tree species. The taxon experts also recorded the quality of the data used to assess the level of association between the ash-associated species and the alternative tree species. Data were first classed as 'expert judgement' (level of association based on 'expert knowledge' of the species habitat requirements rather than on literature, frequently used for the likely, no and unknown categories of association.) and then as 'peer-reviewed', or 'non-peer-reviewed'. 'Peer-reviewed' covered a broad range of data sources and included anything that had received some form of quality control: published text books, scientific literature and databases that were quality controlled. The 'peer-reviewed' and 'non-peer-reviewed' categories were further sub-divided depending on whether the data were based on UK information or not. This was done because there is evidence that some associated species use different tree species in the UK than in other countries. Taxon experts were asked to note specific issues with identification of use of alternative tree species by the ash-associated species.

Traits

The aim was to collect data for all 48 alternative species for the following traits: bark pH, deciduous or coniferous, floral reward, fruit type, height, leaf dry matter content (LDMC), leaf shape, length of flowering time, mycorrhizal association, pollen vector and specific leaf area (SLA). As separate trait data were available for the two *Betula* species (*B. pendula* and *B. pubescens*) and the two *Quercus* species (*Q. robur* and *Q. petraea*) this resulted in 50 assessments in total.

The primary sources of data used for the tree traits in this study were: Barkman (1958), Klotz et al. (2002), Kattge et al. 2011, Hill et al. (2004) and Kleyer et al. (2008). However, not all traits for all tree species were covered by the above sources. Gaps in the data were filled on a case-by-case basis where possible, and using a range of literature. In some cases data from congeners was used. The data sources for each tree by trait combination are listed in Tables S2 and S3. Where there were multiple values for any one tree/trait combination in a database the average value was used.

Many of the alternative tree species match *F. excelsior* when assessed by individual traits, but ideally any alternative tree species should match *F. excelsior* in a high proportion of traits. Analysis across multiple traits could be carried out using a similarity index; however the calculation of similarity indices is not possible with missing data (as is the case here). Therefore for categorical traits (deciduous, floral reward, fruit type, leaf shape, mycorrhizal association and pollen vector) the alternative trees can be classed according to whether they are the same (i.e. occur in the same category as *F. excelsior*) or dissimilar to *F. excelsior* (occur in a different category to *F. excelsior*).

For traits with continuous variables the data were standardised.

$$[1] \text{ Standardised data} = ((\text{Fex-Alt})/\text{Fex})^2$$

Where Fex = value for *F. excelsior* and Alt = value for alternative tree.

The standardization allowed comparisons across traits measured in different units and assigned a value of zero for *F. excelsior*, with higher values indicating a greater difference between the alternative tree and *F. excelsior*. Alternative species were then classed as similar to *F. excelsior* (0-0.005); intermediate (>0.005-0.24) or dissimilar to *F. excelsior* (≥0.25). The cut-off between the different groups is essentially arbitrary but does allow species very different from *F. excelsior* to be identified. The number of traits classed as the same or similar then provided a measure of how similar the alternative was to *F. excelsior*.

Site requirements

The site requirements of the alternative trees were assessed in three different ways: 1) their occurrence within northern/southern and upland/lowland Britain, 2) detailed climatic and soil nutrient requirements and 3) natural suc-

cessional processes.

The site requirements of the alternative species were first assessed using the National Vegetation Classification (NVC) for Great Britain (Rodwell 1991). Semi-natural UK broadleaved woodlands with a medium to high amount (>10% of cover and >20% frequency) of *F. excelsior* referred to here as 'ash-woodlands' were identified. The alternative species were first assessed by whether they already occurred within these ash-woodlands and an assessment of the climatic constraints was made using information on the amount and distribution of *F. excelsior* present (Forestry Commission, 2012; DARDNI, 2013) and hence the distribution of ash-woodland communities from NVC maps (Rodwell, 1991; Rodwell and Patterson, 1994). Climatic constraints were then further assessed by splitting the UK into northern (Scotland, northern England and Northern Ireland) and southern (southern England and Wales) and into upland and lowland areas. Lowland areas were defined as areas where an accumulated temperature (number of day degrees above 5 degree C) exceeded c.1200 day-degrees with 'uplands' having accumulated temperatures below this threshold. Upland and lowland areas can be found in both northern and southern parts of the UK.

For those species used for production planting in the UK, there is a greater knowledge of their site requirements (climate constraints, preferred soil conditions) from the Ecological Site Classification (ESC) for Great Britain (Pyatt et al 2001). For these species we compared their site requirements to those of *F. excelsior* in more detail. In ESC seven different climate zones have been identified in the UK based on the combined climatic factors of climatic warmth (30-year average of accumulated temperature above 5 degrees C in day degrees) and climatic wetness (30-year average of moisture deficit based on the maximum excess of potential evapotranspiration over rainfall in mm). We compare the range of climate zones in which *F. excelsior* is able to grow with the suitable range of the alternate species, using data from Pyatt et al. (2001). For forestry in the UK, soil condition is described by the availability of water (Soil Moisture Regime - SMR) and soil nutrients (including the influence of pH) for plant growth (Soil Nutrient Regime - SNR). SMR is based on soil texture, stoniness and rooting depth and is divided in to eight classes (Very Wet, Wet, Very Moist, Moist, Fresh, Slightly Dry, Moderately Dry, Very Dry). SNR is derived from lithology, soil type or ground flora composition and the gradient in SNR is arbitrarily divided in to six classes (Very Poor, Poor, Medium, Rich, Very Rich and Carbonate) (Pyatt et al. 2001). We compare the range of SNR and SMR classes suitable for *F. excelsior* with the range of suitability of each of the alternatives from (Pyatt et al. 2001).

Many of the alternative species, particularly when used for conservation purposes, would be maintained by natural regeneration. Information in the literature on the germination success of alternative species and their ability to establish from seedlings or saplings in shade, indicates how well these species may function to replace *F. excelsior* in a woodland setting. A literature review to identify the ecological function of 11 of the alternative tree species was carried out using key-word driven searches undertaken during the 6-24 January 2014 in Web of Knowledge (http://wok.mimas.ac.uk/) (see Mitchell et al. 2104b). Search terms included the Latin name of the tree species together with the keywords: succession, gaps, colonization and light. For each search the abstracts of all the extracted articles were read, and if the abstract was relevant to the project (i.e. including references to more than one tree species and so enabling comparisons to be made) the full manuscript was obtained. The papers were then used to rank the species relative to each other with respect to different successional processes (succession, gaps, colonization and light).

Results

Species use

Identification of ash-associated species

Nine hundred and fifty five species were identified which use *F. excelsior* trees. This included 45 obligate species: 4 lichen species, 11 fungi and 30 invertebrates; and 62 highly associated species: 19 fungi, 13 lichens, 6 bryophytes and 24 invertebrates (Table 1). Details of ash-associated species have already been published in Mitchell et al. (2014a, b) but that work did not report on the difficulties of identifying ash-associated species which is the focus of the results here.

Table 1. Number of species and level of association with *F. excelsior* for six taxon groups. Differences in numbers of species compared to Mitchell et al. (2014a) are due to additional records being added, see Mitchell et al (2014b) for details

Organism	Level of Association					
	Obligate	High	Partial	Cosmopolitan	Uses	Total
Birds			7	5	2	12
Mammals			1	2	25	28
Bryophytes		6	30	10	12	58
Fungi	11	19	38			68
Lichens	4	13	231	294	6	548
Invertebrates	30	24	37	19	131	241
Total	45	62	344	330	174	955

The 955 ash-associated species identified is likely to be an under-estimate of the total number of ash-associated species, due to lack of data for some taxa. Algae, soil invertebrates and micro-organisms were not included in this

assessment as data on their association with tree species is lacking or hard to assess. Even for the six taxon groups that were studied our values are likely to be an under-estimate. Key issues on data availability reported by the taxon experts are summarised below.

The greatest knowledge gaps in relation to fungi associated with *F. excelsior* in the UK is the absence of data on its leaf endophytes and our limited knowledge of the fungi associated with *F. excelsior* that do not produce visible sexual or asexual structures and which are usually only detected in molecular studies. Studies on the continent (e.g. Scholtysik et al. 2012) have found high numbers of taxa from both groups associated with *F. excelsior* but so far there have been few comparable studies in the UK. In addition there are limited data on fungi associated with the below-ground structure of *F. excelsior* trees – the base and structural and feeder roots of the tree. Kubikova (1963) reported a range of common soil fungi associated with *F. excelsior* root surfaces and Summerbell (2005) also mentioned non-specialised soil fungi associated with *F. excelsior* roots.

For some invertebrate groups, in particular for saproxylic species of Diptera, Coleoptera and for Heteroptera, it is known that more species have been recorded on *F. excelsior* than those for which our literature search revealed documentation. For example, Rotheray et al. (2001) record that 69 species of saproxylic Diptera were recorded on *F. excelsior* during their fieldwork in Scotland between 1988 and 1998 but only those with a specified conservation status were named. Similarly Bernard Nau (pers. Comm.) reported finding 63 species of Heteroptera. However, many are likely to be predatory species that show no affinity to particular tree species. Identification of parasite and parasitoid invertebrate species that are associated with *F. excelsior* involved searching for species that have invertebrate hosts that were identified as having an association with *F. excelsior*, making identification of ash-associated parasitic and parasitoid species difficult. Even for the more well studied invertebrate groups, such as the Lepidoptera, there may be incomplete knowledge of plant associations for rarer species because most records are of adults and hence do not reveal information about the food plant used by the larva.

Hole nesting bird species (such as *Cyanistes caeruleus* blue tit, *Poecile palustris* marsh tit, *Sitta europaea* nuthatch, *Ficedula hypoleuca* pied flycatcher, *Dendrocopos major* and *D. minor* great and lesser spotted woodpecker) are well studied, providing quantitative assessments of tree species use compared with availability. Seed eating birds (e.g. *Pyrrhula pyrrhula* bullfinch, and *Coccothraustes coccothraustes* hawfinch) are also well studied with quantitative data on diets. Data are more limited from other bird species (e.g. *Sylvia atricapilla* blackcap, *Phylloscopus collybita* chiffchaff, *Troglodytes troglodytes* wren, and *Muscicapa striata* spotted flycatcher) that have a less direct link with tree species such as association with phytophagous invertebrate biomass or woodland structure.

Identification of use of alternative tree species by ash-associated species

In total 45840 assessments of the level of association between an ash-associated species and an alternative tree species were made (Fig. 1). Of ash-associated species 67% (640 species) are also associated with native *Quercus* species (*Q. robur* and *Q. petraea*). More than 400 ash-associated species are also associated with each of the following tree species: *Fagus sylvatica, Ulmus procera, Acer pseudoplatanus*, *Corylus avellana* and *Betula pendula*/*pubescens* (Fig. 1). Four non-native *Fraxinus* species were included in the assessment: *F. ornus*, *F. americana*, *F. pennsylvanica* and *F. mandschurica*. These species were assessed as 'likely' to support over 200 ash-associated species particularly ash-associated bird, fungi and invertebrate species. Of the non-native alternative tree species considered *Acer pseudoplatanus* (473), *Aesculus hippocastanum* (208), *Larix decidua* (166), *Juglans regia* (149), *Castanea sativa* (148) and *Juglans nigra* (126) support the greatest number of ash-associated species (number of ash-associated species supported in parentheses).

Alternative tree species are unlikely to provide a suitable mitigation measure for obligate ash-associated species, as according to our collated data obligate species only use *F. excelsior.* There may be a few species listed as 'obligate' in our data that would use other alternative species, perhaps non-native *Fraxinus* species, if they had the chance but as these alternatives are rare or not present in the UK there are no records of the obligate species using them, and hence they are classified as obligate. Alternative tree species are a potential mitigation measure for highly associated species and, after obligate species, highly associated species are most at risk from ash dieback. It is therefore important to identify which alternative tree species support the greatest number of highly associated ash species (Table 2). *Quercus robur/petraea, Corylus avellana* and *Ulmus procera/glabra* all support more than 20 highly associated ash species with *Populus tremula* and *Acer pseudoplatanus* supporting more than 15 highly associated ash species. When assessed within taxon group the alternative species that supports the greatest number of highly associated ash species varied. *Acer campestre* and *Aesculus hippocastanum* both support 5 highly associated bryophyte species. *Ulmus procera/glabra* supports 9 highly associated fungi with *Populus tremula* and *Quercus robur/petraea* supporting 7 highly associated fungi. *Fraxinus ornus* supports 6 highly associated invertebrate species and *Ligustrum vulgare* supports 4 highly associated invertebrate species. *Quercus robur/petraea* supports 10 highly associated lichen species and *Corylus avellana* and *Ulmus procera/glabra*

Table 2. Number of ash-associated species supported by 48 alternative tree species, shown for all species together and separately by the different taxon groups and their level of association with *F. excelsior* (high, partial, cosmopolitan, uses)

	All species				Bird		Bryophyte				Fungi		Invert				Lichen				Mammal		
Alternative tree species	High	Partial	Cosmopolitan	Uses	Partial	Cosmopolitan	High	Partial	Cosmopolitan	Uses	High	Partial	High	Partial	Cosmopolitan	Uses	High	Partial	Cosmopolitan	Uses	Partial	Cosmopolitan	Uses
Abies alba	1	26	38	9	2			1	1			1	1			6		22	37				3
Acer campestre	9	157	68	22	1		5	25	10	7	2	4		2	4	11	2	124	54		1		4
Acer platanoides	4	26	15	15	1	3	4	24	10	4				1	2	9							2
Acer pseudoplatanus	17	228	202	26		1	4	26	10	7	6	8		2	5	14	7	191	185	1	1	1	4
Aesculus hippocastanum	9	116	60	23	1	2	5	12	4	1	3	3		4	5	15	1	95	48		1	1	7
Alnus cordata			2	4					2							3							1
Alnus glutinosa	11	164	187	27	1	5	4	24	10	7	4	6	2	6	4	14	1	127	168				6
Betula pubescens/pendula	11	167	208	36	6	4	1	7	5	3	6	9	1	11	5	28	3	134	194				5
Carpinus betulus	7	90	57	15	1	5	4	23	10	4	2	4		2	2	8	1	60	40				3
Carya ovata				1																			1
Castanea sativa	5	61	72	10			1	1	1		4	5		1	3	7		54	68				3
Corylus avellana	21	193	186	28			4	29	10	12	6	3	3	6	6	9	8	154	169	1	1	1	6
Crataegus monogyna	9	155	117	21	1	1	4	23	10	4	3	7	1	5	3	15	1	118	102		1	1	2
Fagus sylvatica	13	222	206	64	5	2	4	25	10	6	5	14		5	5	50	4	172	188		1	1	8
Fraxinus americana	1	5	2	2								2	1	3	2	2							
Fraxinus mandschurica	1	3		2	1							2	1			2							
Fraxinus ornus	6	5	3	10								2	6	3	3	10							
Fraxinus pennsylvanica	2	5	1	2								2	2	3	1	2							
Ilex aquifolium	3	107	129	12	1	2		5	3		1	4		1	1	5	2	95	122	2	1	1	5
Juglans nigra	3	78	43	2					1				1	1	1	1	2	77	41				1
Juglans regia	7	85	50	7			2	5	6		1	1	2	2	3	5	2	77	41				2
Larix decidua		50	106	10	2	1		1		1		2		2	1	3		43	104	1			5
Ligustrum vulgare	8	61	17	6	2			1			3	3	4	9	2	4	1	46	14			1	2
Malus sylvestris	5	140	104	23			3	17	9	2	1			6	5	17	1	116	89		1	1	4
Ostrya carpinifolia		5	3	2				5	3							2							
Pinus sylvestris		60	134	22	4	2						2		2	4	14		51	127	1	1	1	7
Platanus x hybrid	2	60	34					1	2		2	1			2			58	30				
Populus nigra	4	45	17	10			1	3	1		2	2		4	4	9	1	36	12				1
Populus tremula	18	176	150	26	2	5	4	27	10	10	7	3	1	8	5	13	6	136	130				3
Prunus avium	1	48	62	5	1	1		2	5		1	1		2	8	5		42	48				
Prunus padus	2	49	41	3		1		2	5		2	1		2	4	3		44	31				
Prunus spinosa	4	76	71	15	2	2		12	7	2	3	7	1	7	4	11		47	57		1	1	2
Pseudotsuga menziesii		3	4	1		1		1	3			2											1
Pterocarya fraxinifolia			1												1								

Table 2. (Continued)

	All species				Bird		Bryophyte				Fungi		Invert				Lichen				Mammal		
Alternative tree species	High	Partial	Cosmopolitan	Uses	Partial	Cosmopolitan	High	Partial	Cosmopolitan	Uses	High	Partial	High	Partial	Cosmopolitan	Uses	High	Partial	Cosmopolitan	Uses	Partial	Cosmopolitan	Uses
Quercus cerris	3	29	21	17		3		10	6	2	3	3		5	2	13		11	10				2
Quercus robur/petraea	23	271	276	70	7	5	4	28	10	11	7	11	2	17	10	44	10	207	250		1	1	15
Quercus rubra	1	13	4	10				4	3		1	2		7	1	8							2
Salix caprea	7	44	19	35		1	4	28	10	11	3	8		8	8	17							7
Salix cinerea	4	39	17	31			4	28	10	11		4		7	7	13							7
Sambucus nigra	6	53	26	10	4	3	3	20	9	4	1	4	2		1	3		24	12		1	1	3
Sorbus aria	1	51	38	10								1		1	6	5	1	49	31			1	5
Sorbus aucuparia	9	166	192	20	3	5	3	6	7	5	2	6	1	7	4	11	3	143	176		1		4
Sorbus torminalis	2	1	1	3		1		1			2					3							
Taxus baccata		53	36			1			1			3			2			50	32				
Thuja plicata		13	1	3														13	1				3
Tilia cordata	7	37	18	22	1	5	4	23	10	4	2	6	1	7	3	15							3
Tilia platyphyllos	4	136	94	8			2	2	2			1		4	1	8	2	129	91				
Ulmus procera/glabra	21	248	183	24	2	4	4	21	10	5	9	12		15	7	16	8	197	162	2	1		1

each support 8 highly associated lichen species. No highly associated bird or mammal species were identified.

Data availability

There was more data available on ash-associated species associations with alternative tree species that are native to the UK than for those that are non-native (the unknown category in Figure 1 indicates that the data is not available to make the assessment). Most native trees had information on species use for 75% of ash-associated species. The exceptions to this were *Populus nigra*, *Salix caprea*, *Salix cinerea*, *Sambucus nigra*, *Sorbus torminalis* and *Tilia cordata*, which, although native to the UK, had information for less than 35% of ash-associated species. Most non-native tree species only had information for less than 35% of ash-associated species. The exceptions to this were *Acer pseudoplatanus*, *Aesculus hippocastanum*, *Castanea sativa*, *Juglans nigra*, *Juglans regia* and *Larix decidua*, where information was available for over 75% of ash-associated species. Thus generally, and due to a lack of data, there is lower confidence in the use made by ash-associated species of non-native tree species than native tree species.

The taxon experts also identified a number of reasons why data were lacking for the assessment of the suitability of alternative trees by ash-associated species. These can be grouped into four main issues:

Tree species not recorded:

In studies of bats and birds that use trees to roost in and/or breed in, the tree species is often not recorded. Most studies of the characteristics of bat roosts focus on the physical attributes of tree holes and their entrances (Kanuch 2005), their origins, and particularly their thermal characteristics (Jenkins et al. 1998; Ruczynski 2006; Smith and Racey 2005) without necessarily reporting the tree species involved. Birds are a well-studied taxonomic group with a wide and long established literature. If there were strong associations with *F. excelsior* for any bird species this is likely to have been noticed and remarked upon. However, most studies of both bird communities and individual species have looked at the effects of woodland structure and tree species composition, (e.g. MacArthur and MacArthur 1961, Lewis et al. 2009, Broughton et al. 2012) rather than associations with particular tree species. It is therefore often assumed that for birds and bats it is the physical attributes rather than the tree species that are important; however, this has yet to be tested with respect to using alternative trees to replace diseased tree species.

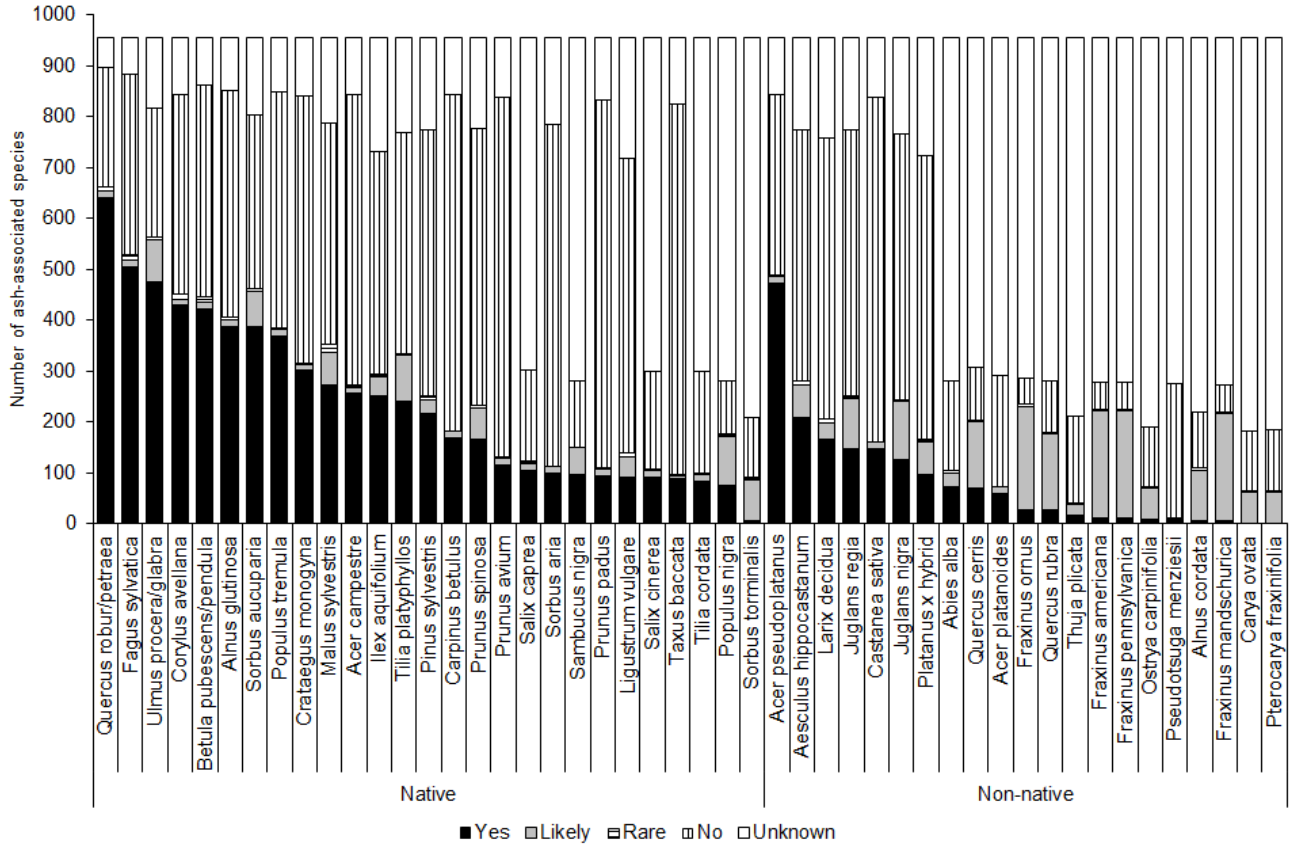

Figure 1. The use made of 48 alternative tree species by ash-associated species. Tree species ranked according to whether they are native or non-native to the UK and then by the number of ash-associated species known (yes) to use them.

Only tree genera recorded:
The lichen, bryophyte and invertebrate taxon specialists all recorded problems of only tree genera, not tree species being recorded e.g. in the British Lichen Society database the use of *Salix caprea* and *S. cinera* by ash-associated lichens was not available at the tree species level but were grouped under *Salix* spp. and records for *Tilia cordata* were only available for *Tilia* spp. (*T. cordata* and *T. platyphyllos* combined). This reduces the level of precision available when discussing which tree species may be suitable as replacements for ash.

Unclear which tree species an ash-associated species is associated with:
This problem is unique to fungi that fruit on the ground on mycelia associated with the tree roots. The Fungal Records Database of Britain and Ireland (FRDBI) records the nearest associated organism, in this case resulting in the nearest tree species being recorded. However the nearest tree may not actually be the tree whose roots the fungi is growing on. This can result in spurious associations being made between fungi and other organisms.

Unsystematic recording:
The majority of the records of species distribution in the UK are collected by volunteers, which results in unsystematic recording with the distribution of data in databases such as the FRDBI heavily weighted to those areas that have been extensively recorded. This means that when using distribution and abundance data to calculate the level of association of a species with *F. excelsior*, data from less well studied areas are likely to under represent actual species occurrence with *F. excelsior*. The data are also biased to taxa forming large obvious fruiting bodies. Information will be less complete for rare species or those that are rarely studied or documented. If there are only a small number of records of the plant species on which an invertebrate has been found, this may have the effect of making any association appear to be stronger simply through a lack of sufficient data from alternative plants. In such situations, apparent feeding preferences may be biased by the recording activity of one or a very few entomologists or may show geographic bias according to the distribution of entomological studies. The majority of records of plant-invertebrate associations are based on unsystematic observations and undoubtedly there will be many uses made of plants by invertebrate species that are not documented at all.

Data quality

Levels of association between the ash-associated species and the alternative tree species classed as 'yes' (will use the alternative tree species) generally have a high level of confidence associated with them: 91% of 'yes' records are based on peer reviewed data from the UK (Table 3). Associations that were classified as 'likely' are largely based on expert judgement (74% of likely records). These records therefore have a lower confidence associated with them, and this should be taken into account when considering which tree species to plant to promote ash-associated biodiversity, with tree species classed as 'yes' being prioritised over those classed as 'likely'. Eighty-seven percent of associations classed as 'no' were based on peer-reviewed data from within the UK, with 10% based on expert judgement. Associations classed as 'unknown' were predominantly based on expert judgement, with 70% of unknown associations in this category. Therefore, if the aim is to conserve ash-associated biodiversity, planting of alternative tree species with a level of association 'unknown' is not recommended.

Table 3. Relationship between levels of association with alternative tree species and data quality. Number of records in each class are shown. Expert judgement = level of association based on 'expert knowledge' of the species habitat requirements rather than on literature, PR = peer-reviewed data, NR = not peer-reviewed, UK = data from UK, Non-UK = data not from the UK

	Level of association					
Data quality	Yes	Likely	Rare	No	Unknown	Total
Expert judgement	94	2056	61	1755	12602	16568
NR-NonUK	87	104	1	42	117	351
NR-UK	285	377	27	283	1454	2426
PR-NonUK	279	122	16	102	164	683
PR-UK	7402	111	103	14561	3635	25812
Total	8147	2770	208	16743	17972	45840

Traits

Comparison of traits between alternative tree species

The trait values collected are available as part of the published AshEcol spreadsheets (Mitchell et al. 2014b). Table 4 ranks the alternative trees by the number of traits coded as the same or similar to *F. excelsior*. Of the eleven traits assessed, *Ulmus procera* is the most similar native tree to *F. excelsior* with eight of the traits being the same or similar; *Betula pendula* had six of the traits the same or similar. The other 27 native trees assessed had five or fewer traits the same *F. excelsior*. Twenty-four of the native trees have five or more traits classed as dissimilar with *Crataegus monogyna, Malus sylvestris, Salix cinerea* and *Tilia platyphyllos* all having six traits classed as dissimilar to *F. excelsior*. Thus when assessed by the traits most of the native tree species were not very similar to *F. excelsior*. Many of the non-native trees had similar traits to ash: *Fraxinus americana* has eight traits the same/simliar, *Juglans regia* and *F. pennsylvanica* have seven traits the same/similar and *Juglans nigra* and *Fraxinus mandschurica* have six traits the same/similar. Of the non-native trees assessed the most dissimilar species to *F. excelsior* were *Larix decidua* with seven and *Acer platanoides, Pseudotsuga menziesii* and *Abies alba* each with six of the eleven traits classed as dissimilar.

For the continuous variables of height, LDMC, SLA and length of flowering time the data shows that there are a large number of tree species that are intermediate in their similarity to *F. excelsior*. The niche breadth of the associated species and the mix of species that *F. excelsior* is growing with will determine the point at which the alternative species are no-longer suitable alternatives along this continuous gradient.

Data limitations

Trait data for many tree species were missing. Of the 50 tree species considered, there were only data for all 12 traits for 25 species. Despite searching international trait databases, appropriate data were unavailable for many of the non-native tree species. The proportion of traits with data for each tree species may be used as a measure of confidence in the data (Table 4). Data for all tree species were only available for the following traits: deciduous or coniferous, fruit type, height, leaf shape, mycorrhizal association and pollen vector. The number of trees out of 50 that had data for the other traits were bark pH 29, floral reward 47, LDMC 40, length of flowering time 36, SLA 38.

Relationship between tree traits and species use?

There was no correlation between the number of species supported and the number of traits that are the same as for *F. excelsior* (Table 4). Although the results may be influenced by missing data for some non-native tree species, analysis of native tree species, for which there are good data also showed no clear pattern. For example *Q. robur/petraea* supports the greatest number of ash-associated species (640) but only has four or five (*Q. robur* and *Q. petraea* respectively) of the eleven traits that are the same as for *F. excelsior*. *Ulmus procera* supports 477 ash-associated species and has eight of the eleven traits the same as for *F. excelsior*, yet *Fagus sylvatica* supports 505 ash-associated species but only has four traits the same as *F. excelsior*.

Table 4. Similarity of alternative trees to ash for 11 traits. S= traits the same or similar, I = traits intermediate, D = traits not the same or dissimilar, x = no data available. Tree species ranked according to whether they are native or non-native to the UK and then by the number of traits that are similar to *F. excelsior*. Trait confidence is the proportion of traits for which data was available. The number of ash-associated species supported is shown for comparison

Tree Species	Bark pH	Deciduous	Floral reward	Fruit type	Leaf shape	Mycorrhizal association	Pollinator	Height	LDMC	Flowering time	SLA	Trait confidence	No. ash species supported
Native species													
Ulmus procera/glabra	S	S	D	S	D	S	S	I	S	S	S	1.00	477
Betula pendula	D	S	D	S	D	D	S	S	S	S	I	1.00	423
Alnus glutinosa	D	S	D	S	D	D	S	I	S	S	I	1.00	389
Populus tremula	S	S	D	D	D	S	S	I	I	S	I	1.00	370
Betula pubescens	D	S	D	S	D	D	S	I	S	S	I	1.00	423
Sambucus nigra	S	S	D	D	S	S	D	D	I	S	D	1.00	96
Salix caprea	S	S	D	D	D	S	D	D	S	S	I	1.00	105
Carpinus betulus	D	S	D	S	D	D	S	I	S	S	D	1.00	169
Sorbus aucuparia	D	S	D	D	S	S	D	I	S	S	I	0.91	387
Tilia cordata	D	S	D	S	D	D	S	S	I	S	D	1.00	84
Prunus avium	D	S	D	D	D	S	D	S	I	S	S	1.00	116
Fagus sylvatica	D	S	D	S	D	D	S	I	I	S	I	1.00	505
Populus nigra	x	S	D	D	D	S	S	I	I	x	S	0.82	76
Prunus spinosa	x	S	D	D	D	S	D	D	I	S	S	0.91	167
Acer campestre	D	S	D	D	D	S	D	I	I	S	S	1.00	256
Salix cinerea	S	S	D	D	D	S	D	D	D	S	I	1.00	91
Corylus avellana	S	S	D	S	D	D	S	D	I	D	I	1.00	430
Ligustrum vulgare	x	S	D	D	D	S	D	D	S	S	I	0.91	92
Sorbus torminalis	x	S	D	D	D	S	D	I	S	S	D	0.91	7
Taxus baccata	D	D	S	D	D	S	S	I	S	D	I	1.00	89
Prunus padus	D	S	D	D	D	S	D	I	I	S	I	1.00	95
Malus sylvestris	x	S	D	D	D	S	D	D	S	D	I	0.91	272
Crataegus monogyna	D	S	D	D	D	S	D	D	I	S	I	1.00	302
Ilex aquifolium	x	D	D	D	D	S	D	I	S	S	I	0.91	251
Tilia platyphyllos	S	S	D	S	D	D	D	I	I	D	D	1.00	242
Quercus robur	D	S	D	S	D	D	S	I	I	D	I	1.00	640
Pinus sylvestris	x	D	S	D	D	D	S	I	D	S	x	0.82	216
Quercus petraea	D	S	D	S	D	D	S	I	I	D	I	1.00	640
Sorbus aria	D	S	D	D	D	S	D	I	I	S	I	1.00	100
Non-native													
Fraxinus americana	S	S	S	S	S	S	S	S	D	x	x	0.82	12
Juglans regia	x	S	S	D	S	S	S	S	S	D	I	0.91	149
Fraxinus pennsylvanica	x	S	S	S	S	S	S	S	x	x	D	0.73	12
Juglans nigra	x	S	S	D	S	S	S	I	S	x	x	0.73	126
Fraxinus mandschurica	x	S	S	S	S	S	S	I	x	x	x	0.64	6
Pterocarya fraxinifolia	x	S	x	S	S	S	S	I	x	x	x	0.55	1
Fraxinus ornus	x	S	D	S	S	S	S	I	x	x	I	0.73	29
Platanus x hybrid	D	S	S	S	D	S	S	D	x	x	D	0.82	96
Aesculus hippocastanum	x	S	D	D	D	S	D	I	S	S	I	0.91	208
Alnus cordata	x	S	S	S	D	D	S	I	x	x	x	0.64	6
Acer platanoides	D	S	D	D	D	S	D	I	S	S	D	1.00	60
Quercus rubra	x	S	S	S	D	D	S	I	I	x	I	0.82	28
Castanea sativa	D	S	D	S	D	D	S	I	S	D	I	1.00	148
Acer pseudoplatanus	S	S	D	D	D	S	D	I	S	D	I	1.00	473
Pseudotsuga menziesii	D	D	S	D	D	D	S	D	x	S	x	0.82	8
Ostrya carpinifolia	x	S	x	S	D	D	S	I	x	x	x	0.55	10
Carya ovata	x	S	x	D	S	D	S	I	x	x	x	0.55	1

Table 4. (Continued)

Tree Species	Bark pH	Deciduous	Floral reward	Fruit type	Leaf shape	Mycorrhizal association	Pollinator	Height	LDMC	Flowering time	SLA	Trait confidence	No. ash species supported
Quercus cerris	x	S	D	S	D	D	S	I	I	D	I	0.91	70
Thuja plicata	x	S	D	D	D	S	S	D	x	x	x	0.64	17
Abies alba	D	D	S	D	D	D	S	D	I	x	x	0.82	74
Larix decidua	x	D	S	D	D	D	S	D	D	D	x	0.82	166

Using traits to predict changes in ecosystem function

Many tree traits are linked to ecosystem functioning; thus some of the changes that would occur in ecosystem functioning if *F. excelsior* were replaced by one of the alternative tree species may be predicted. Most of the alternative trees assessed are deciduous and will therefore continue to produce a similar seasonal pattern of shading and litter fall to ash, if they replace *F. excelsior*. The exceptions to this are *Abies alba, Ilex aquifolium*, *Pinus sylvestris*, *Pseudotsuga menziesii* and *Taxus baccata*; if these tree species replace *F. excelsior* then there will be a change to a continuous canopy with heavy shade all year and a switch to a more continuous litter fall. These changes will influence nutrient cycling and ground flora species richness is likely to decline due to lack of light (Mitchell et al. 2014a).

The structure of the wood in terms of tree height will change least if *Betula pendula, Fraxinus americana, F. pennsylvanica, Juglans regia, Prunus avium* or *Tilia cordata*, replace *F. excelsior* as these tree species are generally (subject to local growing conditions) similar in height to *F. excelsior*. *Corylus avellana, Ligustrum vulgare* and *Prunus spinose* are generally smaller trees/shrubs than *F. excelsio*r and *Abies alba, Larix decidua, Platanus* x hybrid and *Pseudotsuga menziesii* are usually taller trees than *F. excelsior,* thus a very different woodland structure will develop if any of these species replace *F. excelsior*.

Leaf dry matter content (LDMC) is related to decomposition rates (Fortunel et al. 2009). *F. excelsior* has a low LDMC compared to most native UK trees. If *F. excelsior* is replaced by species with a high LDMC such as *Acer campestre, Fagus sylvatica, Prunus padus, P. avium* and *Salix cinerea* decomposition rates will be slower which in turn will increase carbon storage and slow down nutrient cycling.

Most temperate European woodland trees form ectomycorrhizal associations (ECM) with a wide range of soil fungi, whereas *F. excelsior* forms only arbuscular mycorrhizal (AM) associations with a more restricted group of fungi. Thirty of the alternative tree species assessed also form AM associations, but 20 of them form ECM. More soil carbon is stored in systems dominated by ecotomycorrhizal associations than in ecosystems dominated by AM-associated plants (Averill et al. 2014). Therefore if there was a major change to a system dominated by trees with ECM associations this would increase the amount of carbon stored in the system.

Site requirements

Occurrence within northern/southern and upland/lowland UK

Thirty-one of the alternative tree species are listed as components of ash-woodlands by Rodwell (1991) (Table 5). Twenty-eight species are native to the UK but three (*Tilia platyphyllos, Acer pseudoplatanus* and *Castanea sativa*) are considered non-native but regularly occur in semi-natural ash-woodlands. These 31 alternative species can all occur in ash-woodlands throughout the UK except for *Sorbus aria, S. torminalis* and *T. platyphyllos*, which all have a more southern distribution and are generally absent from Scotland, Northern England and Northern Ireland (Table 5). Relatively fewer of the alternative tree species are found in the upland regions. Most of the alternative species (22) occur in the lowlands of southern UK, whereas only half occur in upland areas of southern UK. Only one species (*Prunus avium*) is absent from lowland regions of southern UK. Four of the 27 alternative species which are found in northern UK are absent from the uplands of that region. Throughout the UK, 17 alternative species may be encountered in some but not all 'ash woodlands' (indicated as 'infrequent' in Table 5). For the four species which are non-native, this reflects the availability of nearby planted sites to provide a source of seed for natural regeneration of the species in semi-natural ash woodlands. For the remaining 13 species which are native to the UK, their lower frequency is often due to reduced ability to produce seed under UK climatic conditions (e.g. *Tilia cordata*) or exacting germination requirements being infrequently met (e.g. *Populus nigra*) (Pigott 1991; Cottrell 2004).

Table 5. Summary of production and distribution information available for alternative tree species. Native species: Y= species found in native ash woodlands (Rodwell 1991), N = species not generally found in native ash woods, nn = non-native species found in native woodlands, this reflects the availability of nearby planted sites to provide a source of seed for natural regeneration of the species in semi-natural woodlands. Production information: Y = species used for production and the site requirements of the species is well understood, N = species used occasionally for production but the site requirements of the species are not well understood, O = species used for amenity planting. NU = species not currently used for production in the UK. For native species distributional information is provided indicating climatic constraints to their growth based on their range in semi-natural ash-woodlands in the UK: F = a frequent species in ash woods in this region, I = species present infrequently in ash woods in this region, No = species not present in ash woods in this region. Northern = Scotland, northern England and Northern Ireland, Southern = southern England and Wales. Upland =Accumulated temperature ≤ 1200 day degrees, Lowland = Accumulated temperature >1200 day degrees. Distribution of *Ulmus* was not included due to major declines in abundance caused by Dutch Elm disease

Tree alternative	Native species?	Production information?	Distribution information					
			Northern	Upland	Lowland	Southern	Upland	Lowland
Abies alba	N	N						
Acer campestre	Y	NU	✔	No	I	✔	I	F
Acer platanoides	N	Y	✔	I	I	✔	I	I
Acer pseudoplatanus	nn	Y	✔	F	F	✔	F	F
Aesculus hippocastanum	N	O						
Alnus cordata	N	N						
Alnus glutinosa	Y	Y	✔	F	F	✔	F	I
Betula pendula	Y	Y	✔	F	F	✔	F	F
Betula pubescens	Y	Y	✔	F	F	✔	F	F
Carpinus betulus	Y	Y	✔	No	I	✔	No	F
Carya ovata	N	N						
Castanea sativa	nn	Y	✔	No	I	✔	No	F
Corylus avellana	Y	NU	✔	F	F	✔	F	F
Crataegus monogyna	Y	NU	✔	F	F	✔	F	F
Fagus sylvatica	Y	Y	✔	I	I	✔	I	F
Fraxinus americana	N	N						
Fraxinus mandschurica	N	N						
Fraxinus ornus	N	N						
Fraxinus pennsylvanica	N	N						
Ilex aquifolium	Y	NU	✔	F	F	✔	F	F
Juglans nigra	N	N						
Juglans regia	N	N						
Larix decidua	N	Y						
Ligustrum vulgare	Y	NU	✔	No	I	✔	I	F
Malus sylvestris	Y	NU	✔	I	I	✔	I	F
Ostrya carpinifolia	N	N						
Pinus sylvestris	N	Y						
Platanus x hybrid	N	O						
Populus nigra	Y	Y	✔	I	I	✔	I	I
Populus tremula	Y	NU	✔	I	I	✔	I	I
Prunus avium	Y	Y	✔	F	F	✔	F	No
Prunus padus	Y	NU	✔	I	I	✔	I	F
Prunus spinosa	Y	NU	✔	F	F	✔	F	F
Pseudotsuga menziesii	N	Y	✔	I	I	✔	I	I
Pterocarya fraxinifolia	N	N						
Quercus cerris	N	N						
Quercus petraea	Y	Y	✔	F	F	✔	F	F
Quercus robur	Y	Y	✔	F	F	✔	F	F
Quercus rubra	N	N						
Salix caprea	Y	NU	✔	F	F	✔	F	F
Salix cinerea	Y	NU	✔	F	F	✔	F	F
Sambucus nigra	Y	NU	✔	F	F	✔	F	F

Table 5. (Continued)

Tree alternative	Native species?	Production information?	Distribution information					
			Northern	Upland	Lowland	Southern	Upland	Lowland
Sorbus aria	Y	NU	×			✔	No	F
Sorbus aucuparia	Y	NU	✔	F	F	✔	F	F
Sorbus torminalis	Y	NU	×			✔	No	I
Taxus baccata	Y	NU	✔	I	I	✔	I	F
Thuja plicata	N	Y						
Tilia cordata	Y	Y	×			✔	I	I
Tilia platyphyllos	nn	NU	×			✔	No	I
Ulmus procera/glabra	Y	Y						

Table 6. Soil conditions considered suitable for the productive growth of alternative species and *F. excelsior* (Y) taken from Pyatt et al. 2001. Soil moisture regime: VW = Very Wet, W = Wet, VM = Very Moist, M = Moist, F = Fresh, SD = Slightly Dry, MD = Moderately Dry, VD = Very Dry. Soil nutrient regime: VP = Very Poor, P = Poor, M = Medium, R = Rich, VR = Very Rich, C = Carbonate

	Soil Moisture Regime								Soil Nutrient Regime					
	VW	W	VM	M	F	SD	MD	VD	VP	P	M	R	VR	C
Fraxinus excelsior		Y	Y	Y	Y	Y					Y	Y	Y	Y
Acer platanoides			Y	Y	Y	Y	Y				Y	Y	Y	Y
Acer pseudoplatanus		Y	Y	Y	Y	Y	Y			Y	Y	Y	Y	Y
Alnus glutinosa	Y	Y	Y	Y	Y					Y	Y	Y	Y	
Betula pendula		Y	Y	Y	Y	Y	Y		Y	Y	Y	Y	Y	
Betula pubescens	Y	Y	Y	Y	Y				Y	Y	Y	Y		
Carpinus betulus		Y	Y	Y	Y	Y				Y	Y	Y	Y	
Castanea sativa			Y	Y	Y	Y					Y	Y	Y	
Fagus sylvatica				Y	Y	Y	Y			Y	Y	Y	Y	Y
Larix decidua			Y	Y	Y	Y	Y			Y	Y	Y	Y	Y
Pinus sylvestris			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
Populus nigra		Y	Y	Y	Y						Y	Y	Y	
Populus tremula		Y	Y	Y	Y	Y				Y	Y	Y	Y	
Prunus avium			Y	Y	Y	Y					Y	Y	Y	Y
Pseudotsuga menziesii				Y	Y	Y	Y	Y		Y	Y	Y	Y	
Quercus petraea			Y	Y	Y	Y	Y			Y	Y	Y	Y	
Quercus robur		Y	Y	Y	Y	Y				Y	Y	Y	Y	
Thuja plicata		Y	Y	Y	Y	Y	Y			Y	Y	Y	Y	Y
Tilia cordata			Y	Y	Y	Y					Y	Y	Y	Y
Ulmus glabra		Y	Y	Y	Y	Y				Y	Y	Y	Y	

Detailed climatic and soil nutrient requirements

Site requirements (climatic constraints, soil moisture and soil nutrient regime) were available for nineteen of the alternative species; these were compared to the requirements of *F. excelsior* (Table 6).

Climate:

F. excelsior is ecologically suitable in five of the seven climate zones identified in the UK (Pyatt et al. 2001). Only one of the alternative species (*Betula pubescens*) is suitable in a broader range of climatic conditions than *F. excelsior* as it can still be productive where climatic warmth is as low as c.475 day degrees.

Soil conditions:

F. excelsior will grow productively where the SMR is 'Wet' through all the intervening classes up to and including 'Slightly Dry'. While 11 of the alternative species can be productive in as wet or wetter soil conditions than *F. excelsior*, and 17 species can grow well on soils as dry or with drier soil conditions, only seven species are suitable to grow on the same range of SMR classes as *F. excelsior*. *F. excelsior* is ecologically suitable where the SNR is 'Medium' to 'Very Rich' and also 'Carbonate soils', which typically occurs with rendzina soils on limestone and chalk lithologies. There is a better match between the soil nutrient requirements that are suitable for the growth of *F. excelsior*

and the alternative species. All of the 19 alternative species can tolerate 'Medium' or poorer soils and all but one species (*Betula pendula)* are ecologically suitable on sites with a 'Very Rich' SNR. However, only seven species are suitable on soils classed as 'Carbonate'. When both the SNR and the SMR requirements of the alternative species are considered, only two species, *Acer pseudoplatanus* and *Thuja plicata*, are suited to sites with the same range of soil conditions as those required by *F. excelsior*.

Natural successional processes

Information on natural successional processes is available for *F. excelsior* and eleven of the alternative species. *F. excelsior* germinates well and shows a medium to good ability to grow at the seedling or sapling stage in shade (Table S4-S6). *A. pseudoplatanus, F. sylvatica, P. avium* and *T. cordata* are reported to germinate well in shade and along with *Quercus petraea/robur* to grow well in shady conditions. Providing conditions were suitable for natural regeneration (e.g. available seed bed and low browsing pressure) these six species could be managed to replace *F. excelsior* in woodlands by natural regeneration. *Alnus glutinosa, Populus tremula* and *B. pubesens/pendula* all require higher light levels for seed germination and for early stage of tree growth compared to *F. excelsior* and would therefore not be as easy to manage by natural regeneration in place of *F. excelsior*.

Two further alternative species, both of which are non-native in the UK, were included by Mitchell et al. (2014b) due to their possible role in supporting ash-associated species. None of these species are considered as having production potential: *Aesculus hippocastanum* is threatened by disease (Laue et al. 2014) and *Platanus x hybrid* although being present as part of amenity planting for nearly a century has never been adopted by mainstream forestry in the UK.

The 13 remaining alternative species have been suggested for use on sites which currently support *F. excelsior*. However we have little experience of these species growing in UK conditions.

Discussion

Methods to assess the suitability of alternative trees

An awareness of the potential ecological impact of tree diseases is increasing with both predicted and actual declines in species populations now documented (Boyd et al. 2013, Ellis et al. 2012; Pautasso et al. 2013; Lohmus and Runnel 2014; Mitchell et al., 2014a). Once the potential species at risk due to tree loss are identified the next stage is to identify mitigation measures such as possible alternative trees. Here we have assessed the suitability of 48 alternative tree species to *F. excelsior* by three different methods, a far greater number than previously assessed by Mitchell et al. (2014a, 2016) where only 22 and 11 species were assessed. Our results show that it is possible to begin to make an assessment of the suitability of alternative trees based on their associated species, their traits and their site requirements. However there are a number of challenges with these approaches. When such ecological assessments are made it is important that these limitations are taken into account and where possible, additional data collected to fill the knowledge gaps.

The methods attempt to compare alternative tree species using ash-associated species, their traits and their site requirements. Ideally an alternative tree species should be similar to *F. excelsior* in all three of these categories, however different tree species were shown as most or least similar to *F. excelsior* depending on the method used (Table 7). This issue was identified by Mitchell et al. (2016) when comparing species use and ecosystem function for 11 tree species. Which ranking of alternative tree species is used may depend on the site objectives. If the aim is to conserve ash-associated biodiversity then using associated species to assess the most suitable alternative tree species will be most acceptable. However, if the aim is to preserve the visual attributes of the forest, or the ecosystem function then ranking by traits may be more useful. Finally if timber production is the objective, then site suitability may be the over-arching factor to consider. Ideally methods such as that proposed by Mitchell et al. (2016) to combine multiple types of assessment should be used.

Autoecological knowledge of species suggests that the phenotypic characteristics of a tree (traits) will influence the suite of associated species. In theory it should therefore be possible to use the phenotypic traits to predict if an ash-associated species will use any given alternative tree species. Ideally one would wish to find a correlation between certain traits and the number of ash-associated species supported. This might allow the prediction of which alternative trees would support the greatest number of ash-associated species. However, our data did not show any simple relationship between the number of traits that were the same as *F. excelsior* and the number of associated species supported. Thus while the traits of trees may be useful for assessing the use by individual ash-associated species, or groups of species (e.g. the relationship between bryophytes and lichens with that of bark pH); at the moment it is not possible to make broad generalizations about traits and the number of ash-associated species supported. This may be due to lack of data on traits for some tree species or traits other than those assessed being important in determining which ash-associated species use the alternative trees. In addition it may be the presence or absence of a few traits that determine the number of ash-associated species supported, rather than the overall number of traits that are the same.

Table 7. The 5 most suitable and 5 least suitable native and non-native tree species as alternatives to *F. excelsior* out of 48 assessed as assessed by species, traits and site requirements. ? = many species of intermediate similarity and difficult to rank them

Alternative tree species	Species use	Traits	Site requirements
Native species			
Most suitable	*Quercus robur/petraea*	*Ulmus glabra/procera*	*Quercus robur/petraea*
	Fagus sylvatica	*Betula pendula*	*Populus tremula*
	Ulmus procera/glabra	*?*	*Betula pendula*
	Corylus avellana	*?*	*Ulmus glabra/procera*
	Betula pubescens/pendula	*?*	*Carpinus betulus*
	Salix cinerea	*?*	*Fagus sylvatica*
	Taxus baccata	*Crataegus monogyna*	*Alnus glutinosa*
	tilia cordata	*Malus sylvestris*	*Populus nigra*
	Populus nigra	*Salix cinerea*	*Pinus sylvatica*
Least suitable	*Sorbus torminalis*	*Tilia platyphyllos*	*Betula pubescens*
Non-native species			
Most suitable	*Acer pseudoplatanus*	*Fraxinus americana*	*Acer pseudoplatanus*
	Aesculus hippocastanum	*Juglans regia*	*Thuja plicata*
	Larix decidua	*Fraxinus pennsylvanica*	*Acer platanoides*
	Juglans regia	*Juglans nigra*	*Larix decidua*
	Castanea sativa	*Fraxinus mandschurica*	*?*
	Pseudotsuga menziesii		*?*
	Alnus cordata	*Acer platonoides*	*?*
	Fraxinus mandschurica	*Pseudotsuga menziesii*	*?*
	Carya ovata	*Abies alba*	*Castanea sativa*
Least suitable	*Pterocarya fraxinifolia*	*Larix decidua*	*Pseudotsuga menziesii*

Limitations of approaches

In the final ranking of tree species (Table 7) all ash-associated species are treated equally and all traits are treated equally. However as shown in the results which alternative tree species are considered the most suitable does depend on how the tree species are ranked – whether by all ash-associated species, just by highly associated species or by individual taxon groups. Similarly, some traits maybe more important than others in maintaining ash-associated species or ecosystem functioning similar to *F. excelsior* and these traits could be prioritised in the assessment. However further work is needed to identify which traits would be prioritised. Some traits are known to have greater intra-specific variation influenced by environmental conditions e.g. specific leaf area. Therefore the similarity of tree species to *F. excelsior* when assessed by such traits may vary depending on the environmental conditions. All three approaches are essentially based on a ranking of species which takes no account of the quality of the data or the amount of missing data. Ideally someway of combining the quality/availability of data together with the ranking would represent an improvement.

Some of the traits were used to indicate how ecosystem functioning might change if there was a change to that alternative tree species. An alternative method to using traits is to use direct measurements of ecosystem functions such as litter quality, decomposition, soil chemistry taken from a literature review. However, when this was done by Mitchell et al. (2016) for 11 alternative tree species knowledge gaps were still a major problem with data missing for many tree species/function combinations.

Data availability and quality

Data availability was a major issue for all three types of assessment, particularly in relation to the assessment of the suitability of non-native tree species. There is a statistical basis with the more widespread and abundant alternative tree species being more likely to have had ash-associated species recorded using them when using data from volunteer recording rather systematic comparisons. Lack of data means there is a risk that an alternative tree species may be wrongly classed as ecologically inappropriate due to lack of data, but if planted without an appropriate assessment there is the potential to initiate large changes in species composition and a precautionary principal is advised. Data limitation also resulted in the data being collated at two different scales. Species composition used data that was predominantly collated from the UK (although for some invertebrates their use of non-native alternatives was assessed using non-UK data) while the trait data was col-

lected from international datasets. As mentioned earlier some traits may change with site characteristics with the potential for traits collected from outside the UK to be invalid.

The concept of alternative tree species raises questions over the role of non-native species. If the objective is to climate-proof our forests, in addition to making them more resistant to diseases, then in some countries/regions the ecological suitability of non-native tree species will have to be considered. Non-natives may provide the best alternatives which will ultimately ensure the sustainability of our woodlands and forests. However for many non-native tree species there is insufficient data to make an accurate assessment of their suitability as alternatives.

It is unlikely that we will ever have all the ecological data one would require to make a full ecological assessment of the most appropriate alternative tree species. However recording the species found in association with non-native trees in parks/gardens/arboreta would fill some of the knowledge gaps identified above and provide a better understanding of the potential of these species as alternative trees. The collecting and sharing of information on trait data is a growing area of study and it is likely that many of the gaps in our knowledge of traits may be filled in the next few years. Studying the growth requirements of non-native tree species in their native countries may help fill gaps related to growing conditions.

Data quality seemed to be less of an issue than data availability although the issue of recorder bias was raised. Recorder bias is almost inevitable when using volunteer recording schemes although recent studies are addressing this problem (e.g. Isaac and Pocock 2015). In terms of this study, while acknowledging that the biases are present, the data from volunteer recording schemes provides an invaluable data source for making such assessments, particular since these data are not available from other sources.

Conclusion

In relation to *F. excelsior*, our study shows that no one species is suited to all the site types associated with *F. excelsior*, nor will any one tree species support a high percentage of the ash-associated species while also matching many of the *F. excelsior* traits. The approaches used here can provide broad guidance on the suitability of alternative tree species to replace *F. excelsior* but when making decisions at individual sites, a site based approach such as that used by Broome et al. (2014) is required, taking into account the ash-associated species present at the site, the site management approaches and which tree species will grow productively a site (site requirements – climate and soils). Compromises will have to be made during the selection of alternative tree species concerning whether to replicate traits/species use or to compromise on the site requirements and perhaps accept tree species that are less productive.

The methods applied here to identify species associated with a particular tree species and then the suitability of alternative trees via an assessment of species use and traits could be applied to any other tree/tree disease combination. However, one of the main aims of this work was to collate information on the quality and availability of data. This study involved data from a country (the UK) with a global reputation for high quality biological records associated with a common tree species yet we still had issues associated with lack of data. Such issues are likely to be even more prevalent in countries with less well documented biological records and/or less common tree species.

When new diseases arrive in a region/country, there is often a requirement for a rapid assessment of the potential impact and the suitability of alternative tree species. The issues outlined above in terms of data availability need to be made clear to politicians and policy makers, particularly when such rapid assessments are required. In the longer-term, this case study highlights the need for the collation of biological records, that document the use of tree species so that rapid assessments of the potential ecological impacts of the loss of any given tree species and the suitability of their alternative tree species can be made.

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Data accessibility

This work synthesised existing data. All trait data and the species use data are summarised in an Microsoft Excel file available from Natural England called AshEcol: http://publications.naturalengland.org.uk/publication/5273931279761408.

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Supplementary material

Table S1. Methods used to asess level of species associated with *F. excelsior* and alternative tree species.

Species group	Data sources and criteria used to assess association
Lichens	For all lichen species which had been confirmed as recorded on *F. excelsior* within the British Lichen Society database (1960-2010), the number of times that each species had been recorded on *F. excelsior* as a proportion of the total number of all records across all substrata (including corticolous, terricolous and saxicolous records, etc) was calculated. The 'level of association' for a species was considered *obligate* if 100% of records were from *F. excelsior*, *high* if >50% of records were from *F. excelsior*, *partial* if >11.16% of records are from *F. excelsior*, and *cosmopolitan* if the number of records from *F. excelsior* trees <11.16%.
Bryophytes	The British Bryological Society (BBS) records and the bryophyte atlases (Hill et al., 1991, 1992 and 1994).
Fungi	The species assessed was limited to the fungal taxa in The Fungal Records Database of Britain and Ireland (FRDBI) http://www.fieldmycology.net/FRDBI/FRDBI.asp which matched the criteria: more than 10 records with an associated organism of which 25% or more were with *F. excelsior*, or had a species epithet suggesting a strong affinity with *F. excelsior*. The degree of association with *F. excelsior* of these taxa falling within this criterium was assessed as: obligate – 95% or more of the records were with *F. excelsior*; highly dependent – 50-95% records were with *F. excelsior*, the remaining taxa were considered to be partially dependent on *F. excelsior*.
Invertebrates	Initial species selection was guided by Stubbs (2012) together with reference to the Database of Insects and their Food Plants (http://www.brc.ac.uk/DBIF/homepage.aspx). Some species were discounted where the association with *F. excelsior* was from old references and this association had not been repeated in more recent and comprehensive reviews of the species. References to use of *F. excelsior* solely in captive rearing situations were also discounted. The initial list of invertebrate species identified was then supplemented from a wider literature search and consultation with some species group experts.
Mammals	The Handbook of British Mammals (Harris and Yalden, 2008). Retrieved from http://books.google.co.uk/books?id=w_UJNAAACAAJ was used as the main information source regarding the association of mammals with *F. excelsior*, supplemented with additional literature searches, and accessing websites of interested groups and societies for natural-history information.
Birds	The assessment of birds associated with *F. excelsior* trees was primarily based on online searches of peer reviewed literature. Further information was sought from unpublished reviews on the habitat associations and requirements for woodland birds.

Table S2. Data sources for tree traits. Numbers refer to the numbered references listed in Table S3

Tree alternative	Bark pH	Deciduous	Floral reward	Fruit type	Height	LDMC	Leaf shape	Leaf size	Duration of flowering	Mycorrhizal association	Pollen vector	SLA
Abies alba	16	7	5	10	9	4	7			1	5	
Aesculus hippocastanum		4	4	4	6	3	4	3	4	1	4	3
Alnus cordata		7	11	11	9		7			1	11	
Carya ovata		7		12	8		7			1	11	
Fraxinus americana	17	8	11	11	8	5	7			1	5	
Fraxinus mandschurica		8	11	11	15		8			1	11	
Fraxinus ornus		8	5	11	9		8			1	5	3
Fraxinus pennsylvanica		8	11	11	8		10			1	11	3
Ilex aquifolium		4	4	4	6	3	4	3	4	1	4	3
Juglans nigra		8	11	11	8	11	13			1	11	
Juglans regia		4	5	4	6	3	4	3	4	1	4	3
Larix decidua		4	5	4	6	5	4		4	1	4	
Ligustrum vulgare		4	4	4	6	3	4	3	4	1	4	3
Malus sylvestris		4	4	4	6	3	4	3	4	1	4	3

Table S2. (Continued)

Tree alternative	Bark pH	Deciduous	Floral reward	Fruit type	Height	LDMC	Leaf shape	Leaf size	Duration of flowering	Mycorrhizal association	Pollen vector	SLA
Ostrya carpinifolia		8		10	8		10			1	5	
Pinus sylvestris		4	5	4	6	5	4		4	1	4	
Platanus x hybrid	17	8	5	10	9		10			1	5	3
Populus nigra		4	4	4	6	3	4	3		1	4	3
Prunus spinosa		4	4	4	6		4	3	4	1	4	3
Pterocarya fraxinifolia		8		10	9		14			1	4	
Quercus cerris	18	4	4	4	6	3	4	3	4	1	4	3
Quercus rubra		8	5	11	9	3	5	3		1	11	3
Sambucus nigra	19	4	4	4	6	3	4	3	4	1	4	3
Sorbus aucuparia	20	4	4	4	6	3	4	3	3	1	4	3
Sorbus torminalis		4	3	4	6	3	4	3	4	1	4	3
Thuja plicata		4	4	10	6		10			1	5	
Tilia platyphyllos	21	8	5	4	6	3	4	3	4	1	4	3
Ulmus procera/glabra	22	4	4	4	6	3	4	3	4	1	4	3
Acer campestre	2	4	4	4	6	3	4	3	4	1	4	3
Acer platanoides	2	4	4	4	6	3	4	3	4	1	4	3
Acer pseudoplatanus	2	4	4	4	6	3	4	3	4	1	4	3
Alnus glutinosa	2	4	4	4	6	3	4	3	4	1	4	3
Betula pendula	2	4	4	4	6	3	4	3	4	1	4	3
Betula pubescens	2	4	4	4	6	3	4	3	4	1	4	3
Carpinus betulus	2	4	4	4	6	3	4	3	4	1	4	3
Castanea sativa	2	4	4	4	6	3	4	3	4	1	4	3
Corylus avellana	2	4	4	4	6	3	4	3	4	1	4	3
Crataegus monogyna	2	4	4	4	6	3	4	3	4	1	4	3
Fagus sylvatica	2	4	4	4	6	3	4	3	4	1	4	3
Fraxinus excelsior	2	4	4	4	6	3	4	3	4	1	4	3
Populus tremula	2	4	4	4	6	3	4	3	4	1	4	3
Prunus avium	2	4	4	4	6	3	4	3	4	1	4	3
Prunus padus	2	4	4	4	6	3	4	3	4	1	4	3
Pseudotsuga menziesii	2	4	4	4	6		4		4	1	4	
Quercus petraea	2	4	4	4	6	3	4	3	4	1	4	3
Quercus robur	2	4	4	4	6	3	4	3	4	1	4	3
Salix caprea	2	4	4	4	6	3	4	3	4	1	4	3
Salix cinerea	2	4	4	4	6	3	4	3	4	1	4	3
Sorbus aria	2	4	4	4	6	3	4	3	4	1	4	3
Taxus baccata	2	4	4	4	6	3	4	3	4	1	4	3
Tilia cordata	2	4	4	4	6	3	4	3	4	1	4	3

Table S3. Details of references used to obtain trait information for alternative tree species. No. refers to the trait by tree combination number listed in Table S1.

No	Reference
1	Harley, J.L. & Harley, E.L. 1987. A check-list of mycorrhiza in the British flora. *The New Phytologist*, 105 (2 Supplement), 1–102.
2	Barkman, J.J. 1958. *Phytosociology and Ecology of Cryptogamic Epiphytes*. Netherlands: Van Gorcum & Co., 628 pp.
3	LEDA trait database: Kleyer, M., Bekker, R.M., Knevel, I.C., Bakker, J.P, Thompson, K., Sonnenschein, M., Poschlod, P., Van Groenendael, J.M., Klimes, L., Klimesová, J., Klotz, S., Rusch, G.M., Hermy, M., Adriaens, D., Boedeltje, G., Bossuyt, B., Dannemann, A., Endels, P., Götzenberger, L., Hodgson, J.G., Jackel, A-K., Kühn, I., Kunzmann, D., Ozinga, W.A., Römermann, C., Stadler, M., Schlegelmilch, J., Steendam, H.J., Tackenberg, O., Wilmann, B., Cornelissen, J.H.C., Eriksson, O., Garnier, E., Peco, B. (2008): The LEDA Traitbase: A database of life-history traits of Northwest European flora. Journal of Ecology 96: 1266-1274. http://www.leda-traitbase.org/LEDAportal/
4	Bioflora database: Derived from Klotz, S., Kühn, I. & Durka, W. 2002. BIOLFLOR – Eine Datenbank zu biologisch-ökologischen Merkmalen der Gefäßpflanzen in Deutschland. Schriftenreihe für Vegetationskunde 38. Bonn: Budesamt für Naturschutz. http://www2.ufz.de/biolflor/index.jsp
5	Try database: Kattge, J., S. Díaz, S. Lavorel, I. C. Prentice, P. Leadley, G. Bönisch, E. Garnier, M., Westoby, P. B. Reich, I. J. Wright, J. H. C. Cornelissen, C. Violle, S. P. Harrison, P., M. v. Bodegom, M. Reichstein, B. J. Enquist, N. A. Soudzilovskaia, D. D. Ackerly, M., Anand, O. Atkin, M. Bahn, T. R. Baker, D. Baldocchi, R. Bekker, C. Blanco, B., Blonder, W. J. Bond, R. Bradstock, D. E. Bunker, F. Casanoves, J. Cavender-Bares,, J. Q. Chambers, F. S. Chapin, J. Chave, D. Coomes, W. K. Cornwell, J. M. Craine,, B. H. Dobrin, L. Duarte, W. Durka, J. Elser, G. Esser, M. Estiarte, W. F. Fagan, J., Fang, F. Fernández-Méndez, A. Fidelis, B. Finegan, O. Flores, H. Ford, D. Frank, G., T. Freschet, N. M. Fyllas, R. V. Gallagher, W. A. Green, A. G. Gutierrez, T. Hickler,, S. Higgins, J. G. Hodgson, A. Jalili, S. Jansen, C. Joly, A. J. Kerkhoff, D. Kirkup, K., Kitajima, M. Kleyer, S. Klotz, J. M. H. Knops, K. Kramer, I. Kühn, H. Kurokawa, D., Laughlin, T. D. Lee, M. Leishman, F. Lens, T. Lenz, S. L. Lewis, J. Lloyd, J. Llusià, F., Louault, S. Ma, M. D. Mahecha, P. Manning, T. Massad, B. Medlyn, J. Messier, A. T., Moles, S. C. Müller, K. Nadrowski, S. Naeem, Ü. Niinemets, S. Nöllert, A. Nüske, R., Ogaya, J. Oleksyn, V. G. Onipchenko, Y. Onoda, J. Ordoñez, G. Overbeck, W. A., Ozinga, S. Patiño, S. Paula, J. G. Pausas, J. Peñuelas, O. L. Phillips, V. Pillar, H., Poorter, L. Poorter, P. Poschlod, A. Prinzing, R. Proulx, A. Rammig, S. Reinsch, B., Reu, L. Sack, B. Salgado-Negret, J. Sardans, S. Shiodera, B. Shipley, A. Siefert, E., Sosinski, J.-F. Soussana, E. Swaine, N. Swenson, K. Thompson, P. Thornton, M., Waldram, E. Weiher, M. White, S. White, S. J. Wright, B. Yguel, S. Zaehle, A. E., Zanne, C. Wirth. 2011. TRY – a global database of plant traits. Global Change, Biology, 17:2905–2935.
6	Hill, M.O., Preston, C.D. & Roy, D.B. 2004. *PLANTATT - attributes of British and Irish Plants: status, size, life history, geography and habitats*. Abbots Ripton: Centre for Ecology and Hydrology.
7	Mitchell, A. (1974) A field guide to the trees of Britain and Northern Europe. Collins, Glasgow
8	Mitchell, A. Wilkinson, J. (1982) The trees of Britain and Northern Europe. Collins, London
9	Stace C.A. (1995) New Flora of the British Isles.Cambridge University Press
10	Based on descriptions of leaf shape or fruit and then categorised using Bioflora categories (expert judgement)
11	Data based on data from species in same genus due to lack of data from this species
12	http://en.wikipedia.org/wiki/Carya_ovata accessed 3/2/14
13	http://apps.rhs.org.uk/plantselector/plant?plantid=6235 accessed 3/2/2014
14	http://en.wikipedia.org/wiki/Pterocarya_fraxinifolia accessed 3/2/14
15	http://en.wikipedia.org/wiki/Fraxinus_mandshurica accessed 3/2/14
16	Legrand I, Asta J, Goudard Y (1996) Variations in bark acidity and conductivity over the trunk length of silver fir and norway spruce. Trees-Structure and Function, 11, 54-58.
17	Everhart SE, Keller HW, Ely JS (2008) Influence of bark ph on the occurrence and distribution of tree canopy myxomycete species. *Mycologia*, 100, 191-204.
18	Ozturk S, Oran S (2011) Investigations on the bark ph and epiphytic lichen diversity of quercus taxa found in marmara region. *Journal of Applied Biological Sciences*, 5, 27-33.
19	Atkinson, M.D. & Atkinson, E. (2002) Biological Flora of the British Isles. Sambucus nigra L. Journal of Ecology, 90, 895-923
20	Raspe,O., Findlay, C., Jacquemart, A.L. (2000) Biological Flora of the British Isles. *Sorbus aucuparia* L. Journal of Ecology, 88, 910-930
21	Loppi S, Frati L (2004) Influence of tree substrate on the diversity of epiphytic lichens: Comparison between tilia platyphyllos and quercus ilex (central italy). *Bryologist*, 107, 340-344.
22	Juriado I, Liira J, Paal J (2009) Diversity of epiphytic lichens in boreo-nemoral forests on the north-estonian limestone escarpment: The effect of tree level factors and local environmental conditions. *Lichenologist*, 41, 81-96.

Table S4. Species names and codes used in Tables S5 and S6

Latin	English	Code for species
Fraxinus excelsior	Ash	Fe
Sorbus aucuparia	Rowan	Sau
Betula pubescens /pendula	Birch, silver or downy.	Bp/p
Acer campestre	Field Maple	Aca
Acer pseudoplatanus	Sycamore	Aps
Populus tremula	Aspen	Ptr
Quercus petraea/robur	Oak, pedunculate or sessile	Qr/p
Fagus sylvatica	Beech	Fsy
Tilia cordata	Lime	Tco
Alnus glutinosa	Alder	Agl
Juglans nigra/regia	Walnut, black or common	Jn/r
Prunus avium	Wild cherry	Pav

Table S5. Details of references used to obtain hierarchy of the ability of the alternative trees to germinate in shade. Tree species aligned between studies where possible.

Poor				Good	Reference
Bp/p					Atkinson 1992
Bp/p				Fsy	Muys et al 1988
Bp=Ptr=Sau=Agl	Qr		Tco		Bobiec 2007
			Sau		Raspe et al 2000
Ptr					Vehmas et al 2009
Ptr					Myking et al 2011
Aca1			Aca2		1.Mathey 1924 (in Jones 1945); 2. Jones 1945
	Sau				Raspe et al 2000
	Agl				Mcvean 1953
		Pav			Petrokas 2010
		Jr			Taugourdeau et al 2010
		Aps		Fsy	Nagel et al 2010
		Fsy	Fe	Aps	Jones 1945
				Tco	Pigott 1991
				Fsy=Aps	Collet et al 2008
				Qp	Brezina & Dobrovolny 2011
			Qp/r	Fsy	Packham et al 2012
				Qp/r	Jones 1959
				Fe=Fsy	Peltier et al 1997
				Fsy	Szwagrzyk et al 2001
				Fe=Fsy	Emborg 1998
				Fsy	Jarcuska 2009

Species codes are shown in Table S4 and references listed below:

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Table S6. Details of references used to obtain hierarchy of seedlings/saplings of alternative trees to grow in shade. Tree species aligned between studies where possible

Low						High	Reference
Agl							McVean 1953
Agl							Ogilvy et al 2006
Bp/p							Atkinson 1992
Pav				Pav*			Petrokas 2010 * may persist into older forest due to its suckering abilities.
Bp			Aca=Aps=Fe		Qp/r=Fsy		Van Couwenberghe et al 2010
Bp		Ptr		Qr			Portsmuth & Niinemets 2007
	Ptr*						Raspe et al 2000. *But evidence of regeneration in old growth forest so must manage with small gaps… not well studied (Vehmas et al 2009)
		Ptr					Myking et al 2011
		Ptr				Fsy	Wittmann et al 2001
		Jr					Taugourdeau et al 2010
			Sau				Raspe et al 2000
			Aca			Fsy	Diaci et al 2012
				Aps			Hein et al 2009
					Aca		Jones 1945a
					Aps		Jones 1945b
						Aps	Hein et al 2009
				Aps	Qp	Fsy	Kazda et al 2004
				Aps	Fe	Fsy	Petritan et al 2007
				Qp	Tco	Fsy	Pigott 1991
				Qp/r			Jones 1959
				Qp		Fsy	Ligot et al 2013
				Qr		Fsy	Mountford et al 1999

Table S6. (Continued)

Low			High	Reference
	Qp			Brezina & Dobrovolny 2011
	Qr		Fsy	Rozas 2003
	Qp		Fsy	Petritan et al 2013
		Qp/r		Von Lupke 1998
		Qr	Fsy	Welander & Otterson 1998
	Fe=Fsy			Peltier et al 1997
	Fsy			Szwagrzyk et al 2001
			Fsy	Packham et al 2012
			Fsy	Jarcuska 2009

Species codes are shown in Table S4 and references listed below

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Introduction Part 2. Consequences of Ash Dieback: Damage Level, Resistance and Resilience of European Ash Forests

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Kjær E.D. 2017. Introduction Part 2. Consequences of Ash Dieback: Damage Level, Resistance and Resilience of European Ash Forests. *Baltic Forestry* 23(1): 141-143

Global warming combined with increased risk of introducing new pests and pathogens through international trade is a global concern for forest health (Pautasso et al. 2010). The outbreak of ash dieback on European ash species (especially *Fraxinus excelsior*) is a recent example of a severe infectious pathogen that has developed into a major problem across the natural distribution of the host species within a few decades. The disease is caused by the fungus *Hymenoscyphus fraxineus* (Kowalski 2006, Bakys et al. 2009, Gross et al. 2014), which is native to Asia, but has been introduced to Europe – perhaps with plants of Mandschurian ash (Drenkhan et al. 2014). Later, dispersal of seeds, seedlings and timber products may have contributed to the rapid spread across Europe, in addition to long distance wind dispersal of ascospores (Solheim and Hietala 2017). Ash dieback is an example of an emerging infectious disease that challenges native tree species of large ecological and economical importance in the new environment. This new threat stresses the importance of forest assessment to determine the current severity of damage to our native ash forests and to foresee the damage caused in the decades to come. Another important aspect to clarify will be to what extent European ash trees will be able to respond to the new disease through selection and adaptation. Will ash populations be sufficiently resistant to survive in the short run, but only at specific habitats? Will European ash suffer severely in the short run, but be resilient and recover after generations of high selection pressure for trees with natural resistance? Previous examples of emerging infectious pest and pathogens have shown that forest trees species often possess evolutionary potential to respond to new diseases (Budde et al. 2016), but will this be the case for *Fraxinus excelsior* challenged by *Hymenoscyphus fraxineus* across Europe*?*

Several studies report high mortality in young stands, but in general there is a fraction of trees that tends to remain healthy in areas that are otherwise heavily infected (Pliūra et al. 2011, Kjær et al. 2012, Enderle et al. 2013). The short term mortality of mature trees tends to be lower, and the situation can vary substantially among sites (Marçais et al. 2016). Studies of disease development among related trees (half sib families) in field trials have shown that phenotypic variation in susceptibility reflects genetically controlled resistance with moderate to high narrow sense heritability (reviewed in McKinney et al. 2014, Muñoz et al. 2016). The findings are supported by results from controlled infection assays (McKinney et al. 2012, Lobo et al. 2015) and recently from genomic and metabolomics studies (Harper et al. 2016, Sollars et al. 2016). The findings have led to optimism regarding presence of natural genetic resistance and to initiation of testing and breeding activities in several countries (see details in Vasaitis and Enderle 2017). But how will existing forests develop? Will damaged stands be regenerated by natural recruitment? A number of interesting studies that shed light on these important questions is included in the present issue of Baltic Forestry.

Highlights of this issue

Several studies deal with different aspects of the disease development from areas where the disease is relative new and areas where disease symptoms have been reported since the 90'ties. Solheim and Hietala (2017) document in detail how the disease has spread in Norway from the southeast towards the northwest with 25-78 km per year.

Norwegian regulations stopped movement of seedlings from infected areas, but the spread of the disease has continued showing the efficiency of dispersal by airborne spores. Timmermann et al. (2017) report how the disease has influenced the ash trees in different parts of Norway. High mortality was observed among young and intermediate sized trees, while the disease development has been slower in mature trees. A similar picture is presented by Marçais et al. (2017) based on results from a number of survey plots in different parts of France and Belgium. Besides the role of tree age, these studies shed new light on the role of environmental factors for the severity of the symptom development. Many details in this regard are also presented by Havrdova et al. (2017) based on a survey from the Czech Republic. Also here, the authors find damage level to be negatively correlated with tree height, and statistical correlations were observed with different environmental parameters, *i.e.* tree density, site class, species composition, presence of watercourses in the area, and distance to other ash stands. The role of other pathogens is also discussed in several of the papers. Pacia et al. (2017) studied damaged trees in the Wolica Nature Reserve in Poland and provide specific details on the presence and potential role of *Phytophthora* species. The ash trees in this Nature Reserve were genotyped with genetic markers, and the authors also report interesting genetic differences among trees in different damage levels. Similar results have previously been reported from Germany (Fussi and Konnert 2014) and Heinze and Fussi (2017) therefore present baseline information on genetic diversity in Austria based on seed samples collected prior to first observation of the disease in the country. The authors also describe how genetic markers can be an effective tool for monitoring the origin and level of diversity in seedlots.

Pušpure et al. (2017b) analyzed the natural regeneration in 90 different stands across Latvia. *Hymenoscyphus fraxineus* has probably caused significant health problems for at least 15 years in this region and therefore may already have triggered selection for more resistant trees and caused high level of damage on young trees. A very positive finding of the study was presence of relatively abundant healthy 2-6 years old ash seedling/saplings in many ash forests located on soils suitable for the species. The situation varied among sites depending on several factors and the authors note that further monitoring is required. Still, the results provide new optimism for the future of the species. Enderle et al. (2017) studied the situation in three stands in South West Germany, and based on their findings they also conclude that promotion of natural regeneration can prove an important supplement to breeding activities based on the presence of healthy individuals in the regeneration. The disease history is shorter at the German sites and the ash saplings were severely affected by the disease. However, a fraction of the recruitment remained healthy and even saplings with some symptoms remained fast growing and so far survived competition in dense regeneration in the German sample plots.

Previous genetic studies have identified a statistical relationship between phenology and susceptibility to the disease. In this relation, Nielsen et al. (2017) report that *F. excelsior* seedlings inoculated with the pathogen prior to budburst developed stronger dieback compared to seedling inoculated after budburst. Diminic et al. (2017) provide results from an inoculation study of *F. angustifolia* clones and also find an apparent relationship between phenology and susceptibility where the early flushing also was associated with lower susceptibility. Only few studies have so far targeted the specific interaction between susceptibility and year-to-year variation in temperature and participation, but this aspect is addressed by Pušpure et al. (2017a), who compare increment cores from heathy and unhealthy trees at four sites in Latvia. They find that variation in annual fluctuations of temperature and precipitation in general has had higher influence on increment of unhealthy trees compared to healthy ones. Interestingly, the authors also find that the sampled unhealthy trees in general were older than the sampled healthy trees. The authors argue that older trees have higher maintenance cost making them more prone to damage by the fungus, and based on the increment patterns they also suggest that social status of the trees have had an effect. Given that older trees in general exhibited fewer symptoms compared to young trees in other studies (as discussed above), one can speculate if the result from Latvia also can be a result of higher past mortality among young trees compared to old trees in these stands given their relative long disease history. If so, old susceptible trees may remain longer in the studied stands. More studies are required to test such and other relevant hypotheses, but the findings indicate interesting on-going dynamics in the European ash forests. Many questions remain to be addressed regarding these dynamics and the short and long term consequences of ash dieback on the European ash trees are therefore still difficult to predict. However, the new findings presented in this thematic issue provide a valuable new contribution based on observations across Europe. The new knowledge will allow better prediction of disease development under various conditions, and thereby guide foresters in development of wise management and intervention activities.

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Spread of Ash Dieback in Norway

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Abstract

Ash dieback, caused by the ascomycete *Hymenoscyphus fraxineus*, was first observed in the eastern and southernmost Norway in 2008. Based on the age of stem bark lesions, it was concluded that the fungus had arrived to the region no later than 2006. Since 2008 the annual spread of the disease northwards along the west coast of Norway has been monitored. The registration was done each year during early summer around a disease frontier recorded in the previous year. The occurrence of necrotic bark lesions in the previous-year shoots and dieback of these shoots, and isolation of *H. fraxineus* from the discoloured wood associated with necrotic bark lesions were used as signs of ash dieback. These records indicate an annual spread of ash dieback in the range between 25 km and 78 km, and a mean annual spread of 51 km. The cause of the spread is discussed.

Keywords: Ash dieback, *Hymenoscyphus fraxineus*, monitoring disease spread, west coast Norway

Introduction

Ash dieback was first observed in Poland in the early 1990ies (Przybył 2002). The disease spread first to the neighbour countries such as Lithuania and has later reached most stands in Europe where common ash (*Fraxinus excelsior* L.) is growing (Timmermann et al. 2011, McKinney et al. 2014). The cause of ash dieback was for a long period unknown, but in 2006 the anamorphic fungus *Chalara fraxinea* T. Kowalski was described as the cause (Kowalski 2006). The teleomorph was assigned to genus *Hymenoscyphus*, and the species was first named *H. pseudoalbidus* (Queloz et al. 2011). The new codex rules adopted at the International Botanical Congress in 2011 advocated the abandoning of dual naming system for pleomorphic fungi. As a result, the oldest epithet *C. fraxinea* was combined with the teleomorph genus and the species was renamed as *H. fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (Baral et al. 2014).

The ash dieback pathogen can spread to new geographic regions via several pathways. One is trade or movement of various material infested by the fungus (Sansford 2013, McKinney et al. 2014). Seedborne spread (Cleary et al. 2013, Drenkhan et al. 2016) and transfer and planting of seedlings infected in the nursery conditions provide spread pathways (Schumacher et al. 2010, Kirisits et al. 2012) that may have facilitated the disease spread to UK and Ireland (Sansford 2013). Even timber transport may be a source of spread of ash dieback, since the fungus is able to establish infection in bark and wood at stem collar in large trees (Husson et al. 2012, Chandelier et al. 2016, Marçais et al. 2016). Airborne spores provide a pathway for natural spread of most fungi, but the effective distance of this route depends on many factors (Ingold 1971). An example of fungi with long distance spread of spores is species in genus *Heterobasidion*, whose basidiospores have been sampled more than hundred kilometres away from the nearest possible source (Rishbeth 1959, Kallio 1970). During the sporulation season, ascospores of *H. fraxineus* are simultaneously liberated in vast amounts in the early morning, presumably due to active discharge. The maximum sporulation of *H. fraxineus* occurs in July and August (Hietala et al. 2013, Chandelier et al. 2014, Dvorak et al. 2016). Their amount in air is drastically reduced already a few hundred meters away from a diseased ash stand (Chandelier et al. 2014, Steinböck 2013). Since the first European recording of ash dieback in Poland around 1992, most of the common ash forests in Europe have become infested by *Hymenoscyphus fraxineus* (Figure 1).

In Norway, ash dieback was first recognized in 2008 (Talgø et al. 2009) in a plant nursery which had imported ash plants from Sweden. The nursery manager had observed shoot dieback on ash seedlings already in 2007, but thought, as many others, that it was damage caused by winter frost, since these symptoms were present in a large area covering a distance of more than 350 km measured on the map. However, when searching for disease symptoms in 2008, also bark necroses where the vascular cambium had died already in 2007 were found, and it was concluded that the first infections had taken place already in 2006 (Sol-

heim 2012). The current paper describes the spread of ash dieback in Norway in the period between 2008 and 2016 along the west coast in Norway.

Material and methods

Every year after leafing, at turn of May- June or later in the summer, ash trees up to 8-10 meters height were inspected for shoot dieback and presence of bark lesions: the survey was started at the place where the disease front had been observed the previous year, and extended into the region considered as healthy the previous year until no signs of ash dieback were observed. A few times the previous year front had to be redefined due to the discovery of old bark lesions in an area considered as healthy the previous year. The distance between an old and a new front was measured as a straight line on the map.

Shoots with shoot dieback or stem bark lesions, sampled at the new front, were brought to the laboratory for fungal isolation. The shoots were split and small tissue pieces taken at the edges of discoloured wood were placed on malt extract agar (MEA, 1.5% malt and 1.5% agar) in Petri dishes. The Petri dishes were inspected regularly for the presence of phialophores characteristic to the vegetative phase of *H. fraxineus* (Kowalski 2006). Representative isolates were later grown on a cellophane membrane placed on malt agar. The harvested mycelia were ground in liquid nitrogen using a mortar and subjected to DNA isolation with Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification was carried out with ITS1 and ITS4 primers (Gardes and Bruns 1993) in 50-µl-reaction volumes using HotStarTaq™ Plus DNA Polymerase (Qiagen) according to the manufacturer's instructions. After gel electrophoresis, the amplicon from each reaction was purified with MinElute PCR purification Kit (Qiagen) and sequenced in both directions on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Contigs were assembled with Seqman software (Lasergene, DNASTAR Inc., Madison, WI) and queried against ITS sequences in NCBI GenBank Sequence Database.

Results

During the first search in 2008, symptoms characteristic to ash dieback were recognized in a huge area that extended 360 km south from Hedmark county (Ringsaker mucipality, Nerlia 60.83494423°N, 10.91020265°E). The south-easternmost finding was ca. 1 km in Rogaland county (Lund municipality, Skår, 58.44918°N, 6.56468°E) (Table 1, Figure 2). The site in Rogaland was an open area near the main road, where a few diseased ash saplings, obviously naturally regenerated, were observed. The nearest forest habitat with ash dieback was located along a small river near the fjord close to Flekkefjord town in Vest-Agder county.

In 2009 the front of ash dieback was observed in Eigersund municipality (Litle Hogstad) in Rogaland county ca. 25.5 km northwest of the previous year's front (Table1, Figure 2B). At this site a few diseased ash saplings were growing along a river in an open landscape with agricultural fields on both sides of the river. A young ash stand with few diseased saplings was located about 1.5 km east of the front.

In 2010 the new front was found in Gjesdal municipality in Rogaland (Ålgård) (Table 1, Figure 2B), 29.5 km away from the previous year's front. Here a few ash saplings showed stem lesions or shoot dieback in two places in the village and a few locations outside the village around the lakes Edlandsvatnet and Limavatnet.

In 2011 a few diseased saplings were observed just north of the front of 2010, but further north near Jørpeland (59.0170353N, 6.04809061E) also two-year-old bark lesions were observed in ash saplings. At Hogganvika in Vindafjord municipality (59.47992973°N, 5.92234053°E) even older necroses were found and especially one site showed dead small ash trees and saplings with symptoms

Table 1. Spread of ash dieback at the west coast of Norway with information about the year observed, county, municipality, site, and coordinates of the deduced diseased fronts. The column distance indicates the distance of the deduced disease front site to the disease front observed the previous year

Year	County	Municipality	Site	North°	East°	Distance (km)
2008	Rogaland	Lund	Moi	58.44913233	6.56457068	
2009	Rogaland	Eigersund	Litle Hogstad	58.54115054	6.16421514	25.5
2010	Rogaland	Gjesdal	Ålgård	58.7692211	5.91129864	29.5
2011	Sogn og Fjordane	Askvoll	Rivedal	61.36118896	5.24321179	(52.5-70)
2012	Sogn og Fjordane	Flora	Sunnarvåg	61.65853028	4.99647677	36
2013	Møre og Romsdal	Herøy	Nykreim	62.25528828	5.79300963	78
2014	Møre og Romsdal	Ørskog	Sjøholt	62.55258389	7.67142575	59
2015	Møre og Romsdal	Averøy	Bruhagen	63.05479556	7.63431558	76
2016	Møre og Romsdal	Aure	Våg	63.28595052	8.5410134	53
Mean 2008-16						51

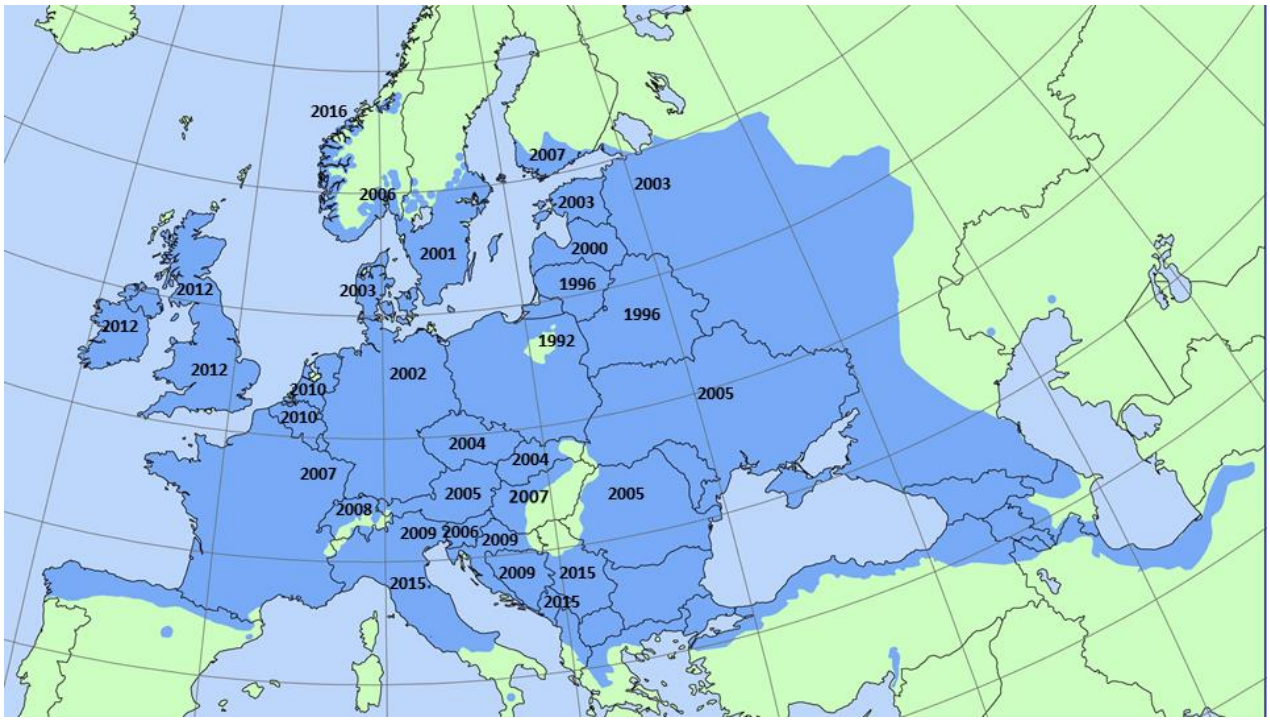

Figure 1. Map of Europe showing the distribution range of *Fraxinus excelsior* (in blue) and the year of the first observation of symptoms of ash dieback or the year it was supposed to have arrived in the country for the first time. Most information is based on references. In addition Dr. R. Vasaitis and Dr. N. Keca have contributed with personal information from some countries. The distribution map of common ash was kindly provided by EUFORGEN (2009)

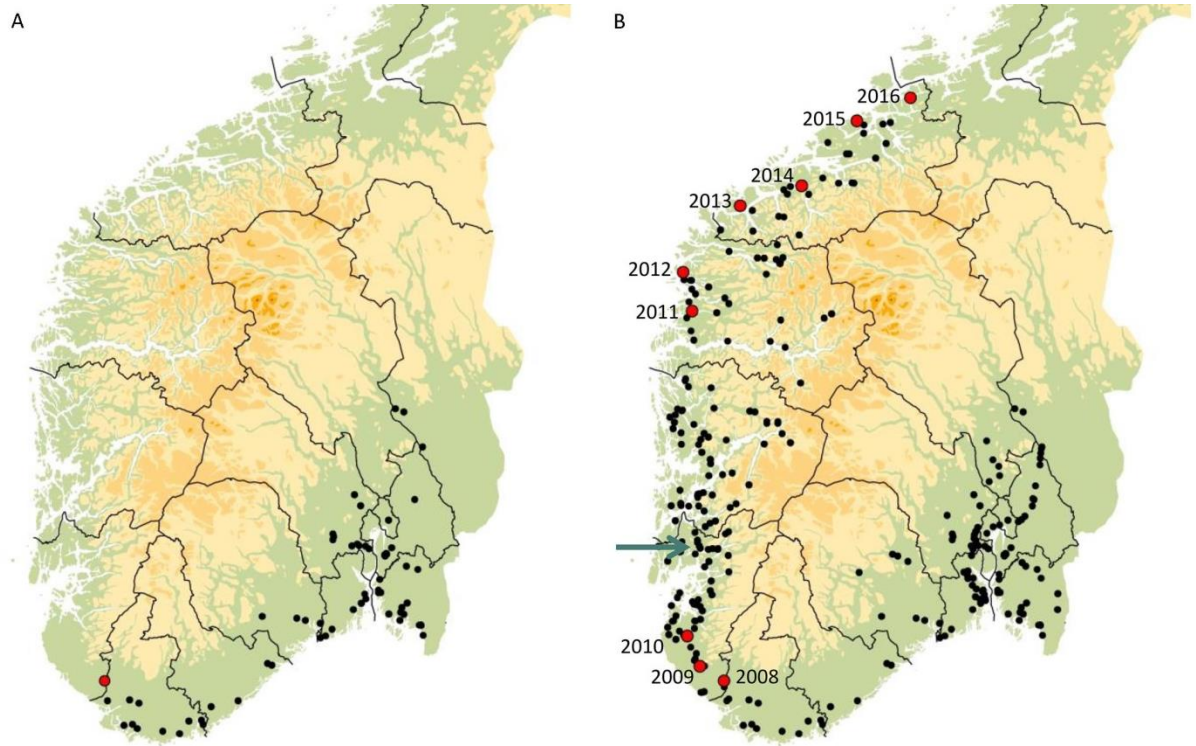

Figure 2. A. The distribution of ash dieback in Norway in 2008. All dots are verified with isolations and many also with sequencing. B. The known distribution of ash dieback in Norway in 2016. Red dots are front sites each year at the west coast. Green arrow shows the area in Vindafjord municipality (Hogganvika), where a separate introduction is supposed.

that were concluded to be 3-4 years old (Figure 2B, big arrow). Even further north, one-year-old necroses were found up to Askøy, just outside Bergen.

So in 2011 the new disease front had reached Sogn og Fjordane county, and thereby passed Sognefjorden, the longest fjord in Norway. The front was in Askvoll municipality (Rivedal) (Table 1, Figure 2B). Here the fjord Dalsfjorden was surrounded by small patches of agricultural fields, and small ash trees were found scattered in the area. Only one stem bark lesion was found here. The distance of the site to Hogganvika is 210 km. Since it most probably has been an own introduction to Haugalandet, the distance of spread in 2011 could not be estimated. If the spread from Hogganvika had taken for example 3 or 4 years, the annual spread had been 70 km or 52.5 km, respectively.

In 2012, the disease front site in Sogn og Fjordane was just south of Sunnarvåg (Table 1, Figure 2B) in Flora municipality in a mixed broadleaved stand with ash saplings and small plants growing in a cove a few meters from the Atlantic Ocean. This stand was first recognized in 2013, but some of the lesions observed then were concluded to be one year old. The distance of this site to the previous year's front in Rivedal, Askvoll is ca. 36 km (Table 1, Figure 2B).

In 2013 the disease front was observed in Herøy municipality (Nykreim) in the county of Møre og Romsdal (Table 1, Figure 2B). The site hosted ash saplings and small trees, two of which had symptoms of ash dieback. The distance to the 2012 front at the coastline is 78 km.

In 2014 the disease front in the coastal region of the Møre og Romsdal county was at Sjøholt in Ørskog municipality (Table 1, Figure 2B), locating 59 km north of the 2013 disease front. Here there were as many as 20 ash trees with one or several stem bark lesions or shoot dieback, and this was the most severely affected site at the front any year.

In 2015 the northernmost site with diseased ash along the coast was in Averøy municipality near Bruhagen (Table 1, Figure 2B), locating 76 km north of the previous year's disease front. The site hosted many big and small ash trees in an agriculture area.

In 2016 the northernmost finding of ash dieback symptoms was near Våg in Aure municipality (Table 1, Figure 2B), 53 km north of the previous year's disease front. Here one of the small ash trees growing along a small river (Vågselva) had a necrosis on the main stem.

Based on vegetative morphology, a total of 106 isolates obtained from symptomatic tissue of common ash were identified as *H. fraxineus* (Table 2). Out of the 34 *H. fraxineus* isolates subjected to molecular identification by ITS rDNA sequencing, 32 showed 100% sequence similarity to the holotype of *Chalara fraxinea* (FJ597975), while one of the remaining isolates showed a one-nucleotide-insert and the other a point mutation within the ITS1 region.

Table 2. Number of *Hymenoscyphus fraxineus* obtained from annual disease fronts and the subset subjected to ITS rDNA sequencing

Year	Isolates obtained	Isolates sequenced
2009	12	5
2010	9	5
2011	51	16
2012	4	0
2013	20	5
2014	9	3
2015	0	0
2016	0	0

Discussion

The survey for the occurrence of ash dieback was done annually during early summer. The examined stem bark lesions were deduced to be < 1 year old if the lesion area and adjacent healthy tissue showed the same number of year rings. In such cases the infection was concluded to originate from previous year, since the sporulation period of *H. fraxineus* in Norway extends from the end of June to October, and the peak sporulation occurs in the period between mid-July – mid-August (Hietala et al. 2013). The dieback of shoots is easy to see, but this may be caused also by some other fungi and by winter frost damage as well. To be sure that shoot dieback or a stem lesion was caused by *H. fraxineus*, tissue samples taken at a deduced disease front were always brought *to* the laboratory for fungal isolations. The identification of *H. fraxineus* was based on the typical anamorph characters of the species (Kowalski 2006), and in addition the identity of many of the isolates was confirmed by sequencing of the ITS rDNA gene cluster.

To search for ash dieback over a huge area is complicated, especially in the west coast of Norway that is shaped by fjords and mountains and is often poorly accessible by roads. Therefore there is obviously uncertainty whether the exact front of ash dieback was recognized every year, and indeed sometimes older necroses were found up to a few kilometres north from the disease front deduced during previous year's survey. In such a case the front was "moved" to the new site. Generally, the recognized front was probably not far from the real front. The distances of the estimated annual disease spread are thus not exact, but rather approximate.

The rapid spread of ash dieback through Europe is still not fully understood, but many factors may have contributed, including trade and movement of infected seeds (Cleary et al. 2013), ash seedlings and other plant material

(Schumacher et al. 2010, Kirisits et al. 2012, Gross and Holdenrieder 2013, Sansford 2013), timber (Husson et al. 2012), and natural spread of windborne ascospores over unknown, but probably substantial dispersal distances. The first observation of the pathogen in the UK in 2012 was made on seedlings imported from the continent. However, subsequent spatial mapping of infected trees in natural stands implies that *H. fraxineus* spread to the UK by wind-dispersed spores crossing the North Sea from continental Europe, infecting natural stands particularly in the south-eastern and eastern parts of the country (Sansford 2013, www.ashtag.org).

Regarding Norway, in 2008 the Norwegian Food Safety Authority laid down regulations with the aim to prevent the spread of ash dieback to areas in Norway deemed to be free of infection. These regulations divided the country into a quarantine zone (southeastern counties), an observation zone (Rogaland county) and an infection-free zone (the rest of the country). These regulations concern ash plants, propagating material and wood, and prohibit import of such material from the quarantine zone into observation and infection-free zones. Moreover, it must be officially verified that no symptoms of ash dieback have been observed on the plant material to be placed on the market in the infection-free and observation zones (https://lovdata.no/dokument/SF/forskrift/2008-09-08-1005?q=FOR-2008-09-08-1005). After detection of ash dieback in 2008 in Norway (Talgø et al. 2009), there was extensive publicity around this disease that reduced greatly the demand for ash seedlings for planting. Nurseries with ash seedling production destroyed ash plants to give space to saleable plants. An exception was a nursery located in Trøndelag far away from the diseased area. Ash dieback has not reached Trøndelag even in 2016, and this nursery is still selling ash plants locally, but very few (Wormdal nursery, pers. comm.). Because of regulations and stopping of commercial ash seedling production, it is likely that the spread of ash dieback recorded now in the west coast relates to airborne spread of the fungus or alternatively, originates from previous transfer of infected plant material.

Our records indicate an annual spread of ash dieback in the range from 25 km to 78 km, with a mean of 51 km each year. These estimates are well in line with the annual spread of 75 km estimated for *H. fraxineus* in Europe (Gross et al. 2014). A similar spread distance has also been observed in northern Italy (Luchi et al. 2012). They reported a spread of ca 50-60 km each year westward near the border to Slovenia.

The annual 75 km disease spread estimated by Gross et al. (2014) is the total spread which could have been facilitated by movement of plants from nurseries as seen in UK and Ireland (Sansford 2013). Trade-facilitated spread was probably more important in the beginning of the disease spread, since the cause of ash dieback by *Chalara fraxinea* was first described in 2006 (Kowalski 2006). At that time the disease had been in Europe for more than 10 years (Przybył 2002) with free movement of ash plant material.

Steinböck (2013) showed that the ascospore amount of *H. fraxineus* in air reaches a low plateau already by 160 m away from an infested stand. Similar results were obtained by Chandelier et al. (2014) who recorded a comparable background level of pathogen ascospores 50 to 500 m away from an infested stand when the wind speed was 20 km/h (5.6 m/s). In line with the now recorded spread of ash dieback along the west coast of Norway, the prevailing wind direction in this region is from south or south-west and the average wind speed during the pathogen sporulation season in July and August may be at a similar level (20 km/h). The effective dispersal distance the ascopores of *H. fraxineus* remain viable and the amount of ascospores required for shoot infection of common ash remains to be clarified.

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Progression of Ash Dieback in Norway Related to Tree Age, Disease History and Regional Aspects

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Abstract

Ash dieback, caused by the ascomycete *Hymenoscyphus fraxineus*, has been spreading throughout Europe since the early 1990s, threatening European ash at a continental scale. Little is known about the development of the disease in individual forest trees and in different age classes. In this study we monitored ash dieback on trees of different diameter classes in five permanent plots in ash stands in south-eastern Norway from 2009 to 2016, and from 2012 to 2016 in three plots in western Norway with a shorter disease history. Our results showed that more than 80% of the youngest and more than 40% of the intermediate future crop trees in the plots in south-eastern Norway were dead by 2016, while the disease development in large, dominant trees was slower. Although less damage has been observed in the plots in western Norway, the trend for the juvenile trees is the same as in south-eastern Norway with rapidly increasing damage and mortality. Most dead trees in south-eastern Norway were found at sites with high soil moisture and showed symptoms of root-rot caused by *Armillaria* species. Infected trees, both young and old ones, are weakened by the disease and appear to be more susceptible to other, secondary pathogens, especially under unfavourable site conditions.

Keywords: Ash dieback, *Hymenoscyphus fraxineus*, monitoring, year-to-year disease development, crown damage

Introduction

Ash dieback was first reported from Poland in the beginning of the 1990s (Przybył 2002), and has since spread to most areas in Europe where European ash (*Fraxinus excelsior*) occurs (Timmermann et al. 2011, McKinney et al. 2014). The cause of the disease is the pathogenic ascomycete *Hymenoscyphus fraxineus* (syn. *H. pseudoalbidus*, anamorph *Chalara fraxinea*) (Kowalski 2006, Queloz et al. 2011, Baral et al. 2014). Fruit bodies of the fungus are formed between late June and late September (under east Norwegian climatic conditions) on ash leaf litter overwintered in the forest floor, releasing vast amounts of ascospores with a seasonal peak from mid-July until mid-August (Hietala et al. 2013). The spores are infecting the compound leaves, and fungal hyphae are thought to spread to branches and stem before leaf shed late in the season (Gross et al. 2014). Infection initially causes necrotic lesions in leaves and petioles, and later in the bark of branches and stems, leading to leaf wilting, defoliation and shoot or even top dieback (Gross et al. 2014).

Ash dieback threatens European ash at a continental scale, and European ash is now red-listed in many European countries, including Norway (Henriksen and Hilmo 2015). Trees of all ages and at all sites, also outside forest stands, are affected. Younger trees are usually killed faster by ash dieback than intermediate or older, dominant trees, but there is large variation in susceptibility and disease development among individual trees, irrespective of age class. As a consequence of ash decline, a shift in forest species and biodiversity and changes in forest management can be expected in the long run (Pautasso et al. 2013). In forest management, special concern is expressed for the fate of the future crop trees, the next generation of ash trees (Keßler et al. 2012). Furthermore, insect and fungal species associated with European ash may be impacted indirectly when their host is declining (Pautasso et al. 2013, Cross et al. 2016, Thomas 2016).

European ash is commonly distributed in Norway from the Oslo fjord area in the east along the coast in southern and western Norway, with fragmented populations up to ca 64°N around the Trondheim fjord region in Mid-Norway (Figure 1), where the species reaches its northernmost European distribution border, but scattered occurrences of (mostly planted) ash trees are found even farther north. It grows up to 720 m a.s.l. in the temperate broadleaf and mixed forest biome in Norway, mostly in mixture with other deciduous tree species. Larger, pure stands of ash are rare in Norway, although Scandinavia's largest ash forest is found west of the Oslo fjord (Fjugstad nature reserve, monitoring plot FU in Figure 1). Until the first half of the 20th century, ash was widely planted in Norway and the fast-growing, yet strong and flexible hardwood was used for manufacturing of e.g. skis, tool handles and parquet floors. The importance and thus the economic value of ash wood products declined through the second half of the 20th century. Ash received again increasing attention in some regions in Norway at the beginning of this century due to its drought tolerance, and some forest owners west of the Oslo fjord planned to replace Norway spruce with European ash at drought-exposed sites – before ash dieback invaded the country and altered the prospects.

The presence of ash dieback in Norway was confirmed for the first time in 2008 (Talgø et al. 2009) and was at that time already widespread in south-eastern Norway. Based on older stem lesions it was concluded that the disease must have been present in the region at least since 2006 (Solheim et al. 2012). Ash dieback has since been spreading along the coast in Norway at a considerable pace, reaching the southernmost parts of the west coast in 2008/2009. By 2013, ash dieback had spread through most parts of the continuous distribution range of European ash in Norway. In 2016, only the region around the Trondheim fjord was still free of the disease (Solheim and Hietala 2017).

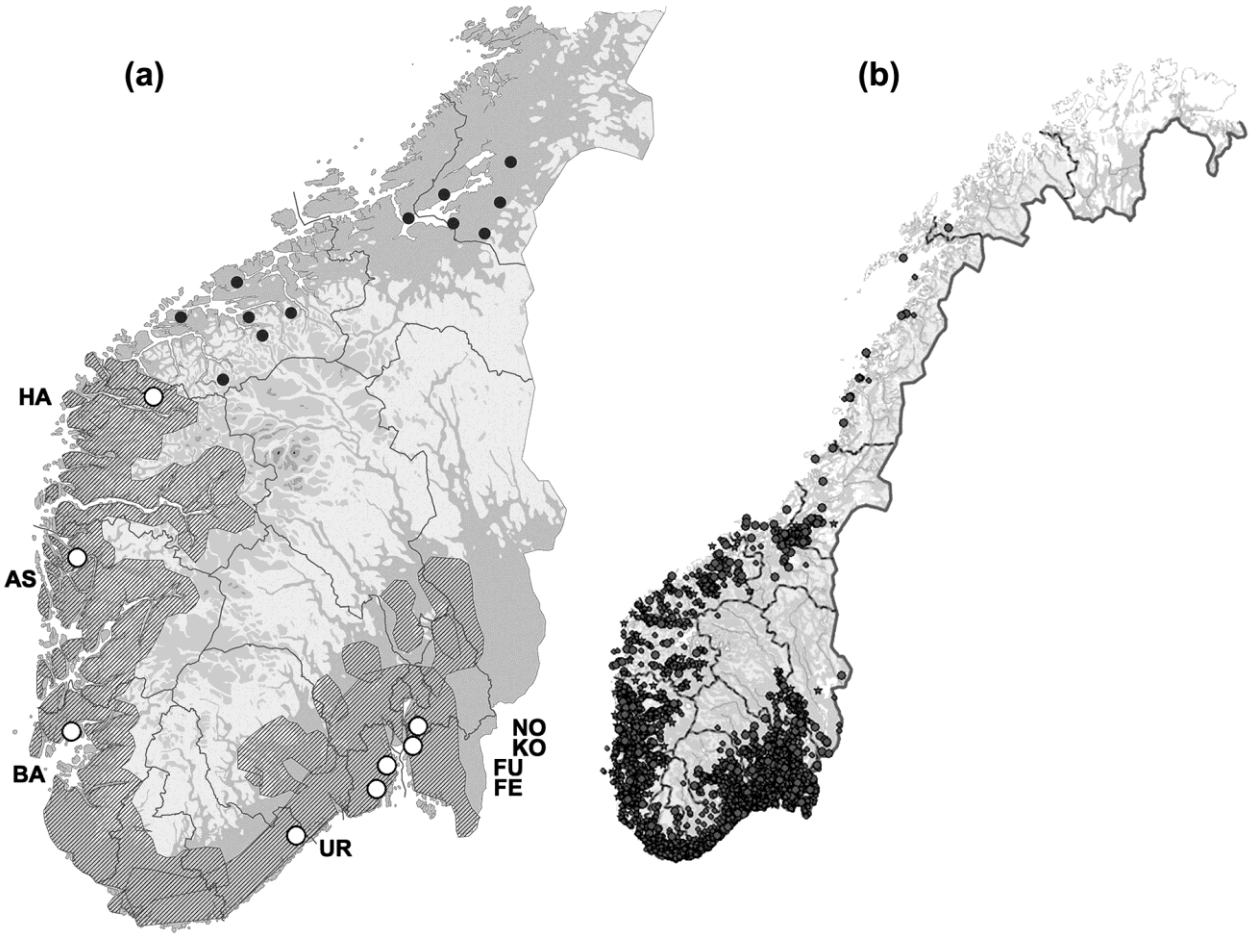

Figure 1. (a) Location of monitoring plots (white dots) and distribution of European ash in forested areas in Norway (shaded area: continuous ash populations; small black dots: fragmented ash populations) according to EUFORGEN 2009 (www.euforgen.org). (b) Single observations of European ash in Norway including park and garden trees according to the Norwegian Biodiversity Information Centre/GBIF Norway 2016 (www.artskart.artsdatabanken.no)

The objectives of the present study were (i) to evaluate disease development in individual ash trees of varying age and to confirm the intensity of ash dieback in ash stands in Norway, and (ii) to compare disease progression in regions with different lengths of disease history. To meet these aims, we assessed disease symptoms such as defoliation and other crown damage parameters annually in trees of different diameter classes on five permanent monitoring plots in south-eastern Norway with a disease history presumably back to 2006, and in three plots in western Norway with a shorter disease history (Table 1).

Materials and methods

Monitoring of ash dieback in Norway has been conducted at eight study sites; five situated in south-eastern Norway and three at the Norwegian west coast (Figure 1). Four of the monitoring plots in south-eastern Norway were established in 2009 (NO, KO, FU, FE) and the southernmost (UR) in 2010, while the western plots (BA, AS, HA) were established in 2012 (Table 1, Timmermann et al. 2013). All plots are situated in ash dominated forest stands, with stand area ranging from a few 100 m^2 to almost 30 ha (Table 1).

In four plots, showing distinct age classes, 10 large, dominant trees with varying degree of crown damage and 40 healthy appearing young trees were subjectively selected, 50 trees in all (Table 1). In the other four plots, with more even age distribution, 40 trees in total were selected. All trees were marked and numbered for long-term monitoring.

Weather and climate data for each monitoring plot were obtained from the nearest weather observation station providing long-term measurements (the same weather station was used for NO and KO, and for FU and FE, respectively. Data were retrieved from the online weather service www.yr.no). In 2015, mean annual temperature and total annual precipitation was in the range from 7.1 to 8°C and 1035 to 1622 mm in the eastern region, and from 8 to 8.7°C and 1716 to 3102 mm in the western region. Mean summer (June–August) temperature and average summer precipitation in 2015 was 15.0°C and 365 mm in the east, and 13.7°C and 363 mm in the west. In the winter months (December–February), mean temperature and average precipitation was 1.3°C and 325 mm in the east, and 3.5°C and 1016 mm in the west (Table 1).

Visual assessments of crown condition and damage symptoms were conducted on individual trees once per year on every plot (between 15th of June and 15th of August) based on standardized methods developed by ICP Forests (UNECE 2010). Parameters assessed were defoliation, leaf discolouration, shoot and leaf wilting, dead branches and tops, dieback, fruiting, and epicormic shoots. A field calibration was performed in 2012 before monitoring in western Norway started to ensure that assessments were done similarly by the two field workers.

Table 1. Overview over study sites and monitoring plot data. TS15 and PS15: Mean temperature and total precipitation for summer months 2015 (Jun.–Aug.). TW15 and PW15: Mean temperature and total precipitation for winter months 2015 (Dec.–Feb.). Deviations from standard reference period 1961–1990 are shown in parentheses. Weather and climate data were obtained from the online weather service www.yr.no. ADB infect.: Presumable year of first ash dieback infections in the surroundings of each monitoring plot

Plot ID	Plot start	Coordinates	Plot area (m^2)	Stand area (ha)	No of trees	Altitude (m a.s.l.)	TS15 (°C)	PS15 (mm)	TW15 (°C)	PW15 (mm)	ADB infect.
NO	2009	59.680847 N 10.776908 E	378	3.4	50	100	14.8 (-0.5)	349 (117)	0.7 (5)	214 (77)	2006
KO	2009	59.536433 N 10.711131 E	1036	1	50	40	14.8 (-0.5)	349 (117)	0.7 (5)	214 (77)	2006
FU	2009	59.361899 N 10.457551 E	792	26.7	50	40	15.3 (-0.2)	327 (80)	1.9 (5.1)	319 (94)	2006
FE	2009	59.198004 N 10.235486 E	595	0.1	40	100	15.3 (-0.2)	327 (80)	1.9 (5.1)	319 (94)	2006
UR	2010	58.783128 N 09.121598 E	114	0.02	40	100	14.9 (0.2)	420 (134)	1.3 (4.3)	441 (166)	2006/7
BA	2012	59.345577 N 05.838939 E	n.a.	n.a.	50	20	13.7 (0.8)	425 (19)	3.4 (3.4)	1079 (615)	2007/8
AS	2012	60.615296 N 05.468731 E	n.a.	n.a.	40	15	14.1 (0.2)	430 (-40)	4.2 (2.5)	1218 (641)	2010
HA	2012	61.861023 N 06.339424 E	n.a.	n.a.	40	110	13.1 (-0.5)	235 (15)	3 (3.2)	751 (376)	2013

In the result part, defoliation scores, considered to be the most important damage variable (UNECE 2010), were used to group the trees into five damage classes (Table 2), comparable to the classes used by McKinney et al. (2011). Growth measurements were carried out twice. Diameter at breast height (DBH, all trees > 50 mm) and tree height (only for dominant trees) were measured in 2009 (south-eastern plots) and 2014 (all plots), and the trees were grouped in three diameter classes according to their DBH in 2014: Juvenile or small trees with DBH ranging from 10 to 50 mm, intermediate, future crop trees with DBH 50 to 125 mm and large, dominant trees with DBH above 125 mm (Table 3). Although tree heights are overlapping between diameter classes, diameter reflects tree age to a certain degree, especially for the youngest trees (K. Andreassen, pers. comm.).

Table 2. Grouped damage classes according to percentage of defoliation

Damage class	Defoliation (%)
1 Healthy trees	0–10
2 Slightly damaged	11–25
3 Moderately damaged	26–50
4 Severely damaged	51–99
5 Dead trees	100

Table 3. Diameter classes according to measurements of diameter at breast height (DBH) in 2014, tree height and number of trees in the plots in south-eastern Norway (SE) and western Norway (W)

Diameter class	DBH	Height	No of trees	
	(mm)	(m)	SE	W
Juvenile	< 50	1–10	111	64
Intermediate	50–125	8–16	67	34
Dominant	> 125	12–34	52	32

The percentages of ash trees in the different damage classes were calculated for each diameter class. Annual mean values are presented combined for the five plots in south-eastern Norway, and combined for the three plots in western Norway. One-way analysis of variance with least significant difference *post hoc* test was used to analyze significant differences in defoliation between years for means of each damage class by using the SPSS program (IBM SPSS Statistics 22.0). Differences with $P < 0.05$ were regarded as statistically significant.

Results

Disease progression in south-eastern Norway 2009-2016

In 2009, 86.1% of the juvenile trees on the monitoring plots in south-eastern Norway appeared healthy (0–10% defoliation) or only slightly damaged (11–25% defoliation), and 4.6% were severely damaged (50–99% defoliation) (Figure 2a). In 2016, the proportion of healthy or slightly damaged trees was significantly reduced to only 8.1%, while 89.2% of the young trees were severely damaged or dead, representing a significant increase in dead trees and a mortality rate of 82%. Only few year-to-year changes within each damage class were significant. At plot level, disease development among the juvenile trees has been devastating in plot NO during the monitoring period, leaving 35 of 38 juvenile trees dead by 2016, one dying and two severely damaged.

For the intermediate trees disease development has not been as rapid as for the juvenile trees in south-eastern Norway, but also in this group there has been a considerable increase in the number of severely damaged trees and a significant increase in the amount of dead trees from 2009 to 2016 (Figure 2b). Based on defoliation assessments, 70% of the trees in this intermediate diameter class were considered to be healthy or slightly damaged and 12.5% to be severely damaged in 2009. In 2016, 69.7% of these future crop trees were either dead or severely damaged, and only 24.2% still healthy or slightly damaged. The mortality rate for the entire monitoring period was 42.4%. None of the year-to-year changes were significant, except for an increase in dead trees from 2014 to 2015. The decrease in number of healthy future crop trees was significant from 2009 to 2016. However, despite an accelerating mortality rate since 2012, the proportion of healthy trees has been relatively stable since 2013 (around 20%).

Defoliation within the group of large, dominant trees did not increase as much as for the younger trees in south-eastern Norway from 2009 to 2016, and annual changes were not significant for any of the damage classes except for dead trees. The proportion of healthy or slightly damaged dominant trees was fairly stable from 2009 to 2014 (around 60%, Figure 2c), but had decreased to 42.3% in 2016, due to a sharp decline in the number of healthy trees from 2014 to 2016. The proportion of severely damaged and dead trees increased from 16.7% to 46.2% from 2009 to 2016, and the mortality rate for that period was 23.1%.

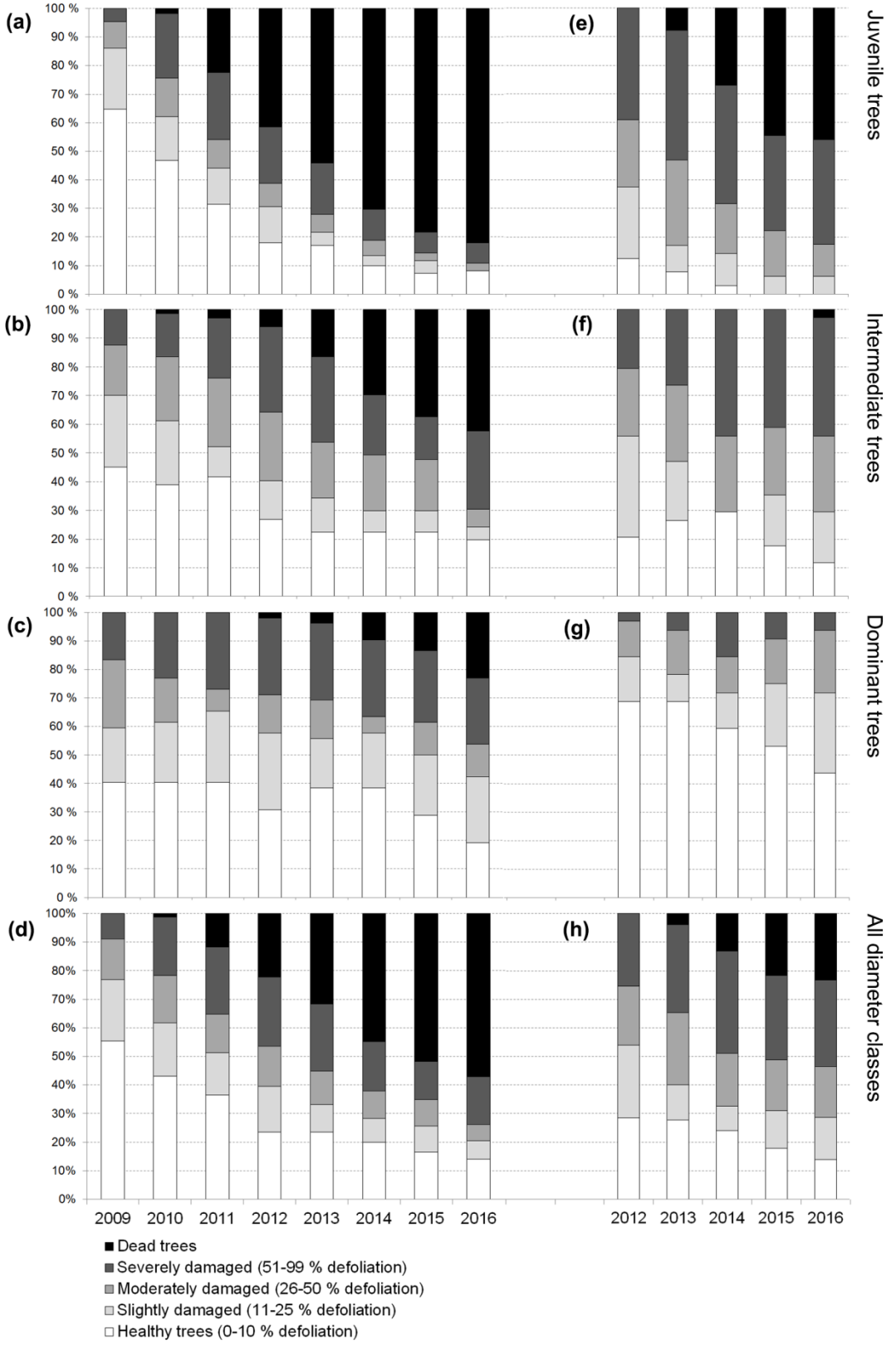

Figure 2. Percentage of ash trees in each damage class. (a–d) Plots in SE Norway 2009–2016; (e–h) plots in W Norway 2012–2016. (a, e) Juvenile trees; (b, f) intermediate trees; (c, g) dominant trees; (d, h) all diameter classes

Of all assessed trees in all diameter classes on the monitoring plots in south-eastern Norway, 76.8 % were considered to be healthy or slightly damaged in 2009, and only 8.9 % to be severely damaged. In 2016, only 20.5 % were still healthy or slightly damaged, 16.6 % severely damaged and 57.2 % were dead (Figure 2d). The increase in number of dead trees from 2009 to 2016 was significant in all diameter classes.

Disease progression in western Norway 2012-2016

In 2012, 37.5% of the juvenile trees on the three plots in western Norway were considered to be either healthy or slightly damaged, and 39.1% severely damaged (Figure 2e). In 2016, no healthy trees were found (a significant decrease) and only 6.3% were slightly damaged. The proportion of severely damaged juvenile trees remained more or less unchanged during the monitoring period, while there was a strong and significant increase in dead juvenile trees to 46% in 2016.

No significant changes in defoliation could be detected for the intermediate trees in the western plots during the monitoring period. However, there was a trend of an increasing proportion of severely damaged trees, which doubled from 2012 to 2016 (to 41.2%, Figure 2f), and the first dead tree in this diameter class was observed in 2016.

No significant differences were found for defoliation in the large, dominant trees in the monitoring plots in western Norway, and no clear trends are visible yet (Figure 2g). The annual changes in the different damage classes were rather small, and no dead trees were found yet in this diameter class. Still, the proportion of healthy trees decreased from 68.8% in 2012 to 43.8% in 2016.

Of all assessed trees in all diameter classes on the monitoring plots in western Norway, more than half (53.8%) were considered to be healthy or slightly damaged in 2012, and 25.4% to be severely damaged. In 2016, only 28.7% were still healthy or slightly damaged, 30.2% severely damaged and 23.3% were dead (Figure 2h).

Discussion and conclusions

The development of ash dieback in individual trees has been monitored on permanent plots in south-eastern Norway since 2009 and in western Norway since 2012. Our monitoring data have shown that disease progression has been fast, even in areas with a short disease history. While there are numerous earlier reports about mortality of ash in relation to ash dieback, there are few prior studies where the health condition of ash has been monitored annually, and related to the duration of disease exposure. The current survey showed that the extent of crown damage has continuously increased in all diameter classes, and that tree mortality has been high, especially among the youngest trees in south-eastern Norway, the region with the longest disease history in the country. But also intermediate and old, dominant trees are attacked, damaged and eventually killed. Diseased large trees can survive for many years due to their extensive foliage and gradual decline in crown condition, usually with small annual changes in defoliation and dieback. Infected mature ash trees often develop epicormic shoots from the stem or main branches to compensate for foliage loss due to the disease. This stress reaction can lead to a temporary increase in foliage, even though the tree crown is severely damaged by ash dieback.

Monitoring projects performed in the Czech Republic 2009–2015 (Vacek et al. 2015) and in Bavaria in southern Germany 2010–2014 (Lenz et al. 2016) found similar patterns in disease development for different age classes and accelerating mortality especially for understorey trees after four years of monitoring. A study conducted in France with 15–20 year-old ash trees showed a mortality rate of only 3% in the period 2010–2014 (Muñoz et al. 2016), while an Austrian survey performed on mature ash trees (Keßler et al. 2012) reported slow disease progression and a low mortality rate (1%) during the monitoring period between 2007 and 2010. In contrast, mortality rate for the mature, dominant trees in our study in south-eastern Norway was 23% from 2009 to 2016, and 42% for the intermediate trees in the same region and period. Even when considering a shorter period (2009–2013), mortality rates in south-eastern Norway were 4% and 16% for mature and intermediate trees, respectively.

Juvenile trees with small DBH are easier killed or severely damaged by the disease than larger trees since a single necrosis on the stem may lead to dieback of the top or even the whole tree, while it will take several years and multiple infections to kill major branches in old trees (Cech 2008). Earlier studies conducted at the monitoring plot NO have shown that during summer enormous amounts of ascospores are released by *Hymenoscyphus fraxineus* ascocarps growing on ash leaf litter (Timmermann et al. 2011, Hietala et al. 2013). It is therefore reasonable to assume that infection pressure for smaller, juvenile trees is much higher than for tall, dominant trees having their foliage 10 or 20 metres above ground. This assumption was confirmed by field experiments conducted in Belgium (Chandelier et al. 2014), where spore traps where placed at different heights in an infected ash stand. Spore density was decreasing significantly with increasing height, and was found to be 5- to 100-times lower at only 3 m height than at the ground. Juvenile trees are also more exposed to competition with other vegetation and thus more predisposed to die off if they in addition are infected by ash dieback (Cech 2008). Healthy appearing juvenile trees in our monitoring plots often have been found dead in the subsequent season.

Large differences between individual trees in crown condition and disease development have been observed in all age classes at our monitoring plots. Although ash dieback has been present in south-eastern Norway for around 10 years and has done large damage to trees of all age classes in the monitoring plots, some trees are still healthy. There is no spatial clustering in the occurrence of trees with different degree of damage in a stand, as healthy, damaged and dead trees are found right next to each other. Both findings indicate that there might be individual, genetically determined differences in resistance to ash dieback and thus possible material for future breeding, thereby supporting the observations of earlier field surveys (Kirisits and Freinschlag 2012, Gross et al. 2014, McKinney et al. 2014). Recent evidence indicates that ash trees without signs of crown damage in stands affected by ash dieback are equally susceptible to leaf infection by ascospores as trees highly affected by shoot dieback and defoliation, and that both healthy and damaged trees support the build-up of pathogen infection pressure in a stand (Cross et al. 2016).

Infected trees, both young and old ones, are weakened by *H. fraxineus* and thus more susceptible to other, secondary diseases like e.g. root-rot caused by *Armillaria* (Skovsgaard et al. 2010), as well as to competition and unfavourable abiotic site conditions such as water-logging, or nutrient deficiency. A combination of all or some of these factors might contribute to the death of the trees, making it difficult to determine the primary cause of death. In plot NO, the cause of death of newly dead trees was assessed in 2014, and most dead trees that had clear symptoms of ash dieback in the crowns in previous years showed signs of *Armillaria* rot in the roots, but no obvious symptoms of *Hymenoscyphus*-infections could be found in the roots or root collar. In 2016, all newly dead, dominant trees in monitoring plots NO and FU showed signs of *Armillaria* infection. Lenz et al. (2016) concluded in their study from Bavaria that *Armillaria* rot in ash trees contributed considerably to the acceleration of mortality. A large-scale crown reduction by *H. fraxineus* in a stand can be anticipated to cause significantly reduced water transpiration from trees. In stands with high soil moisture content this may in turn cause oxidative stress in roots, which again predisposes the trees to infection by *Armillaria* species, fungi commonly associated with trees on water-logged sites (Wargo and Harrington 1991). Furthermore, a degrading root system caused by *Armillaria* rot, alone or in combination with collar lesions affecting the vascular tissue, will impair water and nutrient uptake of the tree and hence contribute to accelerating decline in crown condition (Chandelier et al. 2016). Several studies (Cech 2008, Keßler et al. 2012, Vacek et al. 2015) reported increasing defoliation and dieback on sites with high soil moisture compared to drier, better drained sites. This is in accordance with the results from our monitoring plots where the highest mortality rate for both young and old trees has been registered at a water-logged site (plot NO). At this plot, 50% of the dominant and 90% of the juvenile and intermediate trees were dead in 2016. In contrast, at the most well-drained site at a south-faced slope (plot KO) situated only 16 km air-line distance south of plot NO, most of the dominant trees were still healthy or only slightly damaged in 2016, while 55% of the younger trees were dead. High humidity in the field layer and forest floor is presumably crucial for the formation and activity of *H. fraxineus* ascomata; it remains to be examined to what extent the sporulation period and infection pressure of this pathogen differ between sites that vary in soil moisture, and how this is reflected in the progression of ash dieback in a stand.

Based on observations of older lesions, the first infections of ash dieback in the surroundings of plot BA in western Norway might have taken place as early as 2007 (Solheim 2012, Solheim and Hietala 2017), and in the area nearby plot AS in 2010. In these two plots, clear symptoms of ash dieback were present in 2012 when monitoring started here, and respective damage was recorded in the consecutive years. The northernmost plot (HA) was established one year before ash dieback reached that area, and consequently less damage than in the other western plots and only few clear signs of ash dieback have been detected in this plot so far. However, the presence of *H. fraxineus* in this plot was confirmed by microscopy analysis of fruit bodies sampled in 2015, and the disease has also advanced farther north since 2013 (Solheim and Hietala 2017).

Due to different length of disease history, the ash trees in the west Norwegian plots have experienced varying development of the disease as well, both between the three plots in this region and compared to the south-eastern region. Differences in defoliation patterns in western Norway are larger between plots than between age classes. Plot BA in south-western Norway has the longest disease history of the western plots. As a consequence, most of the dead trees are found on this plot. In contrast, little damage and only two dead trees are found in the northernmost plot (HA) with the shortest disease history. Half of the dominant trees in the west Norwegian sample are found at this site, and all of them were still healthy or only slightly defoliated in 2016. Most of the foliage damage observed in plot HA is caused by mining insects and not by ash dieback (H. Nyeggen, pers. comm.). In the third west Norwegian plot (AS), situated in between the two other plots both in terms of geography and disease history, crown damage is quite extensive, but only a few trees have died so far.

In conclusion, our monitoring data showed that there are clear differences in the progression of ash dieback in trees of different age, *irrespective* of their disease history, but also between stands in different regions *depending* on

their disease histories. Our results indicate that development and severity of ash dieback may be correlated with soil moisture in stands and with *Armillaria* root infections. Better understanding of the genetic background that underlies the differential expression of disease symptoms in ash individuals, and the interactions between *H. fraxineus*, European ash and abiotic and biotic environment, is needed to actively facilitate the survival and eventual recovery of ash populations.

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Estimation of Ash Mortality Induced by *Hymenoscyphus fraxineus* in France and Belgium

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Summary

Ash dieback induced by *Hymenoscyphus fraxineus* has emerged as one of the most serious health problem for European forests in the last ten years. However, precise estimation of the mortality induced by the pathogen is still scarce and this hampers management of affected stands. In this work, we used data of several surveys done since 2010 in France and Belgium to estimate the mortality rate associated with ash decline depending on the time of the pathogen presence in the area; for that a 2 steps procedure was used. First, we did an estimation of the frequency and severity of collar lesions associated with *H. fraxineus* depending on the length of the pathogen presence and for 2 trees size classes (lower or higher than 25 cm dbh). Then the annual mortality rate was estimated depending on collar lesion severity, dbh class (lower or higher than 25 cm) and time since pathogen presence. The global mortality induced by *H. fraxineus* was computed from those 2 types of data by a bootstrap approach. Additionally one survey observing young stands was used from which mortality was computed directly. We find that if mortality is drastic in very young ash stand affected by *H. fraxineus* (less than 5 cm dbh), with annual mortality reaching 35% 5-6 years after arrival of the pathogen in the stand, it is much more moderate for trees with dbh above 25 cm, with annual mortality reaching 3.2% after 8-9 years of pathogen presence. Annual mortality rates are intermediate for trees in the 5-25 cm dbh class and reached 10-11% after 8-9 years of pathogen presence.

Key words: Ash dieback, Fraxinus, mortality, invasive pathogen

Introduction

Ash dieback has emerged as one the most serious health problem for European forests in the 10 last years. The disease is induced by *Hymenoscyphus fraxineus*, an invasive pathogen that originated from East Asia (Gross et al. 2014b). Ash dieback was first reported in the early nineties in Poland and since it spread toward throughout Europe (Gross et al. 2014a, McKinney et al. 2014). In Western Europe, it reached France in 2008 and Belgium in 2010 (Chandelier et al. 2016, Husson et al. 2011). Ash dieback has a life cycle of a foliar disease, overwintering on the leaf rachis in the litter and producing apothecia from the colonized rachis from June to August (Gross et al. 2014a).

However, the pathogen is able to infect shoots from the colonized leaves and causes extensive shoot dieback during the winter season (Gross et al. 2014b). *H. fraxineus* has also been shown to induce extensive lesions in the inner bark of the collar area (Husson et al. 2012). These lesions are secondarily colonized very quickly by *Armillaria* species (Skovsgaard et al. 2010, Bakys et al. 2011, Husson et al. 2012, Enderle et al. 2013, Chandelier et al. 2016), which may explains why root rot has been frequently reported on ash affected by *H. fraxineus*. In addition, it has been noticed repeatedly that deterioration of ash health status is strongly associated with the development of collar lesions, whether those are supposed to be associated with *H. fraxineus* or with *Armillaria* species (Bakys et al. 2011, Chandelier et al. 2016, Lenz et al. 2016).

H. fraxineus has been reported to threaten the future of ash and its associated ecosystem in Europe (Lygis et al. 2011, Pautasso et al. 2013, Lõhmus and Runnel, 2014). However, precise estimations of the mortality induced by the pathogen are still scarce. In young ash seedlings / saplings, high mortality rate have been reported (Pliura et al. 2011, Koltay, 2012, Enderle et al. 2013). Data is far less available in mature stands. Lõhmus and Runnel (2014) report a mortality of about 50% in 4 years in a mature ash stands while Rosenvald *et al* (2015) report annual mortality rate of about 2-5% per year for solitary ash trees retained after timber harvesting. By contrast, Keßler *et al* (2012) report very little mortality associated with ash dieback between 2008 and 2010 in their survey of the disease impact in Austria. The lack in precise knowledge on the level and timing of mortality associated with ash dieback clearly hampers the management of affected stands.

The aim of this work was to use the available surveys on ash dieback impact that were done in France and Belgium to derive estimates on the level of mortality in relation to the time of *H. fraxineus* presence in the area. As data on very young stands are less scarce, the main effort was targeted to mature and pole size trees.

Material and methods

The study was based on several surveys done in France and Belgium over 1-6 years to monitor the health of ash trees affected by *H. fraxineus*.

Plots observed over more than 1 year to monitor mortality

Survey 1. Six young ash stands originating from seeding (2-4 m height) were sampled in NE France and monitored for 3-5 years (see Table1). The Seichamps stands, close to Nancy, originated from seeding in an abandoned field and contained only ash saplings with a very high density (30 000 stem.ha^{-1}). The second, located Gremecey (Moselle) was an oak/maple plantation where ash settled by seeding with a density of 1 400 stem.ha^{-1}. Chaumont (Haute-Marne), Meurcourt (Haute-Saône) and the 2 stands (P19 and P36) of Pompey (Meurthe-et-Moselle) are forest stands originating from seeding with a mixture of ash and oak/beech and has a density of ash of 8 300, 1 260 and 9 400 stems.ha^{-1}, respectively. Depending on density, quadrats of 1-20m² were regularly spaced in each stands and all ash seedlings present in the quadrats were marked and surveyed. Altogether, the number of rated seedlings was of 174 in Seichamps, 203 in Gremecey, 555 in Chaumont, 300 in Meurcourt, 1619 in P19 and 280 in P36.

Survey 2. This survey was done in 2011-12 in Haute-Saône and Vosges area, in the initial focus of ash dieback in NE France. The mean diameter at breast height (dbh) of the selected trees ranged from 8 to 38 cm depending on the plot (3 plots with mean dbh>25cm and 39 with mean dbh<25cm).

Survey 3 was set up in the area of Haute-Saône infected since 2008 with a very high disease severity reported since 2010 (Husson et al. 2011). The aim was to monitor the health of ash trees that remained healthy in 2010. Eighty nine ash trees with no crown or collar symptoms as well as 163 of their dieback affected neighbor were selected and

Table 1. Description of the surveys

Survey	Nb plot	Nb trees	Years	Location	Age (years)	dbh range (cm)	Reference
1	6	3651	2012-16	Grand Est, Franche- Comté	3-10	2-7	-
2	42	1023	2012-13	Haute- Saône, Vosges	-	8-38	Marçais et al. (2016)
3	13	252	2012-15	Haute-Saône	-	12-45	-
4	40	648	2010-15	N and NE France	-	10-60	-
5	17	288	2013-15	Wallonia (Belgium)	-	16-46	Chandelier et al. (2016)
6	1	775	2012-15	Devecey (Doubs)	19	15	Muñoz et al. (2016)
7	60	3265	2010	Haute-Saône	-	10-25	Husson et al. (2011)
8	48	1350	2013	Vosges	-	6-50	-
9	87	2610	2012-15	N and NE France	-	-	-

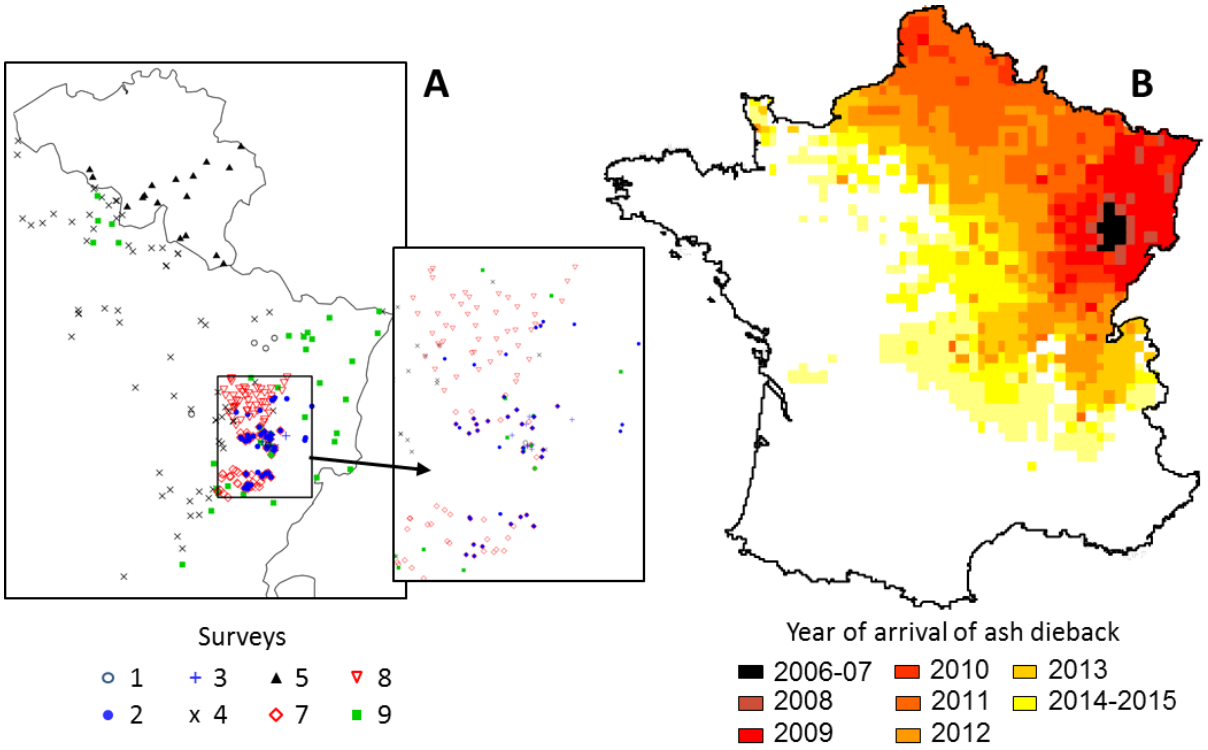

Figure 1. Localisation of studied plots (A) and map of *H. fraxineus* presence (B, source DSF)

marked in 2012 and monitored until 2015. The mean dbh ofselected trees ranged 12 to 45 cm depending on the plot (10 plots with mean dbh < 25 cm and 3 plots with mean dbh >25 cm).

Survey 4-5. These surveys were set up in 2010 by the Département de la Santé des Forêts (DSF) and 2013 by the Department Natural Ecosystem and Agriculture of Wallonia to document the evolution of ash dieback at the tree-scale. From 11 to 20 ash trees were selected and marked in 57 stands scattered in the NE and Northern France in 2010 (24 stands) and 2011 (16 stands) and in Wallonia (17 stands). They were annually monitored for health status until 2015. Mean dbh of the selected trees ranged from 10 to 60 cm depending on the plot with 38 stands having a mean dbh over 25 cm.

Survey 6 was performed in a plantation of ash half sibling families. We did not use the available genetic information in this study. We used data for 2012-2015 where information on the severity of collar girdling by *H. fraxineus* was available.

Surveys 1-6 were used to estimate mortality. In survey 1-6, the trees were separated in 3 categories according to mean stand dbh. Altogether, ash rated in each category was of 3141 seedlings of 1-5 cm dbh (survey 1), 2280 ashes of 5-25 cm dbh (survey 2, 3, 4, 5 and 6) and 707 ashes of 25-60 cm dbh (survey 2, 3, 4, and 5).

Plots observed for just 1 year for estimation of disease severity

Survey 7 and 8 were both designed similarly by the DSF with the aim to estimate the local incidence of *H. fraxineus* (see table 1).

Survey 9 was derived from the DSF data base by extracting all the report of the observation strategy "DE'" that dealt with *H. fraxineus* on ash. This strategy is aimed at quantifying the severity of disease problems by detailed counts of trees in severity classes. We used all the report from 2012 to 2015 that documented the severity of collar cankers associated with *H. fraxineus* and for which an approximate mean stand dbh could be retrieved.

Survey 2-9 were used to estimate the evolution of tree health and in particular of the severity of collar canker, which represents 310 stands The location of the different surveyed stands is showed in Figure 1a. The sampling is dense in the area of NE France where ash dieback was reported for the first time in 2008 but covers north-eastern and northern France as well as southern Belgium. The surveyed stands were separated in 2 size class (lower and higher than 25 cm mean stand dbh). The small tree size class represent 209 stands (7791 ash trees) while the large tree size represent 101 stands (2420 ash trees).

Survey procedure

Trees were rated annually between June and August, depending on the survey, for both crown status and collar canker severity. The crown status was rated as 0, 0-5% defoliation, 1, 5-25% defoliation, 2, 25-50% defoliation and 3, over 50% defoliation. Some surveys included a fifth category (more than 75% defoliation) that was not used because it was not available for all survey. The basal canker was rated for the %collar girdled in 5 categories: as 0, 0-5% girdling, 1, 5-25% girdling, 2, 25-50% girdling, 3, 50-75% girdling and 4, over 75% girdling. In some survey, the total collar circumference as well as the sum of basal lesion widths were both determined and the % collar girdled was computed from those data (surveys 2, 3, 6). In other, the rating was done visually directly in the stands (surveys 4, 5, 7-9). In survey 1, young ashes were rated annually as healthy, affected by *H. fraxineus* or dead. No basal canker was looked at in these regenerations.

For survey 1-8, dbh was determined at least once for each tree observed. For survey 9, the approximate stand dbh was retrieved from the remark done by the DSF observers; only reports where this was possible were included.

Assessment of ash dieback time of arrival in the area

The length of *H. fraxineus* presence in the area is derived from the DSF. The year of first report of *H. fraxineus* is available for all France by quadrats of 16 x 16 km (Fig. 1b). Unpublished observations made in NE France at the time of *H. fraxineus* arrival suggest that the local spread of the pathogen is extremely fast and that it can be assumed that, at a location, all stands are infected within 1-2 years. We thus assumed that *H. fraxineus* was present in a stand in the year it was reported for the first time in the DSF database in the 16 x 16 km quadrat where it is located. Ash dieback was reported in France for the first time in 2008 in the north of Haute-Saône. However, it is very likely that the disease had been present there since few years when first reported because the severity observed in the area in 2008 was already very drastic. Moreover, it is reported in Husson et al (2011) that the first collar cankers could be observed in the area in 2007 while this type of symptoms is usually observed only 2-3 years after arrival of *H. fraxineus* in a stand. Thus, we adapted the year of first presence of *H. fraxineus* in the area. To remain conservative, we thus supposed that the 4 quadrats of north Haute-Saône / south Vosges were colonised in 2006 and the 6 adjacent quadrats in 2007. The time of *H. fraxineus* presence was determined as the year of observation minus the year of first disease report in the quadrat. Thus we may observe 9 years post-infection (2006+9 = 2015). The modified map of *H. fraxineus* presence in France is shown in Fig. 1b.

Data analysis

The influence of crown and collar status on ash tree mortality was studied by mixed logistic regression with the function glmer of R library lmer using data of survey 2-6. The model included the log of time since first report of *H. fraxineus* in the area, crown status as 0-1, 2 or 3, collar status as 0-1, 2, 3, 4 and age category which took 2 levels (mean stand dbh lower than 25cm or above 25 cm) as fixed factor. Two different random factors were tested in a preliminary step, a tree factor and a stand factor. The stand factor was retained for further analysis.

In order to estimate the mortality rate induce by *H. fraxineus* depending on the time of pathogen presence, we proceeded in 2 successive steps, with first i. an estimation of the severity of collar girdling by the pathogen at different years after first report of the ash dieback in the area, using for that survey 2-9 and then ii. Estimation of the mortality per collar girdling classes 0-4, using the data of surveys 2-6. The 2 estimations were done for each of the 2 tree size class (mean stand dbh lower or above 25 cm) and of the 2 periods (less than 5 years of *H. fraxineus* presence in the area or over 5 years).

An underlying hypothesis is that canker severity depends only on tree size and on time since *H. fraxineus* arrival in the stand. Others factors influence canker severity such as site humidity (Husson et al. 2011, Marçais et al. 2016) and likely other local conditions (forest/landscape, road side, river side, site vegetation or understory). But we chose to neglect these site factors because they are not likely to play a major role at the regional level. Tree size was kept because it has important management consequences. Additionally, we were not able to take into account both crown status and collar status in the previous year because one of the major sources of data, the survey 9, gives separate counts for distribution of trees in the crown decline classes and in the collar girdling classes. Collar girdling was chosen for the estimation because it appears more tightly related to mortality.

The estimates of frequency in collar girdling classes, mortality per girdling classes and global mortality per size categories derive from bootstrap samples (5000 samples). The resampling occurred in 2 steps. First, a number of stands equal to the observed number was sampled with replacement in the list of observed stands. Then, within each selected stands, a number of trees equal to the number of trees observed was sampled with replacement in the list of observed trees. For each resampled dataset, the mortality for each canker girdling classes was computed for the 2 dbh classes and the 2 periods of *H. fraxineus* presence. The evolution of canker girdling class relative frequency was computed for each dbh class and each year following *H. fraxineus* arrival in the area. The mortality per year following *H. fraxineus* presence and per dbh class was then computed as:

[1] $\sum CS_i \times Pdead_i$,

with CS_i the frequency of canker girdling class i and $Pdead_i$ $_i$ the mortality associated with the canker girdling class i. The mean and the 0.025 and 0.075 quantiles were then computed.

For young ash stands (survey 1), the mortality was directly estimated from the data of survey 1, using the bootstrap strategy in 2 steps previously described (sampling with replacement of the stands and then in a second step of the trees in the sampled stands). The mortality was then computed from each resampled dataset.

Results

Ash mortality and tree status in the previous year

In a preliminary step, logistic regression was done with a stand and a tree random factor. The variance associated with the tree factor is very small ($1.2\ e^{-12}$) and the deviance drop associated with this random factor is not significant (Chisq = 0.5, p= 0.480) and thus only the stand random factor has been retained in the analysis. The stand factor is associated with a variance of 0.574 and a significant deviance drop (Chisq = 24, $p < 0.001$).

Table 2 gives the result of the logistic regression. All main factors, i.e. time since *H. fraxineus* arrival in the area, tree size class, level of crown decline and of collar girdling by the pathogen are all significantly associated with mortality. By contrast, interactions of age with level of crown decline and of collar girdling and interaction between crown decline and collar girdling are not significant (table 2). Figure 2 shows the evolution of ash mortality with crown status and collar girdling for the 2 tree size class and the 2 length of *H. fraxineus* presence in the stand. Mortality in the 4 years following arrival of *H. fraxineus* in the area is much lower than 5-9 years post pathogen arrival. Mainly trees which have developed collar lesions die during this early period. In the second half of the 9 year period, substantial mortality can be observed for the more severe crown decline and collar girdling classes. Mortality was also much more severe for ash trees in the 5-25 cm dbh size class than in stands with trees over 25 cm dbh.

Collar girdling appears to have a size effect higher than crown decline on mortality: after 5 years of *H. fraxineus* presence, a mortality for trees in the 5-25 cm dbh class with the most severe collar girdling is of 36% [26, 47] compared to 20% [13, 27] for the most severe crown dieback class. For that reason, collar girdling by *H. fraxineus* was selected for the following part of the work.

Evolution of ash health status and mortality with time of presence of H. fraxineus

The severity of collar infection increases steadily with the number of years of *H. fraxineus* presence in the area (Fig. 3), while the status of the crown deteriorates (Fig.4). For the 2 types of symptom, the status deteriorates earlier and stronger for ash in the 5-25 cm dbh class compared to those in the >25 cm dbh class. In a first step, collar girdling remained lower in stands with mean dbh over 25 cm compared to dbh lower than 25 cm. In particular, almost no tree with collar girdling of more than 75% occurred in stands with a mean dbh>25 cm until the seventh year of *H. fraxineus* presence while in stands of smaller trees, ash with a collar girdled more than 75% appeared after only 5 years of the pathogen presence. After 8 years of disease presence, collar girdling did catch up in stands of large trees: we then

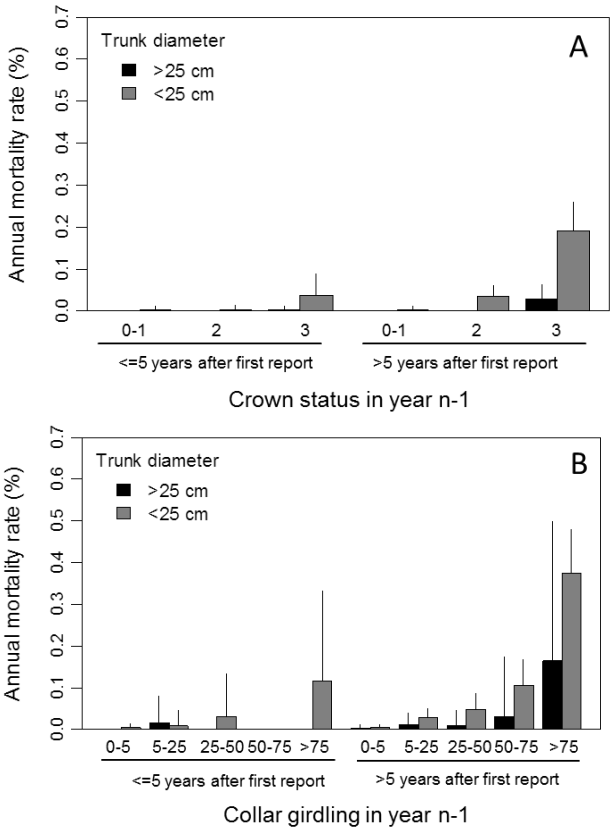

Figure 2. Annual mortality rate of chalara infected ash and status of the tree in the previous year for 2 ash size classes and 2 times since arrival of *H. fraxineus* in the area. A. Crown decline classes. B. Severity of collar girdling (based on bootstrap samples of 5000)

Table 2. Mixed logistic regression analysis of ash mortality

	Df	LR Chisq	P value
Log(time of presence) [a]	1	23.8	<0.001
Tree size (dbh) [b]	2	15.7	<0.001
Crown status [c]	2	118.1	<0.001
Collar girdling [d]	3	93.7	<0.001
Crown status * Tree size	2	0.3	0.99
Collar girdling * Tree size	3	1.2	0.75
Crown status * Collar girdling	6	5.4	0.50

[a] nb year since *H. fraxineus* arrival in the 16 x 16 quadrat

observed a frequency of ash tree with >75% of the collar girdled of about 15-20 % in stands with mean dbh<25 cm and of about 10-15% in more aged stands (>25 cm dbh). This category is the one with really high mortality (Fig. 2).The decrease in frequency of heathy looking crown (rated as 0 or 1) occurs also earlier than increase in the frequency of trees with collar lesions (Fig. 3 and 4). However, a very strong variability existed between stands in the development of collar lesions. About 17% of the stands present no collar canker after 6 years while severe collar infection are present in 20% of the stands after only 3 years (over 75% collar girdled). Altogether, after 8-9 years of *H. fraxineus* presence in the area, about 80-90% of the ash trees show some basal lesions although significant infection with over 25% of the collar girdled then represented about 50-60 % of the tree.

Unlike collar girdling, evolution of crown status is basically similar between the two tree size classes from the second/third year after *H. fraxineus* arrival (Fig. 4). Asymptomatic ash trees frequency (0-5% defoliation) tend to stabilize at 1% after 8 years of disease presence while the most severe crown dieback class is still increasing and represent approximately 40-50% 9 years after the pathogen discovery.

Figure 5 gives the evolution of annual mortality for the 3 age classes. Annual mortality is drastic in the regenerations, quite strong in stands with mean dbh less than 25 cm but remains low in large dbh stands, with about 3.2% 9 years post infection. Mortality appears to reach a plateau in stands of all size categories. This is the most noticeable in stands of 5-25 cm mean dbh with mortality remaining in the 10-11% range (confidence interval of 8-19 %) for times of *H. fraxineus* presence of more than 6 years.

Discussion

This work quantified mortality due to *H. fraxineus* in ash stands in the 9 years following the arrival of the pathogen in France and Belgium. As has been already reported, mortality is very high in young ash seedlings and saplings and to a lesser degree in the 5-25 cm dbh class (respectively 35% and 11% after 6 year of *H. fraxineus* presence). By

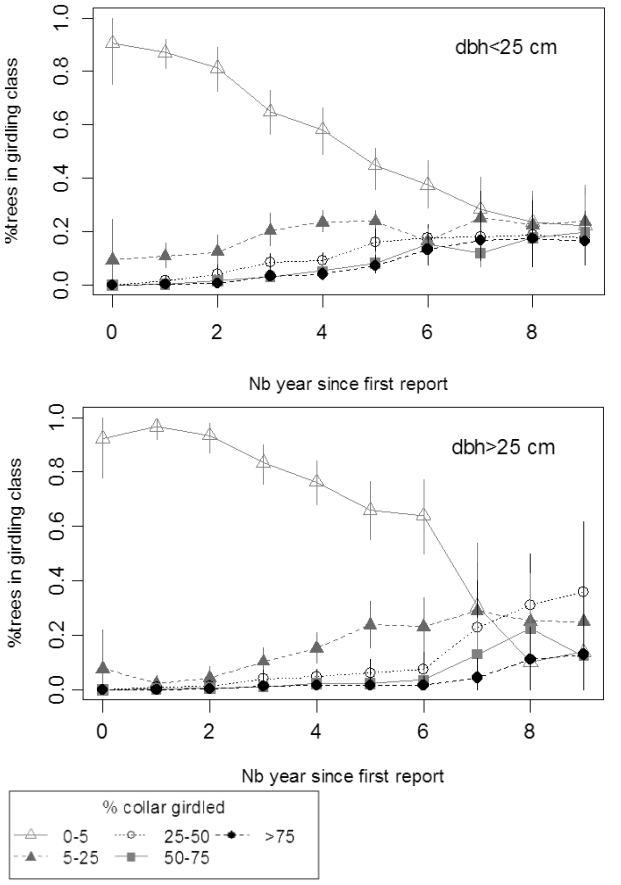

Figure 3. Evolution of level of collar girdling by *H. fraxineus* with time of pathogen presence in the area (based on bootstrap samples of 5000)

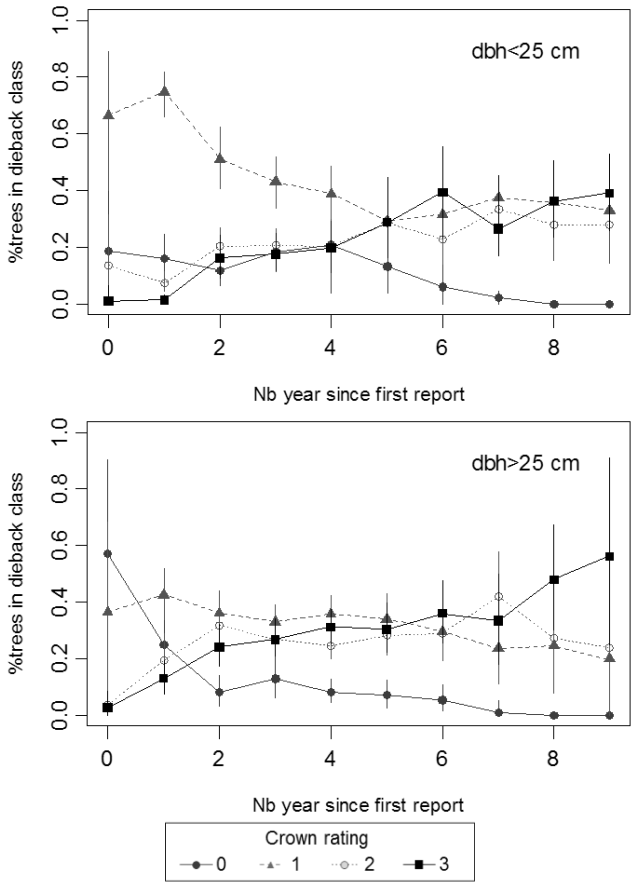

Figure 4. Evolution of the crown status of ash with time of *H. fraxineus* presence in the area (based on bootstrap samples of 5000)

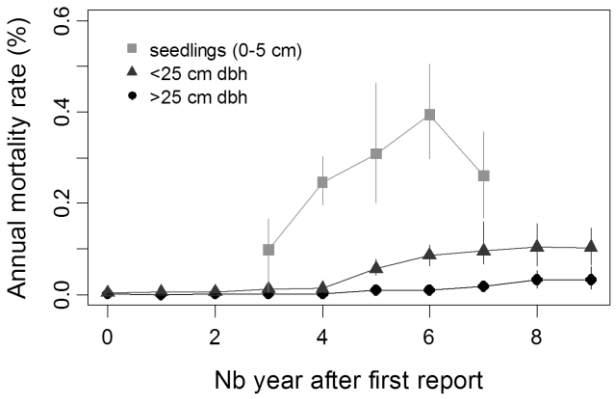

Figure 5. Evolution of mortality linked to *H. fraxineus* after introduction of the pathogen in an area (based on bootstrap samples of 5000)

contrast, we found that mortality of large ash of more than 25 cm dbh remains moderate, at 3.2% per year. A large part of the mortality can be explained by the status of the tree in the previous year, collar girdling by the pathogen and crown decline.

Mortality was influenced additively by crown status and by collar girdling. Both appeared to play an important role. For practical reasons, it was not possible to take these 2 factors into account in the final estimation of the mortality rate. The 2 symptoms have been shown to be tightly correlated at a stand level (Bakys et al. 2011, Husson et al. 2012, Chandelier et al. 2016). Moreover it was suggested that strong crown deterioration often follows the development of severe collar infection (Chandelier et al. 2016). Thus, taking into account only collar status in the computation of global mortality maybe a good compromise although it would probably have improve the estimate to also take into account the crown status. It has been suggested that the development of collar lesions necessitate a strong inoculum from apothecia at the base of the tree and thus appears only after few years of *H. fraxineus* presence in the stand (Chandelier et al. 2016). In agreement with this, we observed in this work an increase in the frequency and severity of collar lesions only 2-3 after the first report of the pathogen in the area.

Our results showed that mortality was strongly influenced by tree size, i.e. low to moderate in large trees, high in pole size ash and drastic in young regenerations. The lower *H. fraxineus* impact on large trees compared to young ash and seedling has already been reported by several authors (Husson et al. 2012, Chandelier et al. 2016, Lenz et al. 2016). The high mortality rate in young stands is in agreement with what was shown in Central and Eastern Europe, in stands originating either from seeds or from planting (Pliura et al. 2011, Koltay, 2012, Enderle et al. 2013, McKinney et al. 2014). By contrast, low mortality rate of mature trees affected by *H. fraxineus* have also been reported by Lenz *et al* (2016). The mortality rate of 3.2% observed in this study for large trees after 8-9 years of the pathogen presence is comparable to the figure reported by Rosenvald *et al* (2015) for solitary ash trees retained after timber harvesting in Estonia.

A large between-stands variability in mortality rate has been observed. Part of the variability is in the severity of the ash dieback symptoms and in particular in the severity of tree collar girdling by the pathogen. The nature of site and stand factors that could explain this variability are beyond the scope of this work. Nevertheless, it was shown that canker frequency and severity could be influenced by both site moisture and by tree size (Husson et al. 2012, Chandelier et al. 2016, Marçais et al. 2016). In agreement, we observed in this work that stands with large trees (>25 cm mean dbh) were affected less and later by collar canker. However, the severity of ash dieback crown and collar symptoms did not explain all mortality as both the stand random factor and as the duration of the pathogen presence in the area explained a significant part of the variability as well.

The difference in tree mortality at a given crown/collar status may be linked to the capacity of the trees to cope with stresses. Trees exposed to *H. fraxineus* suffer chronic infection with high mortality of shoots produced in the previous year. Their ability to cope with this may depend on the site suitability for ash, on their past history and on their exposure to secondary pest such as *Armillaria*. Indeed, mortality is a complex process not yet fully understood. It has been shown that trees may die because a stress such as defoliation or drought has depleted their carbohydrate reserves, potentially leading to carbon starvation (Marçais and Bréda, 2006, Bréda et al. 2006, Sevanto et al. 2014). The vigor of the tree at the time of first infection and it past history including the duration of exposure to *H. fraxineus* both are likely to impact it carbohydrate reserves level and could determine its ability to survive at a given crown / collar status. The fact that the stand random factor was associated with a part of mortality variability much higher than the tree random factor may indicate that individual tree history may be less important that factors acting at the stand level such competition level or site factors. For that reason, in the bootstrap procedure to estimate mortality, we included a first step of resampling of the stands.

The annual mortality appeared to reach a plateau after 6-7 year of *H. fraxineus* presence, especially for pole size trees (5-25 cm dbh). This may be favorable sign for future although annual mortality stabilized at a very high level. Possible future evolution depends on several factors. First, the severity of collar lesions also tended to stabilize after 6-7 years, especially for trees of the 5-25 cm dbh class. This probably happens because the trees the more severely girdled by *H. fraxineus* suffer very high mortality

and are constantly being removed from the population. As it has been showed that genetically based resistance toward the pathogen exists in *Fraxinus excelsior* (McKinney et al. 2010, Pliura et al. 2011) and that resistance to collar infection has also be demonstrated to exist (Muñoz et al. 2016), one may anticipate a progressive increase in the resistance level of the population with time. This might induce a decrease of the mortality rate after a prolonged period of infection. Then, the fact that mortality at a given health status depends on time since infection is not favorable as it may indicate that trees are progressively weakened and more prone to die. It is thus impossible to extrapolate the results of the study to the future.

The consequences of our results for long term impact of ash dieback are important. As was already reported by forest managers throughout Europe, the observed levels of mortality in the stands of 5-25 cm dbh (pole size) jeopardize all possibility of ash stand management. After few year of such high mortality, about half of the stand ash trees are dead. Even a level of 3.2% annual mortality in mature stands with reduced density where very few trees usually dies will preclude any management; moreover a majority of surviving trees are in poor health conditions. The ecological consequences may be less clear-cut. Very pessimistic prospects have been predicted for the ash ecosystem (Pautasso et al. 2013). However, this work shows that affected mature trees do not die fast and that ash dieback may not threaten that much these ecosystems on the short term. By contrast, the impact on ash young stands, especially those with less than 5 cm dbh is drastic and establishing the ash stands of the future appears as the main challenge. The work reported by Lygis *et al* (2011) well illustrates this point. Ash dieback has been managed in the first years in Lithuania before the widespread recognition that the disease was caused by an invasive pathogen and that resistance to the pathogen was present. In this situation, while ash dominated the stands in the previous generation, it became a minor component in the regeneration. Thus retaining ash as a significant component of the new generation stands will require a proactive management. In particular, selecting both the seed trees and the regenerating seedlings on their ability to tolerate the *H. fraxineus* infection is important as it has been pointed out by Mc Kinney et al. 2014. Some studies already started to address this issue (Rosenvald et al. 2015).

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Environmental and Silvicultural Characteristics Influencing the Extent of Ash Dieback in Forest Stands

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Abstract

Extensive investigation of the impact of ash dieback in forest stands in the Czech Republic was conducted in 2013. Data on the defoliation of ash trees were collected from 1169 forest stands within the entire area of the Czech Republic. A set of 37 variables acquired from different databases (State Forests, GIS, Digital Terrain Model) describing silvicultural, environmental and landscape characteristics were used as explanatory variables.A generalized linear model (GLM) explained nearly 26% of the disease data variability. In the model, the extent of the disease was positively affected by the density of stocking, site class, vertical terrain heterogeneity, temperature and the presence and width of watercourse and negatively affected by mean tree height, the altitudinal zone of the forest, and the distance to the nearest ash stand. The model confirmed an important role of tree species composition of stands with ash. The disease extent was the highest in the presence of *Quercus robur* and the lowest in presence of *Acer* spp. and *Abies* spp. This finding is probably due to the different chemical composition of mixed litter and the leaching and translocation of nutrients from maple litter into ash petioles, which could accelerate decomposition, whereas fungistatic tannins and secondary metabolites from fir litter could inhibit microbial growth. The extent of the disease also significantly differed according to edaphic series of forests, and GLM models were successfully developed for them. These models differed from each other and explained 23–37% of disease variability; other factors influencing disease extent were also determined: distance to water, SD of slope, ash area, standing volume, aspect, TPI, landforms and the presence of other tree taxa such as *Pinus* spp., *Quercus petraea*, *Fagus sylvatica* and *Betula pendula*.The results indicated that the disease extent is substantially affected by environmental and stand characteristics and that the development of effective forest management strategies to address the epidemic in European forests (at least in central Europe) is possible.

Keywords: ash dieback, forest stands, disease impact, environmental factors, silvicultural characteristics, litter

Introduction

H. fraxineus (T. Kowalski) Baral, Queloz, Hosoya, the causal agent of ash dieback threatens European ash (*F. excelsior* L.) and narrow-leaved ash (*F. angustifolia* Vahl) in Europe (Gross et al. 2014). European ash is highly susceptible to the pathogen, as can be deduced from the high rate of spread and destructiveness of the ongoing epidemic and the results of infection experiments (Gross et al. 2014). The pathogen is highly virulent because it did not co-evolve with its host (Zhao et al. 2012). The collapse of many European populations and ecosystems in which the dominating or keystone species is *F. excelsior* is repeatedly considered to be a possible consequence of pathogen invasion (e.g., McKinney et al. 2011, Gross et al. 2014, McKinney et al. 2014, Pliūra et al. 2014). This pathogen threatens not only ash, but also the organisms that depend on ash (Pautasso et al. 2013).

The control of established ash dieback in forests is practically impossible, and silvicultural recommendations are limited to avoiding the loss of the value of mature ash stands (Gross et al. 2014). Currently, many healthy or affected ash stands are lumbered in Denmark (Kjær et al. 2012 McKinney et al. 2014), the Czech Republic (Forests of the Czech Republic, state enterprise) and apparently in other European countries. The species is expected to suffer severe decimation as a result of not only high mortality following infection but also intensified logging in forestry (McKinney et al. 2014). Artificial re-establishment of ash stands is not recommended due to the high probability of ash dieback (Pliūra et al. 2014). Furthermore the interest among practitioners to plant the species is limited (McKinney et al. 2014).

Most native *Fraxinus excelsior* trees are highly susceptible to the invasion of *H. fraxineus*, and only approximately 1% of the trees have the potential to produce offspring with an expected crown damage of < 10% under the present disease pressure (Kjaer et al. 2012). The repeatedly detected genetically based resistance of common ash to *H. fraxineus* (e.g., Pliūra et al. 2011, McKinney et al. 2012, Stener 2013, McKinney et al. 2014, Pliūra et al. 2014, Enderle et al. 2015, Harper et al. 2016) has led to the general recommendation of selecting and preserving the most resistant genotypes for use in subsequent breeding. The first orchards to preserve the more resistant genotypes were established in different European countries (McKinney et al. 2014, Pliūra et al. 2014, Havrdová et al. 2015). Following a period of high mortality in natural populations, the selection and breeding of the remaining viable ash trees could provide a route for restoring the role of ash in the landscape (McKinney et al. 2014).

The assisted selection of more resistant genotypes improved by breeding together with still undervalued natural selection *in situ* represents a chance for restoring the species and forests in the future. The restoration of damaged or disrupted forests and other ash stands will take several decades and will be complicated by several other factors such as persisting forestry practices, hindered growth of more resistant trees under high infection pressure, the contribution of susceptible trees to the next generation in reducing the strength of selection (the resistance against *H. fraxineus* is apparently polygenic), and the expected future evolution of the pathogen as a reaction to changes in the host gene pool. Thus, the restoration of ash forests and other ash stands should be facilitated by appropriate methodology.

The appropriate management strategies are still unknown and need to be elaborated (Pliūra et al. 2014). Successful strategies must include thorough knowledge of the biology and epidemiology of the disease (Sakai et al. 2001) however, the effects of only a few environmental and silvicultural factors have been studied in *H. fraxineus* (Gross et al. 2014). The disease extent (crown dieback and collar rot) connects with site humidity (Husson et al. 2012, Enderle et al. 2013, Marçais et al. 2016) and the pathogen is sensitive to high temperatures and dry climate (Hauptman et al. 2013). On mature trees, disease progress is slower than on seedlings or young trees (Kirisits and Freinschlag, 2012; Kowalski and Holdenrieder, 2008). Dieback is more frequent on trees of average or below-average size and the extent of canker in the crown depended on site conditions (Skovsgaard et al. 2010). However, no extensive epidemiological study has been conducted till now. The aim of the present, in cooperation with State Forests of the Czech Republic, was to analyse the distribution and impact of the disease in forests with ash within the area of the Czech Republic and to identify silvicultural, environmental and landscape characteristics potentially affecting the disease epidemiology.

Methods

Study area

The field work was conducted in the entire territory of the Czech Republic, covering 78,866 km^2 with altitudes of 115 – 1603 m.a.s.l. (the mean altitude was 430 m) between 48° 33´ – 51° 03´ N and 12° 06´ – 18° 52´ E. The Czech Republic is located on the territory of four geomorphic provinces: the Bohemian Massif comprising 3/4 of the territory in the west and middle of the country, the Western Carpathians in the east, the Western Panonian Basin in the southeast, and the Middle-European Plain in the northeast of the country. The most common soil types are brown earth in the middle and higher altitudes and chernozem in the lowlands. The area was originally covered mainly by mixed deciduous temperate forests, whereas today´s forestry practices favour the cultivation of coniferous trees. Forests cover 34% of the area, and *Fraxinus* spp. Account for 1.4% of these forests, thus, *Fraxinus* spp. cover approximatelly 36,000 ha (Anonymous 2014).

Plot selection and data collection

The investigated forest stands were uniformly distributed within the entire area of the Czech Republic at altitudes of 150 – 900 m a. s. l. in the area. The data were obtained in cooperation with fieldworkers of the State Forest of the Czech Republic from a total of 1169 ash stands covering the full ecological niche of *Fraxinus* spp. in the area (Figure 1). The field investigations were conducted between 1st July and 31st August 2013 and terminated before the first premature leaf fall caused by ash dieback beginning at the end of August in that year.

Disease detection was performed using information leaflets describing characteristic symptoms of the disease (Havrdová et al. 2013). The percentage of crown defoliation in the surveyed ash stands was observed. Because the

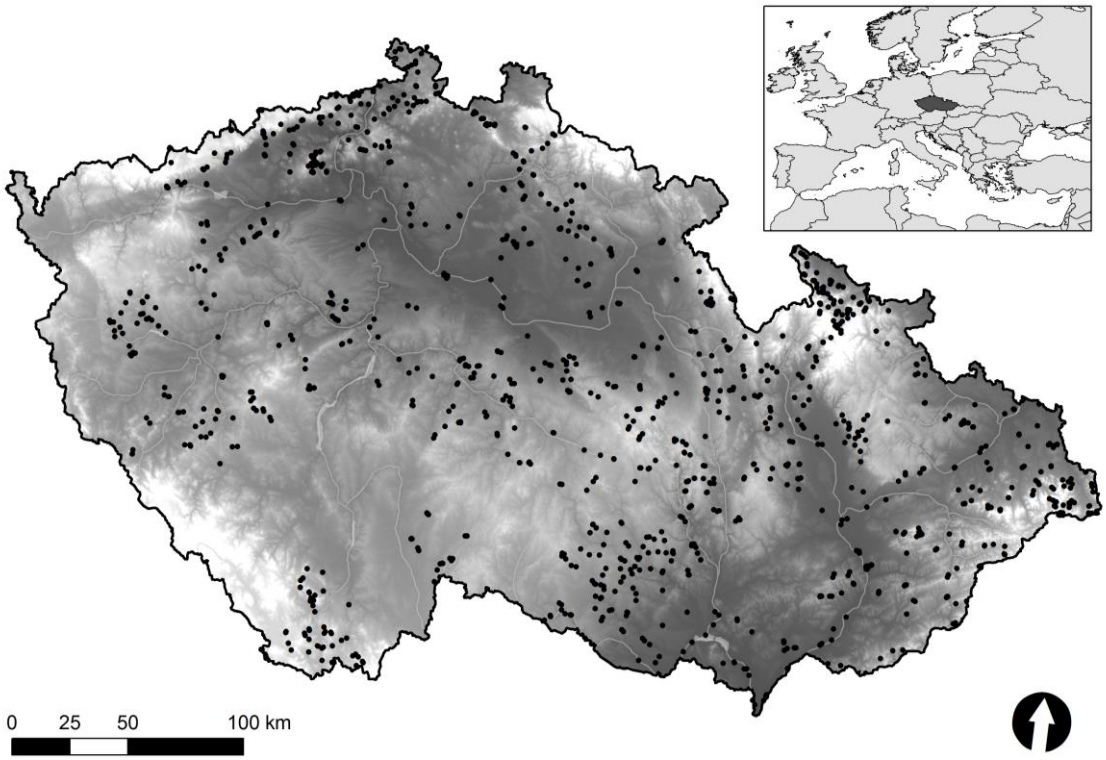

Figure 1. Investigated plots in the Czech Republic

data collection was performed by many people, the data collection methodology was simplified as much as possible. Five damage classes were used to describe ash defoliation: no characteristic symptoms (0%), little crown defoliation (1–10%), medium crown defoliation (11–25%), considerable crown defoliation (26–50%), and high damage (51–100%). The average values of these categories were used for presentation: 0.0, 5.0, 17.5, 37.5 and 75.0% respectively.

Information about silvicultural and forest characteristics such as forest altitudinal zone, age, mean tree height, density of stocking, ash area, site class, standing volume, presence of understory and presence of particular tree species, and forest edaphic series representing trophic and hydric properties (Viewegh et al. 2003) and exact coordinates were obtained from the database of the State Forests of the Czech Republic. In the Czech Republic, the edaphic series are divided into eight categories: extreme (stunted forests), acidic (oligotrophic), nutrient-rich (mesotrophic), humus-enriched (nitrophilous), water-enriched (continually wet with carbonated and oxygenated water), gleyed (alternately waterlogged), wet (permanently waterlogged) and peaty. Because some series were less abundant, the extreme and acidic series were merged into one category and the wet and gleyed series were merged into another category on the basis of similar properties for statistical purposes. The peaty category was omitted because it was not present for ash. Ecological characteristics, including the mean annual temperature, mean annual precipitation, presence and width of watercourse, distance to water and distance to nearest ash stand were acquired using Geographical Information Systems. Information describing landscape morphology, including the mean vertical heterogeneity, mean aspect, mean slope, standard deviation (SD) of slope, topographic position index (TPI; index of the local terrain), SD of TPI and landforms, were obtained using Digital Model of Relief the Czech Republic. An overview of particular variables is presented in Table 1.

Statistical analysis

The statistical analyses were performed using the R plus statistical package (R Core Team 2014). Preliminary data analysis consisted of the evaluation of pair correlations between all continuous variables. Highly correlated variables were identified in the correlation matrix and only one variable was maintained for subsequent analysis from each such group.

To evaluate the mutual influences of individual variables on crown dieback, a general linear model was used,

$$[1] \qquad y_i^{\frac{1}{2}} = \beta_0 + \sum_{j=1}^{m} \beta_j x_{ji} + \varepsilon_i$$

Table 1. Overview of the investigated variables with the codes used in evaluation

Abbreviation	Description of the variable	Units	Category (in categorial variable)
Silvicultural variables			
Defoliation	Crown defoliation		0%; 5% (1–10%); 17.5% (11–25%); 37.5% (26–50%); 75% (51–100%)
Edaphic series	Edaphic series		extreme, acidic, nutrient-rich, humus enriched, water enriched, gleyed, wet
Alt. zone	Forest altitudinal zone		1st oak, 2nd beech-oak, 3rd oak-beech, 4th beech, 5th fir-beech, 6th spruce-beech
Age	Mean age	year	
Height	Mean tree height	m	
Stocking	Density of stocking	%	
Ash area	Ash area	m^2	
Site class	Site class	m	
Volume	Standing volume	m^3 u.b.	
Understory	Understory		1 (1–10); 2 (11–20); 3 (21–30) year old
Abies	Presence of species Abies	%	*Abies alba, A. grandis*
Acer	Presence of species Acer	%	*Acer pseudoplatanus, A. platanoides, A. campestre*
Alnus	Presence of species Alnus	%	*Alnus glutinosa, A. incana*
Ash	Presence of species Ash	%	*Fraxinus excelsior, F. angustifolia*
Betula	Presence of species Betula	%	*Betula pendula*
Carpinus	Presence of species Carpinus	%	*Carpinus betulus*
Fagus	Presence of species Fagus	%	*Fagus sylvatica*
Larix	Presence of species Larix	%	*Larix decidua*
Picea	Presence of species Picea	%	*Picea abies, P. pungens*
Pinus	Presence of species Pinus	%	*Pinus silvestris, P. nigra, P. strobus*
Populus	Presence of species Populus and Salix	%	*Populus tremula, P. alba, P. nigra, Salix* spp. etc.
Quercus1	Presence of species Quercus	%	*Qurcus robur, Q. rubra*
Quercus2	Presence of species Quercus	%	*Quercus petraea*
Salix	Presence of species Salix	%	*Salix caprea*
Tilia	Presence of species Tilia	%	*Tilia cordata, T. platyphyllos*
Ulmus	Presence of species Ulmus	%	*Ulmus minor, U. laevis, U. glabra*
Landscape variables			
Heterogeneity	Mean vertical heterogeneity	m	
Aspect	Mean aspect	degree	N (337.6–22.5˚); NE (22.6–67.5˚); E (67.6–112.5˚); SE (112.6–157.5˚); S (157.6–202.5˚); SW (202.6–247.5˚); W (247.6–292.5); NW (292.6–337.5˚)
Slope	Mean slope	degree	
SD slope	SD of slope	degree	
TPI	Topographic Position Index	index	
SD TPI	SD of TPI	index	
Landform	Landforms		canyons, deeply incised streams; convex concave shapes on slope; plains; open slopes; mountain tops
Ecological variables			
Temperature	Mean annual temperature	ºC	
Precipitation	Mean annual precipitation	mm	
Watercourse	Presence and width of watercourse		presence watercourse; width of watercourse < 1m; width of watercourse > 1m
Dist. to water	Distance to water	m	
Dist. to ash	Distance to nearest ash stand	m	

where y_i is rate of crown dieback in i^{th} stand, the coefficients β_j express the effect of individual factors x_j, β_0 is an intercept and ε_i is an error term [1]. To fulfill the assumption of normality of residuals, a square root transformation of crown dieback rate was performed. In the process of fitting the model [1], a large number of included factors turned out to be insignificant. Thus, a bidirectional step regression method was used to determine a set of factors

important to the crown dieback rate. The optimal submodel of [1] was selected by minimization of the Akaike information criterion.

To evaluate the significance of differences among values of categorical variables, a multiple comparison method with Bonferroni correction was used. The results are presented in the form of homogenous groups.

Results

The damage was identified in 945 of the total 1169 investigated forest stands (80.8%). The average value of crown defoliation was 27.36% (± 0.75%). Particular categories of stands according to the extent of defoliation importantly differed in number of stands. The defoliation category of 17.5% was the most frequent, whereas the category with defoliation of 5% was the least frequent (Figure 2).

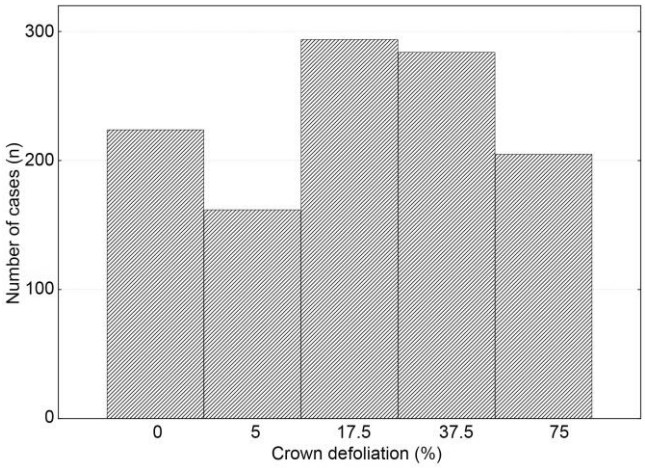

Figure 2. The distribution of forest stands according to the extent of dieback

The most representative of several developed models for all included forest stands was selected (Table 2). The model consisted of 12 significantly contributing environmental variables and explained 25.7% of the disease variability. In the model, the extent of disease was positively affected by the density of stocking, site class, vertical heterogeneity, temperature and the presence and width of watercourse and negatively associated with mean tree height, forest altitudinal zone, and the distance to the nearest ash stand ($P < 0.001$).

The compared edaphic series significantly differed in the proportion of defoliation ($P < 0.001$, Figure 3A, Table 2). The wet and gleyed categories were the least damaged with 23.0% (±2.6) and 11.1% (±3.0) defoliation, respectively, whereas the nutrient category with 34.1% (±1.6) defoliation and the extreme category with 37.5% (±21.7) evaluation were the most damaged.

Table 2. The general GLM model of ash dieback in forests of the Czech Republic

Continuous variable	Estimate	*SE*	*t* value	*P*-value	Strength
(Intercept)	-0.142	2.104	-0.068	0.946	
Stocking	0.135	0.064	2.129	0.033	*
Height	-0.104	0.010	-10.839	< 0.001	***
Site class	0.199	0.032	6.149	< 0.001	***
Heterogeneity	0.040	0.012	3.235	0.001	**
Slope	-0.039	0.022	-1.774	0.076	.
TPI	-0.037	0.023	-1.593	0.111	
Dist. to ash	-0.003	0.002	-2.116	0.035	*
Temperature	0.293	0.123	2.383	0.017	*
Abies	-0.048	0.020	-2.343	0.019	*
Quercus1	0.013	0.006	2.378	0.018	*
Acer	-0.015	0.006	-2.349	0.019	*
Carpinus	-0.044	0.024	-1.808	0.071	.
Categorial variable					
Edaphic series	Estimate	Homogeneous groups			
Extreme	0.000	|	|	|	
Nutrient-rich	-0.700			|	
Humus enriched	-1.587		|		
Water enriched	-1.623		|		
Acidic	-2.018		|		
Gleyed	-2.091	|	|		
Wet	-3.800	|			*
Altitudional zone					
1st oak	0.000		|		
2nd beech-oak	-1.211	|			***
3rd oak-beech	-0.981	|	|		**
4th beech	-0.328		|		
5th fir-beech	-0.905	|	|		*
6th spruce-beech	-0.652	|	|		
Watercourse					
without w.	0.000	|			
width of w. < 1m	-0.163	|			
width of w. > 1m	0.750		|		***

Residual standard error: 2.507 on 1143 degrees of freedom, Multiple R-squared: 0.2572, Adjusted R-squared: 0.241, *F*-statistic: 15.83 on 25 and 1143 DF, *P*-value: < 2.2e-16
Significance codes: *** (0.001), ** (0.01), * (0.05), . (0.1)

The model confirmed an important role for the tree species composition of stands with ash. The disease extent was the highest in the presence of *Quercus robur* and the lowest in the presence of *Abies* spp. and *Acer* spp. ($P < 0.05$).

Three other variables that were included in the model had negative but not statistically significant associations with the disease extent: slope ($P = 0.08$), TPI ($P = 0.11$) and

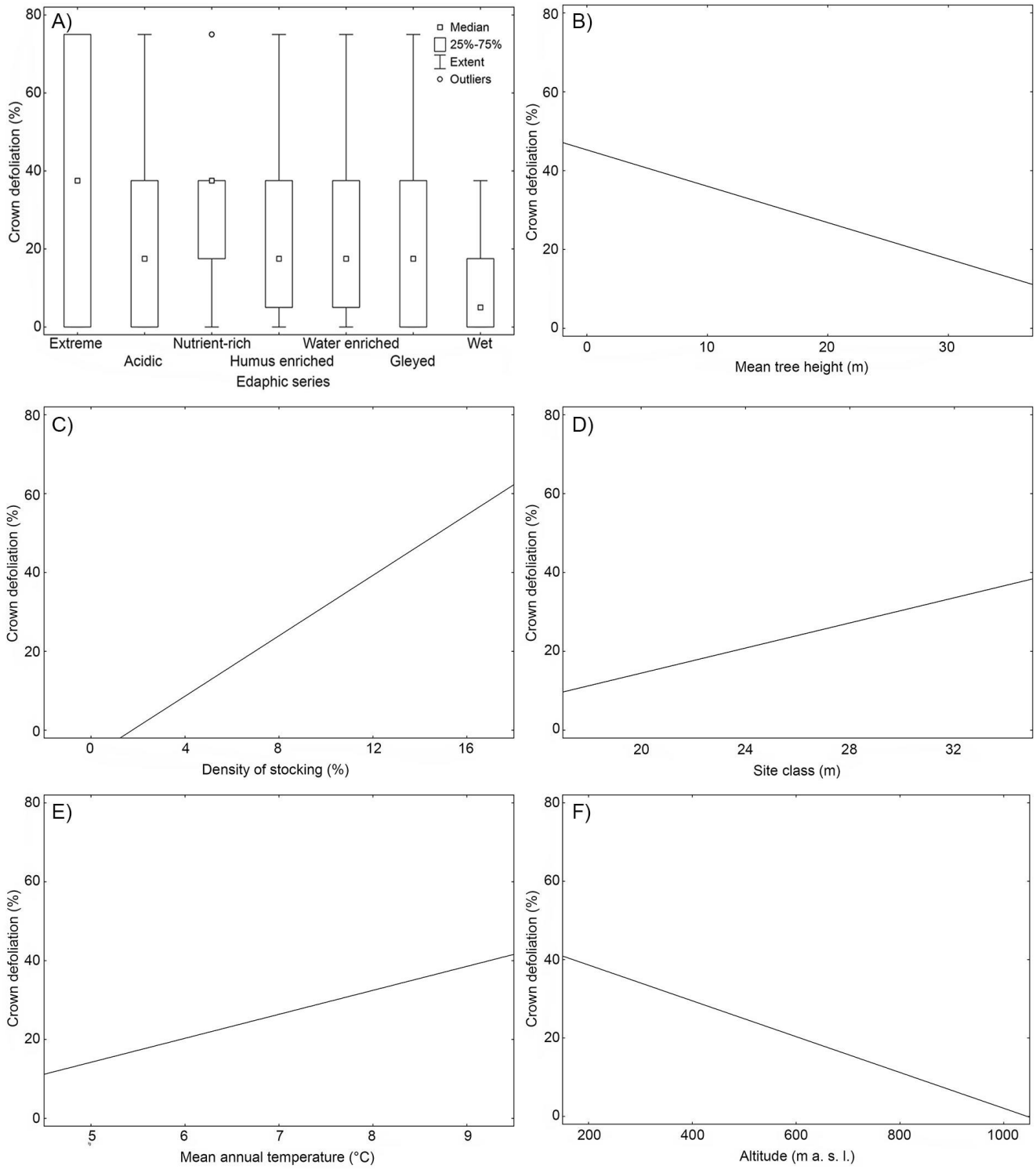

Figure 3. A) Extent of ash dieback in edaphic series in forests of the Czech Republic. B) Correlation between the extent of ash dieback and medium tree height. C) Correlation between the extent of ash dieback and the density of stocking. D) Correlation between the extent of ash dieback and site class. E) Correlation between the extent of ash dieback and the average annual temperature. F) Correlation between the extent of ash dieback and altitude

the presence of *Carpinus betulus* (P = 0.07; Table 2). The negative effect of *Picea* spp. was not identified and, moreover, its partial correlation to disease extent was also insignificant (Table S1).

Overall, the dataset of 35 explanatory variables for the extent of crown damage was evaluated. Twelve variables were included in the GLM model of the disease extent (Table 2); however, four other variables with significant relation to the extent of ash dieback were not incorporated in the model: ash area and the presence of ash with a positive correlation to the disease level and age and the presence of alder which were negatively correlated. The overview of the partial correlations of all quantitative variables is included in the correlation matrix in Table S1. Selected variables with the most influence on the extent of ash dieback are presented in Figure 3.

Five particular GLM models were successfully developed for the edaphic series –nutrient, enriched by humus, enriched by water, wet and gleyed and extreme and acidic series. The last series were merged into pairs (wet and gleyed and extreme and acidic series, respectively) due to the lower number of stands in some series and their resemblance. The models differed from each other in the composition of explanatory variables. These models described 23.2–36.7% of the disease variability (Table 3).

The GLM model for nutrient edaphic series was developed for 272 stands with ash explained 27.9% of the disease variability and contained 9 explanatory variables. Six variables significantly contributed into the model: forest altitudinal zone, mean tree height, the presence and width of watercourse, distance to water, site class and the presence of *Pinus* spp.

The GLM model for the humus-enriched series was constructed with data from 162 stands and explained 36.0% of the data variability. The model contained a total of 8 variables, and 6 of these variables were significant: ash area, mean tree height, standing volume, site class, aspect and landform.

The model for the series enriched by water (alluvial or bottomland series) was based on data from 351 stands and explained 25.6% of the variability of the data in the series. The model contained 11 explanatory variables and seven of them significantly contributed to the model: mean tree height, site class, vertical heterogeneity, distance to the nearest ash stand, temperature, the presence of *Q. petraea* and the presence of *Fagus sylvatica*.

Table 3. Overview of GLM models for particular edaphic series and their groups

Variables	General model	Extreme + Acidic	Nutrient-rich	Humus enriched	Water enriched	Gleyed + Wet
(Intercept)					-	-
E.s. wet	-					
2nd beech-oak	-		-			
3rd oak-beech	-					
5th fir-beech	-					
Height	-	-	-	-	-	-
Stocking	+					
Ash area				+		
Volume				-		
Site class	+		+	+	+	
width of w. > 1m	+	+	+			
Dist. to water			-			
Dist. to ash	-				-	
Temperature	+				+	+
Heterogeneity	+				+	
SD Slope		+				+
TPI						+
Aspect NE				+		
Aspect SW				+		
Landf. Tops				-		
Abies	-	-				
Pinus			-			
Quercus1	+					
Acer	-	-				
Quercus2					+	
Fagus					+	
Betulus						+
Multiple R-squared	0.257	0.232	0.279	0.360	0.256	0.367

+ (positive) and – (negative) influence on the disease impact

The model for the wet and gleyed series was based on 122 stands, explained 36.7% of the disease variability and contained 10 explanatory variables. Five of these variables (mean tree height, temperature, SD of slope, TPI and the presence of *Betula pendula*) contributed significantly to the model.

The last GLM model was prepared for acidophilous and the extreme series. The model was prepared on the basis of 262 stands and was the least successful among the developed models – it explained 23.2% of the disease variability. The model contained 12 variables and 5 of them had significant effects in the model: mean tree height, the presence and width of watercourse, SD of slope, the presence of *Abies* spp. and the presence of *Acer* spp. Particular GLM models are schematically shown in Table 3.

The analysis of particular GLM models (Table 3) found that some explanatory variables play an important role in the all or the majority of models. The variable playing an important role within the entire ecological niche of *F. excelsior* and its pathogen is mean tree height. The variables that significantly explained some disease data in at least two or three models are site class, the presence and width of watercourse, SD of slope, and temperature. Other variables (forest altitudinal zone, ash area, standing volume, distance to watercourse, distance to other ash stand, vertical heterogeneity, TPI, aspect, landforms and the presence of some tree species) were significant in one of the developed models (Table 3).

Table 4. Overview of GLM models for stands divided according to the presence and width of watercourse

Variables	Without watercourse	Width <1m	Width >1m
(Intercept)		-	
2nd beech-oak	-		
3rd oak-beech	-		
4th beech	-		
5th fir-beech	-		
6th spruce-beech	-		
Height	-	-	-
Volume	+		
Site class	+	+	
Dist. to water			-
Dist. to ash			
Temperature		+	+
Heterogeneity	+	+	+
Aspect SW	+		
Abies			-
Carpinus		-	
Multiple R-squared	0.295	0.220	0.222

+ (positive) and – (negative) influence on the disease impact

Because humidity is highly important for spore production, spread and infection in many foliage pathogens (Sinclair and Lyon 2005) including *H. fraxineus* (Hietala et al. 2013; Dvorak et al. 2016) and the influence of water source on the disease is highly significant ($P < 0.001$; Table 2), the disease data were evaluated according to the presence of a watercourse in forest stands. Defoliation was highest (32.2% ± 1.5), in stands with the presence of a watercourse wider than 1 m and was lowest (22.4% ± 1.3) in stands with a watercourse up to 1 m wide. The GLM models explained 22.0 to 29.5% of the data variability: the most successful model was developed for stands without the presence of water. The three models importantly differ in combinations of explanatory variables (Table 4). The model for stands without the presence of water was composed of forest altitudinal zone, mean tree height, standing volume, site class, vertical heterogeneity and aspect with significant value, whereas the models for stands with both types of watercourses were different but more similar to each other and contained mean tree height, temperature, vertical heterogeneity, site class (up 1 m in width), the presence of *C. betulus* (<1 m in width), distance to other ash stand (>1 m) and the presence of *Abies* spp. (>1 m; Table 4).

Discussion

Extensive investigation of ash dieback in Czech forests was performed in 1169 forest stands within the entire area of the Czech Republic in 2013; the average ash defoliation in forest stands was 27.4%. The average crown defoliation registered in the Danish National Forest Inventory increased rapidly from a background level of 10–15% leaf loss to over 40% leaf loss in 2009 (McKinney et al. 2014). This difference between Czech and Danish forests is relatively high and could be due to the differing climate between the two regions. The climate in Denmark is typically oceanic with a high level of precipitation throughout the year, whereas the climate in the Czech Republic is mild and transitional with an increase in continental characteristics in its south-eastern regions (Tolasz et al. 2007). Because spore release and infection processes are influenced by air humidity (Hietala et al. 2013, Havrdová 2015, Dvorak et al. 2016), the difference in disease level in these climatically different areas is understandable.

In total, 224 (19.2%) forest stands included in this study were designated by foresters as "healthy"; however, when a sample of ten "healthy" forest stands throughout the country was thoroughly investigated, the forest stands were found to be diseased, although with very low disease incidence. The other, independent thorough investigation of forest and other stands with ash conducted during 2011–2013 revealed that ca 95% of 1045 trees in 80 investigated plots were more or less affected by the pathogen (Havrdová

2015). Thus, the disease is widespread in the area and affects all or nearly all ash stands in the country.

The most informative GLM model for the disease distribution explained 25.7% of the disease variability in Czech forests. Particular models were also developed for edaphic series with explanatory power from 23.2 to 36.7%. The rest of the (unexplained) variability can be ascribed to the variation in ash sensitivity by genotype (McKinney et al. 2011, Kirisits and Freinschlag 2012, Kjær et al. 2012, McKinney et al. 2012, Stener 2013, Pliūra et al. 2014, Lobo et al. 2015) and provenance levels (Enderle et al. 2013, Havrdová et al. 2016), the variation in pathogen virulence **(**Kowalski and Holdenrieder 2009, Bakys et al. 2011, Husson et al. 2012), the other non-investigated environmental and stand characteristics (Havrdová 2015) and the error.

A total of 23 explanatory variables describing environmental and stand characteristics were found to significantly influence the disease level in the general model or at least in models for particular edaphic series. The variables that positively affected the disease extent were density of stocking, ash area, site class, the presence and width of watercourse, vertical heterogeneity, temperature, NE and SW aspects, TPI, SD of slope, and the presence of some tree species (*Quercus robur*, *Q. petraea*, *Fagus sylvatica*, *Betula pendula*). The variables that negatively affected the disease extent were mean tree height, standing volume, distance to watercourse, distance to nearest ash stand, altitude, landform (mountain tops and ridges) and the presence of *Abies* spp., *Pinus* spp. and *Acer* spp. Furthermore, the disease extent was influenced by edaphic series.

The set of variables significantly affecting disease distribution and intensity comprised of variables at different ecological scales. For example, edaphic series, temperature and altitude were among the variables with an affect at the large landscape scale. Their including apparently connected with the extremely broad ecological niches of the host (Wardle 1961, Dobrowolska et al. 2011) and of the pathogen, which covers the geographical and altitudinal distribution of the host (Queloz et al. 2011, Baral and Bemmann 2014, McKinney et al. 2014). The finding of the lowest disease incidence in the wet and gleyed series in the present study is consistent with the findings of Schumacher (2011) who reported that stands on wet soil with changing moisture had generally lower infection rates.

The next set of variables affected the pathogen distribution at a medium scale – at a range from dozens of metres to a km – aspect, SD of slope, TPI, landform, vertical heterogeneity, medium tree height and density of stocking. These environmental variables described the landscape and stand morphology; thus, they should be ascertained as indirect variables (Franklin 1995). These variables described the shapes and coarseness of the terrain and environment and affected the microclimate including air humidity near the ground in different ways (Bennie et al. 2008, Geiger et al. 2009, Meentemeyer et al. 2012, Pezzopane et al., 2015). Variables such as the presence of a watercourse and its width and the distance to water also affected air humidity including the amount of horizontal precipitation **(**Geiger et al. 2009). Humidity is important for spore production and the release of many pathogens (Sinclair and Lyon 2005); the dispersal pattern of *H. fraxineus* ascospores and the disease level is influenced by air humidity (Havrdová 2015, Dvorak et al. 2016). Because the ascospores are drought-sensitive (Aylor 2003, Gross et al. 2014), leaf wetness from morning dew also protects them against desiccation (Hietala et al. 2013). The presence of watercourses in stands (or connected high water table) could create more friendly conditions for ascomata formation and the production of ascospores due to higher soil humidity, which agrees with the finding of Schumacher (2011) that the disease risk was highest for soils with very (all-season) wet conditions.

Slope usually has a negative effect on air humidity (Geiger et al. 2009), but its standard deviation, which was included in the GLM model of ash dieback in two series, affected air humidity positively. Undoubtedly, this quantity could also describe the terrain coarseness on slopes as vertical heterogeneity in flat landscape forms. Moreover, the slopes closing the valleys and gorges impeded the air circulation and affected the local climate (Geiger et al. 2009).

TPI usually affects the disease level negatively (general model in this study, Havrdová 2015). However, in the wet and gleyed series, the disease level increased in locations with higher TPI, i.e. in places elevated above the surrounding waterlogged areas. The cause of this association is unknown, but this series is relatively less affected by the pathogen and the higher disease impact in drier stands of this series could be caused by the better persistence of ash petioles in drier conditions than in the waterlogged or seasonally flooded conditions. This finding is also in agreement with the findings of Schumacher (2011).

The study also revealed the significant influence of terrain aspects on the disease intensity in one series. The NE aspect is typified by long term sustainable higher air humidity (Geiger et al. 2009), whereas in the most heated SW aspect the strengthened upward airflow can also strengthen the infection pressure of ascospores. These findings generally agree with the outcomes of Havrdová (2015).

The presented outcomes confirmed the importance of air humidity indirectly, but the set of influencing variables (vertical heterogeneity, medium tree height, density of stocking, etc.; Geiger et al. 2009) was nearly the same as in Havrdová (2015), where the correlations between measured air humidity and these variables were confirmed. The direct influence of precipitation on the disease level was not confirmed, but it could be supposed. The influence of precipitation could be confirmed on the whole-European scale especially in the oceanic-continental climate gradient as dis-

cussed above in the comparison with the Danish (McKinney et al. 2014) and Czech forests. Undoubtedly, precipitation plays an important role in disease epidemiology, based on the large amount of new infections in extremely wet summers (for instance in 2011) and the limited number of new infections in dry summers (especially 2015) as we repeatedly observed in the previous decade in the area. Likely, the potential influence of precipitation in presented models could be overshadowed by extreme variability in the length and intensity of wet and dry periods in last years (Daňhelka et al. 2015) or by many other environmental variables affecting air humidity in ash stands (Havrdová 2015) and its elucidation needs further investigation or deeper statistical evaluation.

The other variables of medium scale described the stand characteristics – site class, ash area and density of stocking. These variables were partially intercorrelated and positively influenced the disease level in stands (Table 2, Table S1). The quantity site class describing site productivity is defined as the height of a dominant tree (Avery and Burkhart 2002). These variables positively affected the possibility of colonization of the stands (host area, its concentration and biomass) and, moreover, the amount of *in situ* developed inoculum. The influence of these variables was in concordance with the ash dieback epidemic requiring a sufficient accumulation of susceptible host individuals (Schumacher 2011, Gross et al. 2014) or, better, accessible biomass.

The distance to the nearest ash stand and the mean tree height negatively affected the disease level. Both variables described the distance of the susceptible host tissues (mainly foliage) from the inoculum source. In the first case, a source of primary inoculum was in another stand, whereas in the second case, the distance of the source of "secondary" inoculum on the plant debris on the soil surface from sensitive living tissues directly in the stand was described. The distance of susceptible hosts was highly important to the spreading potential of the pathogen and was determined to be fundamental in landscape models describing spactial patterns of important tree diseases including SOD (Meentemeyer et al. 2011, 2012). Tree height negatively affected the disease level. The younger and smaller trees were usually damaged to a larger extent (Kowalski and Holdenrieder, 2008; Schumacher, 2011; Kirisits and Freinschlag, 2012) because their crowns were closer to the source of inoculum on the ground. Moreover, the infection pressure is lower and leaf quality and/or microclimatic conditions are less suitable for infection in the crown of higher trees (Gross et al. 2014). Standing volume was negatively correlated with the extent of the disease in one series (Table 3). This finding is likely due to the relationship of the standing biomass to the mean tree height, which is important in the epidemiology of ash dieback as elucidated above.

The last group of variables probably influenced the pathogen on a small scale via litter chemistry (Madritsch and Cardinale, 2007) because the disease level was affected by the coincidental presence of other tree species in the stand with ash. The disease level was significantly lower in the presence of *Abies* spp., *Pinus* spp., and *Acer* spp., whereas the extent of the disease was higher in the presence of *Quercus* spp., *Fagus sylvatica* and *Betula pendula*. The influence of these tree species is probably mediated via physical and chemical characteristics of litter and by differences in decomposition rates. For example, maple litter is more quickly decomposed than oak litter (Blair et al. 1990). The decomposition process in one type of litter can accelerate the decomposition of another type in the mixture by translocation of nutrients through diffusion of a water film and/or active transport through invertebrate-microbial interactions (Blair et al. 1990). The difference between the effects of *Acer* spp. and *Quercus* spp. on the disease level could be explained by the different rates of ash petiole degradation in mixed litter with different compounds. On other sites, the coniferous litter containing high amounts of secondary compounds such as tannins, lignin, waxes and terpenoids can leach secondary metabolites and tannins into the surrounding and could directly inhibit microbial growth and activity there (Kraus et al. 2003, Madritsch and Cardinale 2007, Ushio et al. 2013) and thus could also inhibit the development of *H. fraxineus* in mixed litter. The decomposition rate is also affected by C:N ratio in litter, which is the most advantageous in maple litter, worse in oak liter and the less favorable in pine litter (Madritsch and Cardinale 2007). Likely, the structure and size of fallen leaves could also affect the level of diffusion of different compounds into ash petioles. Likely, fine coniferous needles could more tightly surround ash petioles in forest floor, thus the compounds translocation could be more effective in this case than in leaves of oak and other broadleaved trees with relatively higher content of tannin. The significant negative effect of *Abies* spp. and *Pinus* spp. on the disease extent was confirmed in the study, whereas the effect of *Picea* spp. was also negative but not significant.

Conclusions

Extensive analysis of the influence of environmental and stand characteristics on the presence and extent of ash dieback in forest stands in the Czech Republic was conducted. The data on the defoliation of ash trees were collected from 1169 forest stands within the entire area of the Czech Republic in 2013. A set of 37 variables acquired from different databases (State Forests, GIS, Digital Relief Model) describing environmental and stand characteristics was used as explanatory variables.

The general developed model (GLM, R plus) explained nearly 26% of the disease variability. In the model,

the extent of the disease was positively affected by 12 variables. Density of stocking, site class, vertical heterogeneity, temperature and the presence and width of watercourse positively affected the disease impact, whereas mean tree height, forest altitudinal zone, and the distance to the nearest ash stand negatively influenced the disease impact. A direct influence of precipitation was not confirmed. However, a set of environmental and silvicultural characteristics (such as density of stocking, vertical heterogeneity, the height of trees, and the presence and width of watercourse) were determined to be variables that indirectly influenced air humidity near the ground and the infection process indirectly.

The model confirmed a significant role of tree species composition of stands with ash on ash dieback. The disease extent was larger in the coincidental presence of *Quercus robur* and lower in the presence of *Abies* spp. and *Acer* spp. The influence of these trees is likely to be mediated via chemical characteristics of litter and in differences decomposition rates on ash petioles as a substrate for *H. fraxineus* in mixed litter. The decomposition of ash petioles could be accelerated by the coincidental decomposition of maple litter (for instance by the translocation of nutrients from it) in comparison with another litter type (oak). In contrast, secondary metabolites and tannins from coniferous litter leaching into ash litter could directly inhibit microbial growth and activity.

The extent of the disease also differed by edaphic series (wet and gleyed series were less damaged) and particular GLM models were also successfully developed. These models differed from each other and explained 23–37% of disease variability; other factors influencing disease extent were also determined: distance to watercourse, SD of slope, ash area, standing volume, aspect, TPI, landforms and the presence of other tree taxa such as *Pinus* spp., *Quercus petraea*, *Fagus sylvatica* and *Betula pendula*.

The outcome of this study clearly supports the idea that disease management based on the utilization of sources of resistance could be effectively facilitated by appropriate forest and landscape management. Forest management could be useful, at least in more heterogeneous areas with different forest types with ash and in regions with transitional, Mediterranean or more continental climates, which are typically in central, eastern and southern Europe. Of course, in more homogeneous flat regions in north-western and western Europe (for instance Denmark, the Netherlands, the Northern German Plain), the scale of environmental factors affecting the disease impact could be more restricted than in more variable central European landscape. The oceanic climate in west Europe could also support disease development in comparison with more eastern regions with transitional and continental climates.

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Supplementary material

Supplement Table S1a. Correlation matrix of quantitative variables and stand characteristics; partial correlation coefficients are shown; statistical significance ($P \leq 0.05$) is highlighted in grey

	Defoliation	Age	Stocking	Ash area	Height	Volume	Site class	Heterogeneity	Slope	SD slope	TPI	SD TPI	Dist. to water	Dist. to ash	Temperature	Precipitation
Defoliation	1.00	-0.33	0.19	0.08	-0.31	-0.03	0.16	-0.05	-0.12	-0.05	0.02	-0.04	-0.01	-0.03	0.24	-0.02
Age	-0.33	1.00	-0.32	0.02	0.87	0.28	-0.01	0.16	0.18	0.12	-0.04	0.12	-0.08	0,00	-0.16	-0.04
Stocking	0.19	-0.32	1.00	0.04	-0.23	0.02	0.16	0.03	-0.05	0.02	0.06	0.02	0.03	0.04	0.08	0.02
Ash area	0.08	0.02	0.04	1.00	0.02	0.70	0.04	0.05	-0.13	0.01	0.18	-0.02	-0.01	0.15	0.30	-0.04
Height	-0.31	0.87	-0.23	0.02	1.00	0.30	0.27	0.16	0.14	0.15	-0.12	0.12	-0.11	0.03	-0.11	-0.05
Volume	-0.03	0.28	0.02	0.70	0.30	1.00	0.12	0.13	-0.03	0.06	0.12	0.04	0.01	0.14	0.11	-0.02
Site class	0.16	-0.01	0.16	0.04	0.27	0.12	1.00	0.00	-0.06	0.02	-0.14	0,00	-0.15	0.04	0.14	-0.01
Heterogeneity	-0.05	0.16	0.03	0.05	0.16	0.13	0.00	1.00	0.68	0.64	0.08	0.72	-0.09	0.28	-0.35	-0.03
Slope	-0.12	0.18	-0.05	-0.13	0.14	-0.03	-0.06	0.68	1.00	0.68	-0.17	0.77	-0.08	0.04	-0.48	0.00
SD slope	-0.05	0.12	0.02	0.01	0.15	0.06	0.02	0.64	0.68	1.00	-0.13	0.78	-0.14	0.19	-0.28	-0.02
TPI	0.02	-0.04	0.06	0.18	-0.12	0.12	-0.14	0.08	-0.17	-0.13	1.00	-0.05	0.26	-0.04	0.13	-0.05
SD TPI	-0.04	0.12	0.02	-0.02	0.12	0.04	0,00	0.72	0.77	0.78	-0.05	1.00	-0.13	0.19	-0.25	-0.02
Dist. to water	-0.01	-0.08	0.03	-0.01	-0.11	0.01	-0.15	-0.09	-0.08	-0.14	0.26	-0.13	1.00	-0.08	0.12	0,00
Dist. to ash	-0.03	0.00	0.04	0.15	0.03	0.14	0.04	0.28	0.04	0.19	-0.04	0.19	-0.08	1.00	0.01	-0.04
Temperature	0.24	-0.16	0.08	0.30	-0.11	0.11	0.14	-0.35	-0.48	-0.28	0.13	-0.25	0.12	0.01	1.00	-0.04
Precipitation	-0.02	-0.04	0.02	-0.04	-0.05	-0.02	-0.01	-0.03	0,00	-0.02	-0.05	-0.02	0,00	-0.04	-0.04	1.00
Ash	0.09	-0.12	0.06	0.16	-0.10	0.02	0.02	-0.35	-0.17	-0.28	-0.08	-0.30	0.08	-0.27	0.23	-0.01
Picea	-0.03	0.02	0.07	-0.17	0.04	-0.07	0.05	0.31	0.20	0.19	0.08	0.22	-0.04	0.19	-0.28	0.03
Pinus	-0.03	-0.01	-0.07	-0.06	-0.03	-0.04	-0.09	-0.02	-0.07	-0.03	0.07	-0.03	0.05	0.04	0.04	-0.02
Larix	-0.05	-0.04	0.00	-0.01	-0.04	0.00	-0.06	0.03	0.02	0.03	0.08	0.03	0.00	-0.02	-0.06	0.04
Abies	-0.04	-0.04	0.03	-0.06	-0.08	-0.03	-0.02	0.02	-0.02	-0.01	0.05	-0.01	0.00	0.05	-0.06	0.07
Quercus1	0.05	0.07	-0.12	0.15	0.02	0.05	-0.04	-0.08	-0.16	-0.10	0.10	-0.10	0.03	0.06	0.22	0,00
Quercus2	0.03	0.00	0.03	-0.04	-0.02	-0.02	-0.04	-0.01	-0.05	0.02	0.06	0.02	0.04	0.03	0.10	-0.06
Fagus	-0.01	0.13	0.08	0.00	0.07	0.16	0.00	0.44	0.36	0.35	0.10	0.39	0.02	0.06	-0.23	0.00
Acer	-0.11	0.03	-0.03	-0.06	0.04	-0.01	-0.03	0.09	0.19	0.17	-0.12	0.16	-0.09	0.03	-0.16	-0.01
Alnus	-0.06	0.08	-0.10	-0.11	0.12	-0.06	0.04	-0.09	-0.14	-0.05	-0.18	-0.11	-0.12	0.03	-0.05	0.00
Tilia	-0.02	0.03	0.01	0.04	0.08	0.06	0.05	-0.01	-0.08	0.03	0.07	0.01	0.02	0.08	0.11	-0.03
Betula	-0.03	-0.04	-0.05	-0.04	-0.06	-0.05	-0.11	0.03	0.05	0.01	0.06	0.04	0.00	0.02	-0.10	0.01
Carpinus	-0.03	0.05	0.01	0.01	0.05	0.03	0.01	0.06	0.08	0.12	0.05	0.16	-0.03	0.07	0.13	0.03
Populus	-0.02	-0.01	-0.13	-0.01	0.02	-0.01	0.02	-0.06	-0.11	-0.07	0.02	-0.08	0.02	0.00	0.09	-0.02
Ulmus	0.03	-0.04	0.02	0.04	-0.09	-0.01	-0.02	-0.02	0.01	-0.01	-0.02	0.00	0.00	0.04	0.09	0.00

Supplement Table S1b. Correlation matrix of quantitative variables and tree species; partial correlation coefficients are shown; statistical significance ($P \leq 0.05$) is highlighted in grey

	Ash	Picea	Pinus	Larix	Abies	Quercus1	Quercus2	Fagus	Acer	Alnus	Tilia	Betula	Carpinus	Populus	Ulmus
Defoliation	0.09	-0.03	-0.03	-0.05	-0.04	0.05	0.03	-0.01	-0.11	-0.06	-0.02	-0.03	-0.03	-0.02	0.03
Age	-0.12	0.02	-0.01	-0.04	-0.04	0.07	0.00	0.13	0.03	0.08	0.03	-0.04	0.05	-0.01	-0.04
Stocking	0.06	0.07	-0.07	0.00	0.03	-0.12	0.03	0.08	-0.03	-0.10	0.01	-0.05	0.01	-0.13	0.02
Ash area	0.16	-0.17	-0.06	-0.01	-0.06	0.15	-0.04	0.00	-0.06	-0.11	0.04	-0.04	0.01	-0.01	0.04
Height	-0.10	0.04	-0.03	-0.04	-0.08	0.02	-0.02	0.07	0.04	0.12	0.08	-0.06	0.05	0.02	-0.09
Volume	0.02	-0.07	-0.04	0.00	-0.03	0.05	-0.02	0.16	-0.01	-0.06	0.06	-0.05	0.03	-0.01	-0.01
Site class	0.02	0.05	-0.09	-0.06	-0.02	-0.04	-0.04	0.00	-0.03	0.04	0.05	-0.11	0.01	0.02	-0.02
Hetero-geneity	-0.35	0.31	-0.02	0.03	0.02	-0.08	-0.01	0.44	0.09	-0.09	-0.01	0.03	0.06	-0.06	-0.02
Slope	-0.17	0.20	-0.07	0.02	-0.02	-0.16	-0.05	0.36	0.19	-0.14	-0.08	0.05	0.08	-0.11	0.01
SD slope	-0.28	0.19	-0.03	0.03	-0.01	-0.10	0.02	0.35	0.17	-0.05	0.03	0.01	0.12	-0.07	-0.01
TPI	-0.08	0.08	0.07	0.08	0.05	0.10	0.06	0.10	-0.12	-0.18	0.07	0.06	0.05	0.02	-0.02
SD TPI	-0.30	0.22	-0.03	0.03	-0.01	-0.10	0.02	0.39	0.16	-0.11	0.01	0.04	0.16	-0.08	0.00
Dist. to water	0.08	-0.04	0.05	0.00	0.00	0.03	0.04	0.02	-0.09	-0.12	0.02	0.00	-0.03	0.02	0.00
Dist. to ash	-0.27	0.19	0.04	-0.02	0.05	0.06	0.03	0.06	0.03	0.03	0.08	0.02	0.07	0.00	0.04
Tempera-ture	0.23	-0.28	0.04	-0.06	-0.06	0.22	0.10	-0.23	-0.16	-0.05	0.11	-0.10	0.13	0.09	0.09
Precipita-tion	-0.01	0.03	-0.02	0.04	0.07	0.00	-0.06	0.00	-0.01	0.00	-0.03	0.01	0.03	-0.02	0.00
Ash	1.00	-0.57	-0.18	-0.18	-0.12	-0.21	-0.16	-0.27	-0.17	-0.23	-0.12	-0.14	-0.13	-0.07	0.02
Picea	-0.57	1.00	0.02	0.05	0.08	-0.14	-0.06	0.01	-0.05	-0.12	-0.08	-0.04	-0.06	-0.08	-0.06
Pinus	-0.18	0.02	1.00	0.11	-0.01	0.05	0.02	-0.04	-0.05	-0.05	0.02	0.01	0.00	0.01	-0.02
Larix	-0.18	0.05	0.11	1.00	-0.01	-0.03	0.03	-0.02	-0.03	-0.06	0.01	0.00	0.03	-0.02	0.01
Abies	-0.12	0.08	-0.01	-0.01	1.00	-0.03	-0.01	0.05	-0.03	-0.05	-0.02	-0.02	0.00	-0.02	-0.02
Quercus1	-0.21	-0.14	0.05	-0.03	-0.03	1.00	-0.05	-0.08	-0.09	-0.10	0.07	0.01	0.07	0.00	0.05
Quercus2	-0.16	-0.06	0.02	0.03	-0.01	-0.05	1.00	-0.04	-0.06	-0.06	0.02	-0.02	0.11	-0.01	-0.02
Fagus	-0.27	0.01	-0.04	-0.02	0.05	-0.08	-0.04	1.00	0.01	-0.13	-0.06	-0.05	0.01	-0.05	-0.03
Acer	-0.17	-0.05	-0.05	-0.03	-0.03	-0.09	-0.06	0.01	1.00	-0.12	0.02	-0.03	0.03	-0.05	-0.02
Alnus	-0.23	-0.12	-0.05	-0.06	-0.05	-0.10	-0.06	-0.13	-0.12	1.00	-0.08	0.00	-0.02	0.05	-0.02
Tilia	-0.12	-0.08	0.02	0.01	-0.02	0.07	0.02	-0.06	0.02	-0.08	1.00	-0.02	0.10	0.02	0.02
Betula	-0.14	-0.04	0.01	0.00	-0.02	0.01	-0.02	-0.05	-0.03	0.00	-0.02	1.00	-0.02	0.03	-0.03
Carpinus	-0.13	-0.06	0.00	0.03	0.00	0.07	0.11	0.01	0.03	-0.02	0.10	-0.02	1.00	-0.01	0.01
Populus	-0.07	-0.08	0.01	-0.02	-0.02	0.00	-0.01	-0.05	-0.05	0.05	0.02	0.03	-0.01	1.00	-0.01
Ulmus	0.02	-0.06	-0.02	0.01	-0.02	0.05	-0.02	-0.03	-0.02	-0.02	0.02	-0.03	0.01	-0.01	1.00

Common Ash Stand Affected by Ash Dieback in the Wolica Nature Reserve in Poland

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Abstract

The ash stand in Wolica reserve (Poland), affected with ash dieback, was studied. Isolations performed from collected ash tissues and rhizosphere soil samples revealed 28 isolates of *Hymenoscyphus fraxineus* and 27 isolates of *Phytophthora* spp., respectively. The vitality and defoliation of 198 and 176 trees were studied, respectively in 2012 and 2013. In 2013 only one tree was completely vital, while 83 trees were within the degeneration phase. These results suggested that trees being classified in this class are the most vital and the natural genetic resistance should be sought among this vitality class in the future. In total, 112 trees were classified to the stage 2 of vitality, for which large deformation of shoots is typical. Further, monitoring of defoliation in 2013 revealed that the largest number of trees in the stand (126) were moderately damaged trees (defoliation 26-60%), while 47 trees had over 60% of defoliation. The synthetic damage index was 1.58 in 2012 and 1.66 in 2013 indicating that advanced disease processes are occurring in this stand. In addition, sampling, isolation, morphological and molecular identifications of *Phytophthora* species were performed. After the isolation tests, *P. megasperma*, *P.* sp. hungarica, and *P. plurivora* were obtained. These results were confirmed after the PCR and ITS sequencing. This is the first report of *P.* sp. hungarica and *P. megasperma* in the stands of common ash in Poland. The natural genetic variation of the *Fraxinus excelsior* genome was studied to improve understanding of its role in the adaptation and tolerance processes facing ash dieback phenomenon. Six nuclear microsatellite markers and four chloroplast microsatellite markers have been used in order to assess the genetic diversity of *Fraxinus excelsior* stand in Poland, categorized into three different Roloff' classes of vitality 0+1, 2 and 3. We demonstrated lack of correlation between three different vitality classes of ash trees and their nuclear or chloroplast genetic differentiation. Nevertheless, the observed heterozygosity (H_O) value was significantly different between vitality classes 2 and 3 assessed with nuclear SSR markers (p = 0.000183 in HSD Tukey test, $p < 0.05$). Also private (*A*p) alleles distribution of chloroplast SSR markers significantly differ (p = 0.000 in HSD Tukey test, $p < 0.05$) between the vitality classes 0+1 and 3 of ash trees. Those data suggest that DNA differentiation of *F. excelsior* at local spatial scale may be driven by gene based tolerance.

Keywords: Ash dieback, *Fraxinus excelsior*, defoliation, vitality, *Phytophthora* spp., nuclear and chloroplast SSR markers

Introduction

The phenomenon of dieback of European ash (*Fraxinus excelsior* L.) has been observed in the last decade in most European countries (Halmschlager and Kirisits 2008, Szabó 2009, Chandelier et al. 2010, Rytkönen et al. 2011, Husson et al. 2011). This new disease causing mass mortality of trees forced foresters to refrain temporary from cultivating ash as forest tree species, any more. For the first time the symptoms were observed in the early 1990s in North-Eastern Poland and Lithuania (Kowalski 2006, Timmermann et al. 2011). Since that time the disease spread across Europe causing severe mortality of ash trees. The research on ash dieback in Poland was conducted by many researchers but the findings of Professor Tadeusz Kowalski from the University of Agriculture in Cracow led to the discovery and identification of a new fungus species *Chalara fraxinea* Kowalski (Kowalski 2006) an asexual stage of *Hymenoscyphus pseudoalbidus* Queloz. It is accepted to be the cause of mass dieback of ash trees in Europe (Kowalski and Holdenrieder 2009 a, b). The currently recognized scientific name for the fungus causing ash dieback in Europe is *Hymenoscyphus fraxineus* (Baral et al. 2014). According to available data, the last country where *H. fraxineus* was recorded on common ash is Bosnia and Herzegovina (Stanivuković et al. 2014). Next to common ash, narrow-leafed ash was also reported to be susceptible on infections with *H. fraxineus* (Kirisits et al. 2010).

Parallel to studies of *Chalara* in ash dieback phenomenon, the appearance of pathogens from the *Phytophthora* genus in the rhizosphere soil of ash trees was connected with rot of root collars and decay and loss of fine roots, leading consequently to the dieback of many trees (Orlikowski et al. 2011, Akilli et al. 2013). In the last decade, many studies reported the occurrence of soil borne *Phytophthora* species in relation to the severe damage of significant forest tree species. The fungal- or -algae like pathogens of the genus *Phytophthora* are distributed worldwide (Erwin and Ribeiro 1996). Till now, over 140 *Phytophthora* species have been described from a broad range of plant hosts in agriculture and forestry (Abad 2014). Nevertheless, the knowledge about *Phytophthora* species associated with ash trees is still very limited, and there are few reports about *Phytophthora* isolations from ash trees (Orlikowski et al. 2004, Orlikowski et al. 2011). Also, the studies carried out by Orlikowski et al. (2007 a) have demonstrated that *Phytophthora* species were present in the rhizosphere of ash affected by the fungus *H. fraxineus*.

The Wolica nature reserve was established by virtue of the order of the Ministry of Forestry and Wood Industry, of the Government of Poland on August 4, 1984. The area is situated in central part of Poland in Forest District Chojnów (RDLP in Warsaw). Among the large number of different species in this reserve, including ecologically very valuable noble hardwoods, department 374c contains European ash dominated stand. The first symptoms of ash dieback in this stand were recorded during the year 1998. Since then, the number of trees with characteristic ash dieback symptoms has grown every year. This phenomenon continues until present. Also, this nature reserve of lowland forest ecosystem was known as an area to be free from alien plant pathogens of the *Phytophthora* genus.

Genetic structure can play an important role in determining fitness of forest tree populations at equilibrium in changing environmental conditions. Gene-based tolerance is the key in modern forest management actions, often examined at the DNA differentiation level of the individual tree in forest stand (Hamrick et al. 1992, Nguyen et al. 2015). Forest tree species have complex genomes, the biggest in size and still poorly understood in structure and function. Nevertheless, several types of molecular markers have been applied in population genetics studies including reproductive mechanisms, gene flow, hybridization processes or adaptive ecology. The nuclear microsatellite DNA loci are nowadays considered to be the most precise tool to determine the genotype of living organisms (Li et al. 2002, Esudero et al. 2003, Semagn et al. 2006). The microsatellite fragments (simple sequence repeats - SSRs) are built from short nucleotide repetitions, usually comprising from 2 to 9 base-pairs, uniformly distributed over the genome. They are characterized by high polymorphism and high mutation rates in comparison to coding sequences (Epperson 2005). Detailed research based on nuclear microsatellite markers for European ash populations was performed by Heuertz et al. (2003, 2004) excluding, however, the populations of ash from Poland.

Another type of markers, the chloroplast SSR loci were also applied to elucidate the genetic variation of many oak, beech or ash populations (Petit et al. 2002, Vornam et al. 2004, Heuertz et al. 2006). Interestingly, the transition zone between chloroplast microsatellite DNA haplotypes has been previously reported in Poland, proving the genotypic richness of the studied ash populations (Heuertz et al. 2003).

The positive correlation between the level of heterozygosity and the adaptation of population during the evolution of many organisms has been observed. The opposite (negative) trend may also occur, when the elimination of harmful alleles and inbreeding process leads to the impoverishment of a gene pool (Reed and Frankham 2003). The selection agents may favour homozygous alleles in the genome, but sometimes they could favour heterozygous alleles by choosing the genetic information suitable for the advantageous adaptive features of a population (Whitlock 2002).

Nevertheless, little is known about the health status of stands affected with ash dieback and about the ongoing

processes inside the declining stands, especially in the frame of searching for natural genetic resistance against the ash dieback (McKinney et al. 2014). It was recorded in many cases in Poland that stands after appearance of first symptoms and rapid spreading of the disease turn into a stagnation phase according to Roloff vitality classes (Pacia et al. 2011). The application of molecular tools to study the relationship between European ash dieback and natural genetic tolerance/susceptibility has been fairly limited. It seems to be crucial that dead ash trees have to be replaced with more tolerant genotypes before this species completely disappear from forest ecosystem. In this scope, the predictive methodology based on molecular markers may help in successful management of European ash disease mainly caused by pathogenic fungus *H. fraxineus*. Our studies were based on general hypothesis that greater variety of gene pool assessed with SSR markers is positively correlated with vitality class of Roloff among sixty investigated ash trees in Wolica Natural Ash Reserve in central Poland.

Due to the lack of silviculture measurements, which have never been applied in Wolica reserve (Chojnów FD), a study was performed with aims to: i) determine the health status of this ash stand (expressed by defoliation and vitality degrees of trees), ii) confirm the presence of known fungus *H. fraxineus* responsible for ash dieback iii) monitor the occurrence of soil-borne pathogens from the *Phytophthora* genus in the rhizosphere soil of damaged trees, iv) to perform genetic studies on both nuclear and chloroplast SSR markers with the interaction to Roloff vitality classes.

Material and methods

Monitoring of healthiness of ash trees

The study area and inventory of tree health

The stand of ash trees, where the research was conducted is located in the partial nature reserve (coordinates 51.1°N, 10.5°E) in Wolica in central Poland. The total area of the reserve is 50.39 ha, including the separated stand of ash (374c) which makes the object of the research area of 1.77 ha.

Sampling, isolation and identification of H. fraxineus from symptomatic ash tissues

Sampling, isolation and identification were done in 2012, according to the methodology of Kowalski (2006). Samples with symptomatic tissues were collected over the whole stand area from 30 chosen 22-year-old ash trees (2-3 samples per tree). Tissue samples were collected from infected stems and shoots up to 3 m high (of 20 m high trees), by cutting and placing them in separated plastic bags, immediately subjected to the laboratory analysis. Isolation was done in the way that 3-4 mm big pieces were taken between necrotic and healthy areas, using the scalpel sterilized in 70% ethanol and burned on the open flame. Pieces were surface sterilized for 3 minutes in 1% sodium hypochlorite with 4% active chlorine, three times washed in sterile distilled water and plated placed onto Malt Extract Agar media-MEA (18 g/l of Malt Extract (MERCK, Germany), and 18 g/l of agar (BTL, Poland). Petri dishes were incubated at 18-20°C in the dark, and observed daily for the presence of hypha. After appearance of first hypha, they were transferred with sterile mycological needle onto fresh MEA media and incubated at ~20°C in the dark.

Four-weeks-old cultures, incubated at ~20°C in the dark, were observed under the light microscope ZEISS Axioskop 2, equipped with Nikon Ds-fi1 camera, and NIS Elements AhR4® software. In parallel, pure cultures were incubated for four weeks on the MEA media for the purposes of colony shape patterns.

Identification of isolates was done based on the colony shape of pure cultures, incubated for four weeks in the dark at 20°C, the shape and size of phyalides observed in the pure cultures and based on previously registered symptoms. Not to rely solely on morphological characters the molecular based methods were employed for the confirmation of the presence of *H. fraxineus.*

Vitality test based on the extent of trees damage

The vitality test was performed using the method developed by Roloff (1989), in which the vitality is shown as tree growth potential and its ability to regenerate the damaged crown. The basis for the assessment of the vitality is the architecture of shoots produced in the upper part of the crown. This assessment takes into account not only the current condition but also the changes visible in the form of shoot deformation as an effect of ongoing processes over past several years. The vitality assessment of ash trees in department 374c was conducted in May 2012 and 2013. The analyses of vitality and defoliation were conducted by the same observer to minimize subjectivity and biases, and that is why the calibration was not necessary. All the trees were classified into one of four groups distinguished on the basis of differences in vitality (Table 1).

Table 1. Vitality degrees (Roloff 1989)

Vitality degree	Damage level
0	Exploration phase, untouched trees, vital ones
1	Degeneration phase, weakened trees
2	Stagnation phase, damaged trees
3	Resignation phase, badly damaged trees, decaying

Assessment of defoliation

The assessment of crown defoliation of ash trees was carried out in May 2012 and 2013. It was performed according to the criteria ICP Forests protocol shown in Table 2. In our experiment the defoliation was estimated as

the lack of leaves in the canopy at the beginning of the vegetation season in order to avoid damage caused by insects or to be attributed to premature shedding as a result of *H. fraxineus* activity and in this way affecting on our evaluation. Example of ash tree growing in Wolica reserve, described as defoliation 40% (ICP Forests 26-60%) and according to Roloff 2 vitality class (stagnation phase) is shown in Figure 1.

Table 2. Defoliation degrees according to the damage scale

Defoliation (%)	Damage level
0 - 10	0 (no symptoms of damage)
11 - 25	1 (slight damage)
26 - 60	2 (moderate damage)
above 60	3 (strong damage)

Figure 1. Example of ash tree growing in Wolica reserve, described as defoliation 40% (ICP Forests 26-60%) and according to Roloff 2 vitality class (stagnation phase)

The synthetic damage index of trees

Based on the obtained data concerning defoliation and vitality the synthetic damage index of trees was calculated (Dmyterko et al. 2003). Compared to the assessment of vitality and assessment of defoliation applied separately, the synthetic damage index allows for a more objective assessment of the health of trees and stands (Dmyterko 1998). The synthetic damage index was calculated according to the method of Dmyterko et al. (2003), using the following formula:

[1] Syn = 1/2 (0.03 * Def + Wit)

where *Def* – defoliation is expressed in percentage, *Wit* - tree vitality is expressed by the degree of damage. The damage index for the whole separation was achieved following the methodology of Dmyterko (1998):

[2] Syn_wydz = ½ [(0.03 * total sum Def + Wit) / N],

where the *Def* sum - the sum of the percentage of defoliation, *Wit* - the sum of degrees of vitality, *N* - number.

In addition, for assessment of vitality and defoliation, the photographic documentation of the trees was collected from the points which allowed clear separation of crowns from the background of neighbouring trees.

Sampling, isolation and morphological identification of Phytophthora species

Sampling and isolation of pathogens from the *Phytophthora* genus were performed according to the methodology of Jung (2009) and Jung et al. (1996, 2000). Soil and fine roots were collected in the form of soil monoliths ~25×25×25 cm, and two monoliths per tree were taken at the distance of 0.8-1 m from the tree base, and mixed before the isolation. In total, nine symptomatic trees were randomly chosen and sampled from different parts of the stand. Isolation tests were performed using the method of bait (Jung et al. 1996, 2000, Jung 2009), and young pedunculate oak and beech leaves were used as baits. After appearance of the first necrotic spots on the leaves the small pieces of damaged plant tissues were cut with a sterile scalpel and placed onto selective agar media (V8A PARPNH) (Jung at al. 1996, 2000, Jung 2009). The first hypha was taken and transferred on the fresh V8 agar media.

The obtained isolates were morphologically characterized by flooding the pieces of young colonies, grown on CA media, in the non-sterile soil extract, and with observation of four-weeks-old cultures, incubated at 20-22°C in the dark (Erwin and Ribeiro 1996). Sexual and asexual structures were observed at ×400 magnifications using the ZEISS Axioskop 2 microscope, and 50 structures per observed isolate were measured using the Nikon Ds-fi1 camera and NIS Elements AR 4® software. Observed features were compared with identification keys (Stamps et al. 1990, Erwin and Ribeiro 1996), as well as with papers and reports with recently described species (e.g. Jung et al. 1999, 2002, 2003, Brasier et al. 2003, Jung and Nechwatal 2008, Jung and Burgess 2009, Bakonyi et al. 2012).

Molecular identification of obtained Phytophthora spp. isolates

After the detailed morphological classification, small pieces from the edges of young colonies were transferred on liquid V8 media (900 ml/l of distilled water, 100 ml/l of V8 juice (Tymbark, Poland), 3 g/l $CaCO_3$), and incubated at 22-25ºC in the dark. After 3-5 days of incubation, the mycelium was collected and smashed in liquid nitrogen, after washing in sterile distilled water. The DNA was extracted by using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich® GmbH, Germany), according to the company recommendations. ITS amplifications of *Phytophthora* isolates were performed using the ITS4 and ITS6 primers (White et al. 1990, Cooke et al. 2000). The amplification reaction mixture contained 1 x PCR buffer [75mm Tris-HCl (pH 9.0), 50mM KCl, 20 mM $(NH_4)_2$ SO4]; 1×Q solution, 0.2 mM dNTPs, 0.25 mM of each primer; 1 mM $MgCl_2$; 1U of *Taq* Polymerase (Qiagen Ltd., Valencia, CA, USA); and 1 µL of mycelial DNA in a total volume of 25 µL. Reactions were performed in PTC-200™ Programmable Thermal Controller (MJ Research, Inc.) machine, and PCR protocol was as follows: 3 min of initial DNA denaturation at 94°C and 35 cycles of amplification (30 sec of annealing at 55°C, 60 sec of elongation at 72°C), and 5 min of final elongation at 72°C.

Amplified products were analyzed by 1.5% TBE-agarose gel electrophoresis, stained with 6×Orange DNA Loading Dye, and visualized under a UV transilluminator. The presence of a single band (ca. 800 bp) was considered as a positive reaction. The PCR products were cleaned using the A&A Biotechnology (Gdynia, Poland) Clean-up kit, following the manufacturer's protocol. Sequencing was conducted on CEQ™8000 9.0.25 automated sequencer (Beckman Coulter®, Fullerton, USA), using ITS4 and ITS6 primers. The consensus sequences were aligned from two-directional sequencing (Zhang et al. 2000), and compared with sequences in GenBank (http://blast.ncbi.nlm.nih.gov /Blast.cgi) using the ClustalW program (Thompson et al. 1994) and MEGA6 software (Tamura et al. 2013).

Correlation between the DNA differentiation of ash trees and vitality classes in Wolica Reserve

Population differentiation was determined at a local spatial scale in Wolica Ash Reserve, where three different health classes among sixty ash trees were examined, i.e. class 0 and 1 – 0-25 % of defoliation (represented by 23 trees), class 26 – 60 % of defoliation (21 trees) and class above 60 % of defoliation (16 trees, respectively).

From all investigated trees total DNA was extracted using the NucleoSpin® Plant II (Macherey-Nagel), following the manufacturer's protocol except some modifications in volume: 600 µl of PL2, 150 µl of PL3 and 900 µl of PC buffer. Six nuclear microsatellite and four chloroplast loci in two multiplexes (A and B) were amplified. The multiplex A consisted in five loci: Femsatl-4, Femsatl-8, Femsatl-19, ccmp3, ccmp6; and multiplex B in five loci: Femsatl-11, Femsatl-16, M2-30, ccmp7, ccmp10 (Sutherland et al. 2010). The Multiplex PCR Kit (Qiagen®) was used for both multiplexes preparation and the PCR reactions were carried out in 10 µl reaction volumes with 5–50 ng DNA, 10 pmol of each primer labelled with fluorescent WellRED dyes (Beckman Coulter, Inc.), 5 µl Multiplex PCR Master Mix, followed by the PCR thermal amplification profile described by Sutherland et al. (2010). All samples were genotyped and the allele lengths were scored on a CEQ 8000 sequencer (Beckmann-Coulter, Inc.).

The observed (n_a) and expected (n_e) number of alleles, observed (H_E) and expected heterozygosity (H_O), mean Shannon index (I), general heterozygosity (h), inbreeding coefficient of an individual relative to the subpopulation (F_{IS}), inbreeding coefficient of an individual relative to the total (F_{IT}), and fixation index (F_{ST}) were calculated in GenAlEx 6.501 software (Peakall and Smouse 2012). The frequency of private alleles (Ap) was determined for the unique alleles present only once in analysed vitality classes of trees.

For nuclear SSR markers, F statistics was conducted with ANOVA, and the model-based clustering method with STRUCTURE 2.1 software (Pritchard et al. 2000). STRUCTURE assigned individual multilocus genotypes probabilistically to a defined number K from 2 to 3 clusters or gene pools, and was run three times for 100 000 iterations after a burn-in period of 50 000 on the total dataset (n = 60).

The test of departure from Hardy–Weinberg equilibrium was chosen for $P = 0.05$. Gene flow among groups of trees was estimated from F_{ST} using the formula $N_m = 0.25(1/ F_{ST} - 1)$ (where N is effective population size and m is a fraction of migrants per generation).

For both types of markers, the Pearson coefficient (r) of correlation between genetic parameters, i.e. n_a, n_e, I, H_O, H_E and Ap and their distribution in three groups of trees (representing classes: 0 and 1, 2 and 3) was analyzed in STATISTICA ver. 10.

Results

Monitoring of healthiness of ash trees

Registered symptoms, isolation and identification of Hymenoscyphus fraxineus

Different symptoms were recorded on ash trees in this stand, including increased crown transparency, wilting and premature shedding of leaves, dying of shoots and branches, dieback of the top of the crowns, decline of trees, and necrosis and canker on the stems, branches and shoots. The asexual stage of *H. fraxineus* (*Chalara fraxinea*) was isolated from 27 out of 40 plated, randomly chosen sam-

ples, taken from the edges of necrotic zones and surface sterilized as described above. In total, 28 isolates were obtained.

Most of the isolates were of white to light orange colony color, after four weeks incubation at 20°C in the dark, and with hyaline, slightly immersed. Also, few isolates had an intensive orange to brown color of the colonies. Color from the bottom side varied from light orange, light brown to, less often dark brown. Growth of the colonies was slow, and after three weeks at 20°C in the dark on MEA media, total colony diameter averaged 24.4±2.22 mm, with range of 21-28 mm (N=5).

In the four-weeks-old cultures, incubated in the dark at 20°C, numerous phialides were observed on both terminal hypha and on branched or simple phialophores. Phialides were subcylindrical, obclavate and less often spindle shaped, with average total length of 19.7±3.42 µm (range 14.3-25.6 µm). Width of ventral part of phialides averaged 4.4±0.41 µm, with range 3.7-5.3 µm. Phialoconidia were observed in chains, or they were dispersed in droplets. Phialoconidia were hyaline, unicellular, with smooth walls and cylindrical shape, and with one or two oil droplets.

Vitality assessment

The assessment of vitality was performed for 198 ash trees in 2012 and for 176 in 2013. In 2012, only one tree was classified as class 0 – intact - vital class of trees. The trees weakened in the degeneration stage (class 1) included 83 trees (42%). The largest number of trees (100 trees, 51%) was classified into the second class of vitality - trees damaged in a phase of stagnation. The third class (resignation phase, trees badly damaged and dying) included 14 ash trees (7%) (Table 4). In 2013 the vitality dropped in class 1 and 2 reaching values 63 and 92 trees, respectively. In class 3 the number of trees increases from 14 to 20 trees. The obtained results indicate going on long-term stress impact, lasting perhaps even more than a decade.

Assessment of defoliation

The assessment of defoliation was carried out for 198 ash trees in 2012. There were no trees being intact (the degree of defoliation of 0-10 %). Most trees (126 trees) belonged to the range of 26-60 % of defoliation. A large number of trees belonged to defoliation level above 60% (47 trees). Trees slightly damaged (range 11 - 25%) included 25 ash trees. The number and percentage of trees in each class is shown in Table 3.

In 2013, only further negative changes in the health of the examined trees were noticed. The number of healthy trees has been further reduced (Table 3). Of the 198 trees 22 died and 176 left showing different level of crown damage. The year 2013 proved further developing of the disease process.

Table 3. Number of trees representing defoliation classes expressed in percentage in the year 2012 and 2013

Defoliation (%)	2012		2013	
	Number of trees	(%)	Number of trees	(%)
0-10	0	0	0	0
11-25	25	13	16	9
26-60	126	63	121	69
above 60	47	24	39	22
Total	198	100	176	100

Table 4. Number of trees representing vitality classes expressed as percentage of trees in the years 2012 and 2013

Degree of vitality	2012		2013	
	Number of trees	(%)	Number of trees	(%)
0	1	0	1	1
1	83	42	63	36
2	100	51	92	52
3	14	7	20	11
Total	198	100	176	100

Synthetic damage index

The calculated values of the synthetic damage index for each tree are included in the range from 0.8 (the lowest value) to 2.7 (maximum value), with the theoretical, possible range from 0 to 3. The modification of the model for the determination of synthetic damage index for a single tree allowed for the calculation of the synthetic damage index for the entire stand. The value of this index amounted to 1.58 in 2012 and 1.66 in 2013. The obtained result indicates advancing disease processes occurring in the examined stand.

Isolation and identification of Phytophthora species

After the isolation tests, seven out of nine, randomly taken samples, were positive for the presence of *Phytophthora* species, and 27 isolates were obtained. All the isolates were obtained from rhizosphere soil using the baiting techniques. The highest isolation frequency was from samples number 5 and 9, respectively. Positive and negative samples and number of obtained isolates are shown in Table 5.

After the morphological identification, three different species were identified: *P. plurivora*, *P. megasperma* Drechsler and *P.* sp. hungarica (Table 5). *P. plurivora* and *P. megasperma* are well described species in the literature, but little information was available about *P.* sp. hungarica. The species is homothallic with mycelium slightly sparse aerial in the middle and pressed in the edges. Colony was with shape of rosette and with regular edges on V8, CA, and irregular on MEA and PDA.

Table 5. Number of obtained isolates from different samples taken

Isolated species and number of obtained isolates	Sample Number									Total
	No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	
P. plurivora	1	-	1	-	8	3	3	3	3	22
P. megasperma	-	-	-	-	-	-	-	-	3	3
P. sp. hungarica	-	-	-	-	-	-	-	-	2	2
Total	1	-	1	-	8	3	3	3	8	27

Table 6. BLAST analyses of sequenced ITS region for selected isolates

No of Sample	Isolate	Species	GenBank Access Number	The closest sequence in the GenBank	Identities (%)	Query cover (%)	E-value
1	IBL277	*P. plurivora*	JX274427	GU259247	100	100	0.0
2	IBL280	*P. plurivora*	JX274426	KM052583	100	100	0.0
3	IBL282	*P. plurivora*	JX274421	KM052583	100	100	0.0
4	IBL287	*P. plurivora*	JX274420	KF234680	100	100	0.0
5	IBL290	*P. plurivora*	JX274424	EU240194	100	100	0.0
6	IBL293	*P. plurivora*	JX274425	KM052583	100	100	0.0
7	IBL294	*P. plurivora*	JX274422	KM052583	100	100	0.0
8	IBL297	*P. megasperma*	JX274423	HM004230	100	100	0.0
9	IBL301	*P.* sp. hungarica	JX274428	EF522144	100	100	0.0
10	IBL302	*P.* sp. hungarica	JX274429	EF522144	100	100	0.0

Species produced big oogonia with average dimensions of 34.38±3.64 × 34.31±3.63 µm, and range of 27.10-45.30 × 26.58-43.80 µm (N=53). Size of antheridia averaged 15.0±3.40 × 10.5±2.20 µm and range 8.0-22.5 × 5.82-17.3 µm (N=32), and they were mostly paragynous with only few amphigynous recorded. Oospores were aplerotic, and few were plerotic with average dimensions 26.7±3.21 × 27±3.04 µm, and range 20.31-33.82 × 19.88-33.5 µm (N=51). Oospore wall thickness ranged from 0.8-3.3 µm, with mean of 1.84±0.89 µm. Sporangia produced in non-sterile extract were nonpapillate, persistent and with regular, ovoid to obpyriform shape. Dimensions of sporangia were 48.2±6.17 × 31.2±2.95 µm, with range 32.23-62.31 × 24.73-37.35 µm (N=55). Length to breadth ratio averaged 1.55±0.19, and range 1.11-1.94. Growth rate at 20°C on CA media was 3.52±0.24 mm/day. The species belongs to ITS clade 6, *sensu* Cooke *et al*. (2000), and it was identified as *Phytophthora* sp. hungarica. Presence of this species, as well as the presence of *P. megasperma* and *P. plurivora* was confirmed during the molecular identification and ITS sequencing (Table 6).

Used ITS4 and ITS6 primers successfully amplified ITS region of selected isolates, and ~700-1100bp products were obtained, respectively. In total ten isolates were sequenced, seven from *P. plurivora*, two from *P.* sp. hungarica and one isolate from *P. megasperma*. After BLAST analyses of obtained sequences, identity of all the obtained sequences with the closest sequence in the GenBank was 100%, with 0% of gaps and Expect value, respectively (Table 6). Sequences were submitted to the GenBank and assigned accession numbers are shown in Table 6.

This is the first report of *P. megasperma* and *P.* sp. hungarica on *Fraxinus excelsior* in Poland.

Correlation between the DNA differentiation of ash trees and vitality classes in Wolica Reserve

Allele frequency

Among highly polymorphic nuclear microsatellite markers selected for this study, the most variable was locus M2 (27 alleles), while the least polymorphic was locus F16 (5 alleles). Among four chloroplast SSR loci examined, the highest variation was observed in ccmp6 and ccmp10 loci (with 5 different haplotypes), and the lowest in ccmp3 locus (2 haplotypes).

Private alleles were present both in nuclear and chloroplast DNA loci, with prevalence in number of 55 alleles in 5 loci in highly polymorphic nuclear SSR markers in comparison to 5 alleles in 3 chloroplast loci examined.

Genetic variation

A summary of genetic variation measures for six nuclear and four chloroplast SSR loci was given in Tables 7 and 8, respectively. Concerning the nuclear SSR marker variation, the highest numbers of observed (n_a) and expected (n_e) alleles per locus, as well as the highest diversity coefficient of Shannon (I) were found in ash trees belonging to the vitality class 0+1. Conversely, the parameters n_a, n_e and Shannon index (I) had the lowest values in the most damaged class 3 of ash trees (Table 7).

The most heterogeneous trees examined with nuclear SSR markers belonged to the Roloff class 2 (H_E = 0.731)

Table 7. Genetic differentiation parameters based on nuclear SSR markers in studied ash trees

Vitality class	Genetic parameters of nSSR variability							
	n_a	n_e	I	H_O	H_E	F_{IS}	F_{IT}	F_{ST}
0+1	6.611	4.915	1.571	0.694	0.711	0.083	0.128	-
2	6.000	4.544	1.557	0.643	0.731	0.163	0.228	-
3	5.111	3.837	1.373	0.787	0.685	-0.127	-0.006	-
Total:	11.611	6.188	1.954	0.694	0.775	0.142	0.160	0.020**

n_a and n_e, observed and expected number of alleles; *I,* mean Shannon index; H_O and H_E, observed and expected heterozygosity; F_{IS}, inbreeding coefficient relative to the subpopulation; F_{IT}, inbreeding coefficient relative to the total; F_{ST}, fixation index. **Significant deviation from 0: $p < 0.05$

and class 3 (H_O = 0.787). Total mean genetic value of observed heterozygosity was lower than the expected one, which is in accordance with general observations made for different forest tree populations across Europe, e.g. *Fraxinus excelsior* (Heuertz et al. 2004), *Pinus sylvestris* (Kosińska et al. 2007), *Picea abies* (Nowakowska 2009) and *Quercus petraea* (Kremer et al. 2002).

All groups of trees had similar level of genetic differentiation, according to F_{ST} value indicating only 2% differences between vitality classes. The inbreed coefficient F_{IS} and F_{IT} suggest 14.2% and 16.0% of heterozygosity losses respectively between all classes of vitality (Table 7).

Concerning the chloroplast SSR marker variation, the highest observed numbers of alleles per locus (n_a) was found in ash trees belonging to the vitality class 2. Conversely, the highest parameters of: expected number of alleles (n_e), Shannon index (*I*), Nei's (1973) heterozygosity (*h*) and private allele content (*A*p) characterized the most damaged class 3 of ash trees. Because the trees from the class 2 of Roloff had no private allele, their genetic variability was lower than in class 3 and 0+1 (Table 8).

Based on analysis of molecular variance (ANOVA) among trees assessed with nuclear SSR markers, no pairwise Pearson correlation (r = -0.11 – 0.49) was observed among genetic parameters and tree classes of trees (Table 9). The observed heterozygosity (H_O) value was only significantly different between classes 2 and 3 (p = 0.0332 in HSD Tukey test, for p value < 0.05). The other genetic parameters (n_a, n_e, H_E, I and F) showed no statistically significant differences between different classes of trees (p > 0.05).

Similarly, no pairwise Pearson correlation (r = -0.12 – 0.51) was observed among genetic parameters and tree classes of trees assessed with chloroplast SSR markers (Table 9). The only significant statistical differences were observed between private allele presence (*A*p) in the different class of vitality (p = 0.0001 in HSD Tukey test, for p value < 0.05).

Table 8. Genetic parameters differentiation based on chloroplast SSR markers in studied ash trees

Vitality class	Genetic parameters of cpSSR variability				
	n_a	n_e	I	h	*A*p
0+1	2.750	2.151	0.822	0.503	0.178
2	3.000	1.870	0.776	0.459	0.000
3	2.750	2.257	0.854	0.534	0.429
Total:	2. 833	2.093	0.817	0.499	0.607**

n_a and n_e, observed and expected number of alleles; *I,* mean Shannon index; *h*, Nei's (1973) heterozygosity; *A*p, private allele content. *Significant deviation from 0: $p < 0.05$

Table 9. Statistic correlations between vitality classes of trees and genetic parameter values based on Pearson *r* coefficient, N=60, *p* value < 0.05

	nuclear SSR markers					
	n_a	n_e	I	H_O	H_E	F
Vitality Class	0.337455	0.489680	0.271914	-0.114483	0.034685	0.088720
	chloroplast SSR markers					
	n_a	n_e	I	h	*A*p	-
Vitality Class	-0.026841	-0.055929	-0.124202	-0.124202	0.505501	-

n_a and n_e, observed and expected number of alleles; *I,* mean Shannon index; H_O and H_E, observed and expected heterozygosity; *F*, ANOVA correlation coefficient.

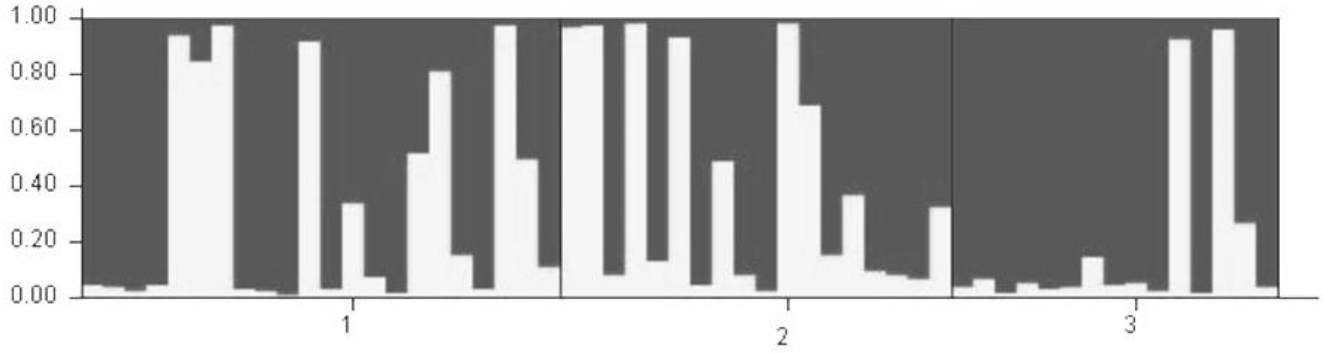

Figure 2. Clustering analysis between three vitality classes of ash trees and their genotypes based on nuclear microsatellite marker data in K-structure algorithm

Surprisingly, the most healthy class of trees (0+1) was not statistically different in any genetic parameter (based on nuclear or chloroplast type marker data) from the other classes.

Genetic differentiation and gene flow

It is generally considered that a value of $N_m > 1$ characterises large gene flow within a given population (Slatkin and Barton 1989). In our investigation, the value of $N_m = 24.72$ obtained indicates a high gene flow between investigated classes of trees.

No particular grouping of investigated ash tree genotypes was revealed by STRUCTURE clustering analysis proving that there is no relationship between the vitality class and genetic structure of a single tree (Figure 2).

Discussion

The examined stand of ash trees is in an advanced disease stage. Different symptoms, indicative for infections with the aggressive and invasive fungus *H. fraxineus* were recorded. After isolation tests, the presence of this pathogen was confirmed and 68% of plated tissue samples were positive for the presence of *H. fraxineus*, what corresponded to the previously published data (Kowalski 2006).

In the Forest District of Chojnów the separated areas with a dominant ash tree amount to 16.85 ha, which accounts for only 0.2% of the total forest area of this Forest District. In the last 10 years within the sanitary cuts more than 500 m^3 of ash timber was obtained, mainly dying trees (according to SILP - State Forests Information System). Since 2000, *Fraxinus excelsior* has not been introduced to forest plantations and its production in nurseries has not been continued. If the situation does not change, probably within the next few years ash species is likely to lose its dominant position on still such a small area. In the Wolica reserve the mortality of trees is high, when we started to count and permanently mark all ash trees in autumn 2011 there were 230 specimens. In 2012 in May when evaluating defoliation and vitality only 198 trees remained and the next year we counted 176 trees, which stayed alive.

Currently, ash dieback is one of the most important phytosanitary problems in Europe (Queloz et al. 2011). However, *H. fraxineus* was present in some areas (e.g. Switzerland) for a long time before the outbreak of the disease (Engesser et al. 2009). After the contamination it might take some years for building up of inoculums and the detection of first symptoms. This probably explains why the ongoing devastating epidemic, particularly on *Fraxinus excelsior*, is still developing.

Concerning the role of particular organisms in ash dieback phenomenon, next to *H. fraxineus* some authors suggested the role of *Phytophthora* species in complex of factors of ash dieback phenomenon (Orlikowski et al. 2011, Akilli et al. 2013). During the research carried out in the Forest District Chojnów in the reserve Wolica the incidence of three species from the *Phytophthora* genus was confirmed, namely, *P. plurivora*, *P. megasperma* and *Phytophthora* sp. hungarica. Their role in the process of dieback is under investigation. Especially, *P. plurivora* is a species highly damaging fine roots of broad-leaved trees, as well as the tissues of hosts belonging to several different genera, including ashes (Jung and Burgess 2009, Orlikowski et al. 2011).

The other two species are new in Poland and found for the first time in ash stands in general as well as little studied. Namely, *Phytophthora* sp. hungarica was previously isolated from soils under the alder trees (Bakonyi et al. 2012), and the role of this species in different syndromes of forest trees decline is little studied. On the other hand, *P. megasperma* was reported as a pathogen of *Malus pumilla* Mill. and *Brassica* spp. (Erwin and Ribeiro 1996). Also, this species was recently reported as being involved in Walnut trees (*Juglans regia* L.) decline in Italy (Belisario et al. 2012). However, the role of these two species in ash decline phenomenon need further research, particularly having in mind that they were isolated from only one out of nine collected rhizosphere soil samples in this stand. The

role of these pathogenic organisms in the decline of observed ash stand in Wolica reserve should be tested through the pathogenicity tests. Because of the fact that their direct impact in ash decline phenomenon was not confirmed in several other studies (Bakys et al. 2009, Schumacher et al. 2010, Husson et al. 2012) the future field surveys in this respect in other declining ash stands in Poland are required. There is also a room for an alternative hypothesis e.g. that these *Phytophthora* species have been detected because we have looked for them, but we could also probably have detected them in ash stands before the arrival of the dieback. However, species from the *Phytophthora* genus were recognized as frequent pathogens in forest and ornamental nurseries from where they could be introduced to newly established plantations and planted forests (Brasier and Jung 2006, Pérez-Sierra and Jung 2013). This is the possible scenario of origin of detected *Phytophthora* species in this ash stand, established in the Wolica reserve c.a. 20 years ago. Water is also a very important source of inoculum of these pathogenic organisms, including both the water from irrigation reservoirs and natural streams (Orlikowski et al. 2007 b, Hulvey et al. 2010, Reeser et al. 2011).

The shoots of examined ashes were infested with *Chalara fraxinea* (anamorph of *Hymenoscyphus fraxineus*) that is why in a further stage of the research it is planned to determine the relationship between the incidence of the fungus *C. fraxinea* attacking stems of trees, and fine root pathogens of the genus *Phytophthora*. Efforts will be also taken aiming at awakening the resistance of trees to these pathogens through the use of preparations containing phosphites. Similar tests were completed successfully in Australia, where they are applied to a large scale against phytophthoras (Jackson et al. 2000, Wilkinson et al. 2001, McCarren et al. 2009), but not yet against *H. fraxineus* in the affected areas.

In addition to the fact that some other studies (e.g. by Schumacher et al. 2010) failed to detect *Phytophthora* in dying ash stands, Husson et al. (2012) suggested *H. fraxineus* as a cause of collar rots on ash trees. Moreover, Enderle et al. (2013) reported the frequent presence and isolation of *Armillaria gallica* Marxm. and Romagn from cankers at the stem base of declining ash trees, and previously Skovsgaard et al. (2010) reported association between the disease and the symptoms of *A. gallica*. It is, however, common that secondary pathogens affect weakened trees, and Enderle et al. (2013) suggested that recorded collar rots on ash trees could be possibly caused by both *Armillaria* species and *H. fraxineus*. However, presence of *Armillaria* species in the necrotic, as well as in asymptomatic tissues close to observed cankers was confirmed in this stand using molecular tools (Oszako, unpublished data), but in the isolation trials we failed to obtain *Armillaria* spp., and future field surveys and isolation trials are required.

One of the most important challenges in silviculture and management of the stands affected with ash dieback is infection spreading and post infection stand status, in the perspective of planning of future measurements. The obtained results showed that the most of the trees (112) were judged to belong to the stagnation phase (stage 2 of the vitality), for which large deformations of shoots is typical. This phase is characterized by slow growth and mainly shoots in clusters that grow in the crown. This fact is confirmed by the results of defoliation, which is a commonly used criterion for assessing the degree of damage to trees and stands. It was found that the largest share in the stand constituted moderately damaged trees (defoliation 26-60%).

Obtained results are very important in the perspective of searching for natural resistance against invasive *H. fraxineus* (McKinney et al. 2014). However, the natural resistance to the ash dieback disease was recorded in several studies in Denmark and Sweden (McKinney et al. 2011, 2012, Kjær et al. 2012, Stener 2013), and the presence of genetic variations of dieback resistance was proved through the progeny inoculation studies (Lobo et al. 2015). Based upon this, presence of highly resistant, not affected trees is very important. Also, and their resistance should be checked via large scale pathogenicity-provenance tests.

Many diversity conservation programs and the forest tree management require the detailed knowledge of the genetic distribution of endangered species, like *F. excelsior* suffering from ash-dieback phenomenon. The richness of the gene pool of each population is basically determined by multilocus allele occurrence in the genome (Escudero et al. 2003).

Our results showed that genetic variation of declining ash trees in the Wolica Reserve was not significantly correlated with the defoliation rate of ashes. The microsatellite DNA-based research on genetic variation in dieback resistance in *Fraxinus excelsior* in Denmark also proved no correlation between inheritability of the Femsatl loci and susceptibility of trees measured by necrosis development due to *Chalara fraxinea* artificial infection, which is consistent with the neutral nature of the SSR markers (Lobo et al. 2015). But the results obtained from the analysis of 60 ash trees from Wolica Reserve naturally subjected to *H. fraxinea* infection indicate that there is a little 2% variation between three Rollof's classes examined, with one significant difference in observed heterozygosity value between the classes 2 and 3. The highest observed heterozygosity in class 3, suggests the highest differences in investigated microsatellite loci among the most damaged trees comparing to other classes. Nevertheless, more studies with bigger number of investigated ashes displaying signs of dieback is needed to confirm this observation.

Total genetic differentiation level of ash trees from Wolica Reserve based on nuclear SSR markers was medium (H_O = 0.694, H_E = 0.775), and comparable to another

studies performed for this species (Yazdani et al. 2003). Previous studies based on nuclear and chloroplast DNA markers showed similar level of genetic variation (G_{ST} = 0.198) in Polish ash populations to the level of G_{ST} = 0.110 found in Angiosperms (Nowakowska et al. 2004, Hamrick 1992). The observed in present study significantly different heterozygosity (H_O) value only between classes 2 and 3 may suggest some genetically based differences between the DNA variation and the degree of damages. Moreover, high gene flow observed among all classes of trees for nuclear SSR markers (N_m = 24.72) may indicate favourable condition for combination of alleles, guaranteeing the survival and adaptation to changing environmental conditions (Reed and Frankham 2003).

In Poland and Lithuania, the preliminary assessment of genetic variability of the population of 13 European ash using RAPD analysis was carried out, but these studies have not assumed correlation analysis of molecular data with the data on the degree of dieback stands (Nowakowska et al. 2004). Detailed studies on the identification of SCARs markers associated with resistance of Polish ash provenances to infection caused by *Hymenoscyphus fraxineus* showed some genetic differences between individuals resistant and susceptible to infection, but the incidence of those molecular markers failed to allow unequivocal identification of the trees resistant throughout the studied populations (Kowalski 2012).

On the other side, the chloroplast SSR markers have been used to determine the postglacial migration routes in Europe thanks to their maternal inheritance in deciduous tree species and low mutation rate. In our study, the significant statistical differences observed between presence of private chloroplast DNA allele (*A*p) distribution in the different class of vitality may suggest some adaptive phenomenon of single trees in the particular Wolica Reserve of ash.

The Polish ash stands studied with cpDNA markers revealed some moderate differentiation due to the presence of only two haplotypes of ccmp genes (Heuertz et al. 2004). Additionally, the same genes were revealed in *F. excelsior* and *F. angustifolia*, suggesting the possible hybridization among ash species (Heuertz et al. 2006). Such phenomenon is commonly observed in *Quercus* (Belahbib et al. 2001), *Betula* (Palme et al. 2004) and *Populus* (Lexer et al. 2005) species. Evolutionary driven forces like hybridization play an important role in tolerance and adaptation processes of ash species in Europe.

Plant genomes contain a large number of resistance genes against various pathogens (Rajesh et al. 2015). During evolution the plants developed several mechanisms against the pathogenic invaders, based on gene-for-gene interaction concept (Manion 1981). Many genes and secondary-metabolite compounds are up- and down-regulated during tolerance / resistance pathway in plants subjected to external physical stress factors (like heavy metals, heat-shock, UV, Nowakowska 1998) or biotic factors (pathogens and bacteria, Jones and Dangl 2006). The resistance genes are constitutively expressed and induced as soon as a pathogen is perceived, which subsequently triggers the plant defence responses (Rajesh et al. 2015). Recently, the associative transcriptomics research performed in Denmark helped to discover specific DNA sequences and gene-expression variants across ash trees scored for disease symptoms and identified markers strongly associated with canopy damage (Harper et al. 2015). The latest study revealed some SNPs differences between susceptible and tolerant Danish *F. excelsior* trees, and even made possible a single-nucleotide based distinction between moderately tolerant ash species (*F. mariensii*) and highly tolerant ash species (*F. mandhsurica*, *F. americana* and *F. ornus*). Such a new method using rapidly identifying molecular markers associated with tolerant trait variation across a set of trees, and taking into account both gene sequence variation and gene expression variation, can be very effective in in stands protection measures.

The further studies based on DNA polymorphism assessment performed on larger group of trees may help to understand the genetic basis of the pathogen tolerance developed by some European ash trees.

Conclusions

New strategies of ash population restitution in Poland may be designed thanks to predictive molecular markers identifying tolerant and susceptible trees, with emphasis on propagation of seeds from the most tolerant-ones. Molecular markers, both nuclear and chloroplast microsatellite loci, constitute good tools to support conservation and management of forest genetic resources, via assessment of the gene-pool diversity of ash populations, and to predict potential tolerance or susceptibility of this species against harmful pathogen like *Hymenoscyphus fraxineus*. More advanced studies, based on resistance-gene identification should be done to elucidate those mechanisms on the molecular level in order to promote marker-assisted silviculture in European ash restitution in Poland.

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Pre-disease Levels of Genetic Diversity and Differentiation among Common Ash (*Fraxinus excelsior* L.) Seedlots in Austria

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Abstract

Ash is an important component of forestry in Austria; its loss due to the dieback disease would be a great challenge for many forest owners. We investigated seed material that was harvested in 2001, just prior to the onset of the disease. Seeds from at least ten (allegedly) separate trees per stand were obtained from commercial harvest lots, from six different stands in Austria. The separate sampling from at least ten seed-bearing trees of ash is a legal requirement in Austria. Levels of genetic differentiation on the basis of six microsatellite markers were low, but somewhat higher than in other typical European forest trees. Stands along the Danube river seemed to share more genetic similarity with each other than with two stands in the Alps. In comparison, within the stands, most single tree seed lots were highly differentiated and they mostly fitted to the stands of origin with their genetic patterns. An attempt was made at reconstructing the unknown genotypes of the mother trees of the seeds from the offspring data. This led to the presumable identification of cases where these mother trees shared more alleles than expected, and their seed lots were closer genetically than on average. It also revealed cases where single seeds did not fit into their lot genetically (as defined by Mendelian rules). The data reported here confirm that detailed information on the genetic background of seed can be obtained from such structured samples, supporting law enforcement. It further confirms that harvesting from a minimum of ten trees leads to seed that more comprehensively reflects levels of genetic diversity in the whole stand. The data presented can be used as a baseline for investigating any genetic effects of the progressing disease in the future.

Keywords: *Fraxinus excelsior*, *Hymenoscyphus fraxineus*, microsatellite markers, genetic diversity, seed, genetic differentiation, forest reproductive material

Introduction

Common ash (*Fraxinus excelsior*) in Europe, the subject of this volume, has some particular features that distinguish it from other hardwood forest trees. In contrast to the more continuously distributed, stand-forming deciduous trees like beech (*Fagus sylvatica*) and the oaks (*Quercus* sp.), ash has a more ecotypic pattern of distribution with some local abundance, but in general, its more scattered distribution (Gömöry et al. 2012) is correlated with that of nutrient-rich soils with good levels of water availability, especially in spring (Weiser 1995). Therefore, the patterns of genetic diversity of this species across the landscape have received some interest in the scientific community. Especially after the publication of several microsatellite DNA markers for this species (Brachet et al. 1999, Lefort et al. 1999), such studies have addressed patterns across countries or regions (e.g. Heuertz et al. 2001, Morand et al. 2002, Ferrazzini et al. 2007, Sutherland et al 2010, Gömöry et al. 2012, Fussi and Konnert 2014, Beatty

et al. 2015) or even across Europe (Heuertz et al. 2004). The peculiar flowering system of the species, with predominantly male, female, or hermaphrodite trees, has also raised attention genetically (e.g. Morand-Prieur et al. 2003, Heuertz et al. 2003), as well as its possible hybridization with the sister species, *Fraxinus angustifolia* (Morand-Prieur et al. 2002, Gerard et al. 2006, 2013; Heuertz et al. 2006, Lexer et al. 2004, Thomasset et al. 2011, 2013). Gene flow in these species has been assessed, *i.a.*, by Heuertz et al. (2003), Bacles et al. (2006); Bacles and Ennos (2008); Gömöry et al. (2012), and Thomasset et al. (2014).

The progress of the disease (see other contributions in this volume) makes it necessary to discern effects of e.g. gene flow and hybridization on the population genetics of this species. For example, it would be interesting to know whether progressive fragmentation (because of tree mortality in affected regions) decreases, or rather, even increases (Bacles et al. 2006; Bacles and Ennos 2008) gene flow; or whether hybridization with *F. angustifolia* affects disease tolerance. For all these questions, comparing genetic analyses before and after the onset of the disease in a particular region would provide valuable data.

The FRAXBACK Cost Action FP1103 (www.fraxback.eu) has brought together scientists with an interest, *i.a.*, in these genetic questions. In the frame of a previous project, 'RAP – Realising Ash' Potential' (contract QLK5-CT-2001-00631 of the EC's Fifth Framework Programme), genetic diversity and relatedness data were obtained also for Austria. The main rationale for this work was an assessment of seed harvesting practices, and whether they would conform to national regulations. These regulations in Austria state that seeds in ash have to be harvested from at least ten different trees in an approved seed stand. A sample of a handful of seeds from each of these trees has to be sent to the BFW research station. For checking conformity with seed harvest regulations, such single seed lots from six different stands were analysed with microsatellite markers. Exactly at the end of the investigation mentioned above, any interest in planting ash stopped more or less completely in Austria (Heinze et al. 2017), and the results have so far remained unpublished. However, the steady progress of the disease now opens new possibilities for presenting the data and re-analysing it in order to provide a baseline for investigating any genetic effects that the disease has on the tree species, now and in the future. Genetic distances between the seed lots, and an attempt at reconstructing the (unknown) maternal genotypes, served for this purpose and are presented here.

Material and Methods

Seed and DNA extraction

Samples of *Fraxinus excelsior* were collected in seed lots in different regions of Austria at the time of harvest (autumn of year 2001) by local forest personnel (Table 1). Seeds from 10 to 13 trees (adult stage – 'mother trees', in total 65 trees) from each of the six seed lots were sent to the BFW research station separately. Seeds from each tree were imbibed in water for softening. Eight to ten seeds per tree (with very few exceptions of only four or seven, or up to 12 seeds) were used in DNA extraction, resulting in a total of 573 seeds (seed stage) investigated genetically. Total DNA was extracted from excised embryos using the SIGMA GenElute^{TM} Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Vienna, Austria).

Table 1. Origin of seed (harvest year: 2001) for this study

Stand	District (province)	Field name	Coordinates (WGS84)	Elevation (m)	Number of mother trees
ASL02	Eferding (OÖ)	Polsing	E 14°05'09.38'' N 48°16'06.68''	279	13
ASL23	Hartberg (STMK)	Riegler-viertel	E 15°49'19.11'' N 47°29'15.71''	928	10
ASL27	Klagenfurt (KTN)	Loiblthal	E 14°15'28.85'' N 46°27'55.73''	775	10
ASL50	Melk (NÖ)	Aggsbach	E 15°30'31.53'' N 48°18'26.40''	466	10
ASL65	Tulln (NÖ)	Langen-schönbichl	E 15°58'51.36'' N 48°19'47.86''	180	10
ASL98	Lilienfeld (NÖ)	Keeramt	E 15°30'04.80'' N 47°49'47.28''	821	12
(total number of trees)					65

Genetic markers

For molecular analysis six primer pairs of microsatellite loci were chosen which showed high polymorphism in initial studies (FEMSATL4, 11, 12, 16, 19, and M2-30; Lefort et al. 1999; Brachet et al.1999). These markers were amplified by polymerase chain reactions (PCR) performed in a mix containing 2 mM MgCl2, 0.5 units Taq Polymerase (Platinum; in Platinum Puffer, Thermofisher, Vienna, Austria) per reaction, 0.2 µM of each primer, 0.2 mM dNTPs, 1 µl of template DNA (approx. 10-50 ng/µL) in a total reaction volume of 20 µl. The thermal cycling profile consisted of an initial denaturing step for 3 min at 94 °C, 10 cycles of 50 s at 94 °C and 1 min at 70 °C, followed by 35 cycles of 30 s 94 °C, 55 °C (annealing temperature) for 50 s, and 2 min at 70 °C. PCR products were separated by electrophoresis in 8 % polyacrylamide gels for 2 h at 40 W and visualized by SYBRGold (Molecular Probes, ThermoFisher) staining on a standard UV–transilluminator (Heinze et al. 2014). Ladders of size standards (10 and 100 bp DNA ladders) were used for sizing bands. Selected alleles were loaded side by side on every gel for additional calibration. Pictures of the gels were taken and alleles recorded with the program Gene Profiler 4.03 (Scanalytics, Inc., Fairfax, VA, USA).

Data analyses

Parameters of genetic variation were calculated for each of the six seed lots separately, and for the total, by the Fstat software (Goudet 1995): (1) the average number of observed alleles A, (2) gene diversity or expected heterozygosity H_E (Nei 1987), (3) Wright´s inbreeding coefficient F_{IS}, (4) observed heterozygosity H_O (for each locus over all populations in seed samples), (5) Weir & Cockerham's F-statistics for describing genetic diversity within (F_{SP}) and between seed lots (F_{ST}). Hierarchical F-statistics were established by computing F_{IT} for comparing individual samples to all samples (individuals to total), along with F_{ET}, calculating the relationship of subpopulations (seeds from single trees) to the total data set, and finally F_{IS}, differentiating individuals within subpopulations. (6) Departure from Hardy-Weinberg-equilibrium, as implemented in the Fstat software (Goudet 1995), was also assessed.

With the program STRUCTURE (vers. 2.1 and 2.3.4, Prichard et al. 2000, Falush et al. 2003, 2007), which applies a widely used model-based clustering method, we attempted to assign individual multi-locus genotypes (the seeds) to a user-defined number K of clusters or gene pools. STRUCTURE (ver. 2.1) was run for 10^4 iterations after a burn-in period of 10^4 on the total dataset of 573 individuals; trial runs for 10^6 iterations and burn-in period runs did not result in substantial differences to the 10^4 finally used for our dataset. The number of clusters were set as K = 1, 2, …, 80, and 10 runs were done at each K. We used the model without admixture in the runs initially (assuming that the analysed stands are situated too far apart from each other for any significant contemporary pollen flow), and repeated these with the admixture model as well. No prior information on the population origin of the individuals was used in these run, and in all runs with STRUCTURE, the "correlated allele frequencies" model was employed.

The runs were repeated separately for each seed lot (in STRUCTURE ver. 2.3.4), this time using 10^6 runs both for burn-in runs and iterations (K = 1 to 20 or K = 2 to 12, 10 runs per K). A similar set of conditions was employed in analysing the whole data set with "population info" set to seed stands and using the "start at population info – locprior" option in STRUCTURE (K = 5 to 15, 10 runs per K). In these cases, both the admixture and no admixture models were used. The most likely number of all these pre-defined K clusters in the various sets of runs was assessed by Pritchard et al.'s (2000) "informal" original suggestion (highest ln(K) with still low variability), and by the more formal criteria of Evanno et al. (2005), as implemented in Earl and von Holdt's (2012) STRUCTURE HARVESTER web service (http://taylor0.biology.ucla.edu/structureHarvester/#).

For assessing similarities versus differences (genetic distances) among entities (seed stands and individual seed lots, respectively), the Q matrices of the STRUCTURE output at the selected K (which give a proportion of ancestry of each actual population in each assumed ancestral cluster) were utilized for calculating simple Euclidian distances between each entity in the analyses (as averages of the ten runs at the same K value).

Reconstruction of maternal genotypes

Lexer et al. (1999) proposed a procedure to infer maternal alleles on the basis of an array of maternal half-sib families (of *Quercus robur*, in their case) with a minimum of eight to ten seeds per tree, using the rules of codominant Mendelian inheritance (Mendel 1866). The same methods are applied to the half-sib families of *Fraxinus excelsior* here. The "rules" how to infer maternal genotypes at a locus, for our specific case, can be described as follows: if a homozygous (single-locus) genotype appears in the seeds, this allele is one of the maternal ones; if there is no such homozygous allele, two alleles are selected so that each of them is present in at least one seed of the lot; if there are more than two homozygous genotypes, the two most frequent alleles among them are selected; if a single allele is present in all seed genotypes, and partially in homozygous condition, no second allele is selected (the maternal genotype is assumed homozygous for the frequent allele); and finally, if a single allele is present in all seed genotypes, but only as a heterozygote, the second most frequent allele is selected as well. An example with two loci is shown in Table 2. However, uncertainties in this inference remain because of technical errors, possible mix-ups of seeds or DNA samples, and stochasticity.

Table 2. Reconstruction of the maternal genotype at an example of 2 loci in population ASL23

Individual	FEMSATL4		FEMSATL11	
23_02_01	192	182	196	190
23_02_02	182	166	196	186
23_02_03	182	166	208	186
23_02_04	182	166	196	186
23_02_05	168	168	196	186
23_02_06	182	168	208	196
23_02_07	240	168	186	178
23_02_08	168	168	196	186
23_02_09	174	168	196	186
23_02_10	202	168	208	186

Offspring individuals of one mother tree (23_02) are arranged in rows, the genotypes at the two loci are listed in columns as allele sizes in base pairs. Maternal alleles as inferred from the offspring are shaded in bright and dark grey

With the inferred data, detection of possibly identical mother trees was done by calculating a mean proportion of shared alleles (POSA) for each seed lot using the pro-

gram MSA (Dieringer and Schlötterer 2003). The same parameter POSA was also calculated for "suspiciously similar" inferred maternal multi-locus genotypes. Because of the uncertainties associated with inference of these genotypes, mother trees belonging to the same seed lot and with POSA 66.7 % and higher were registered as possibly identical.

Results

Basic genetic parameters

Allele size ranges and other basic genetic parameters are listed in Tables 3 and 4, along with data from previous studies in *F. excelsior* which have employed similar sets of microsatellites. On a per locus basis, we observed between seven and 60 alleles. Gene diversity H_E ranged from 0.520 to 0.962 and F_{IS} from 0.165 to 0.608 (not shown). Higher values for H_E and F_{IS} in FEMSATL12 indicate null alleles at this locus, but amplification success was not strikingly different from other markers, and there were not too many homozygous individuals; consequently, it was included in further calculations. Locus FEMSATL16 showed unusually low values in gene diversity and number of alleles, together with high F_{IS}, revealing that this locus is not as polymorphic as the others, with a higher degree of homozygosity.

Other basic genetic parameters on a population basis are given in Table 3. Within populations (seed stage), gene diversity H_E ranged from 0.755 to 0.815, with an excess of homozygotes and a mean inbreeding coefficient (F_{IS} = 0.245) significantly deviating from zero (Table 3). When omitting FEMSATL12 and FEMSATL16 from the calculation of F_{IS}, there was still a highly significant positive mean value (F_{IS} = 0.106 +/- 0.019).

Hierarchical genetic differentiation among seed stands and single tree harvests

Certain alleles strongly differentiated some of the seed lots. This was most pronounced in FEMSATL4 and FEMSATL11, and for seed stand ASL27. In general, differentiation among seed stands was low (F_{ST} = 0.057) but significant (Table 5); *i.e.*, only 5.7 % of the total genetic diversity (H_T = 0.843) arises from among-population (seed stand) differentiation. Among single tree harvests, compared to the total, we calculated a differentiation of 16.9 %. We further assessed the differentiation of single tree harvests within the seed stands, resulting in a slightly lower F_{SP} (between 0.109 and 0.159). Finally, F_{IS} (individual seeds within single tree lots) ranged from -0.116 to 0.514 in the six loci; this means that when collecting seed from a different tree in the stand, or when raising a seedling from a different seed within the same single tree lot (same mother), genetic differentiation is already quite high - 13.3 % and 14.1 % on average, respectively.

The model-based clustering approach implemented in the software STRUCTURE indicated some population structure in the data. The runs of the whole data set with the "no admixture" model (which gave a more meaningful curve of ln(K)), gave "peaks" (according to Pritchard et al.'s 2000, and Evanno et al.'s 2003 criteria) at K = 2 and K = 10, which are close to the actual number of six seed

Table3. Genetic diversity statistics within seed lots and across all seed lots

Population	n	A	H_O	H_E	F_{IS}
ASL02	96	17.8	0.634	0.809	0.234***
ASL23	96	19.7	0.584	0.755	0.235***
ASL27	94	15.3	0.630	0.799	0.227***
ASL50	95	18.7	0.581	0.815	0.294***
ASL65	96	18.0	0.612	0.793	0.237***
ASL98	96	18.3	0.602	0.805	0.257***
mean populations	95.5	18.0 ± 1.45	0.607	0.796	0.245 ± 0.093
all populations	573	33.7	0.603	0.843	0.248***

n, sample size; A, average number of alleles per locus; H_O, average proportion of heterozygotes; H_E, average gene diversity; F_{IS} Wright´s inbreeding coefficient. Deviation from Hardy-Weinberg genotypic proportions: ***P < 0.001

Table 4. Comparison of allele size ranges in various studies using similar sets of microsatellite markers

	Brachet et al. 1999, Lefort et al. 1999		Heuertz et al. 2001		This study		Fussi and Konnert 2014		Gömöry et al. 2012		Ferrazzini et al. 2007	
Locus	A_t	size	A_t	size	A_t	size	A_t	size	A_t	size	A_t	size
M2-30	18	182-248	59	182-294	49	178-282	58	175-301	54	174-290	n.a.	n.a.
FEMSATL4	9	164-228	50	158-251	32	164-243	49	154-254	54	152-268	32	157-205
FEMSATL 11	11	180-226	32	176-266	30	174-238	35	181-255	31	180-242	42	161-234
FEMSATL 12	9	180-262	18	181-264	24	178-265	31	175-229	n.a	n.a.	39	147-261
FEMSATL 16	4	180-200	10	176-204	7	178-203	15	178-208	5	184-200	9	184-214
FEMSATL 19	12	174-214	33	142-229	60	145-243	37	143-223	n.a.	n.a.	55	142-238

A_t, total number of alleles; size of alleles in base pairs; n.a., not analysed

Table 5. Hierarchical F-statistics in the six microsatellite loci

	Overall inbreeding	Differentiation among:			
		seed lots	single trees	single trees within populations	seeds within trees
Loci	F_{IT}	F_{ST}	F_{ET}	F_{SP}	F_{IS}
FEMSATL4	0.256	0.086	0.208	0.159	0.049
FEMSATL11	0.070	0.071	0.158	0.110	-0.116
FEMSATL12	0.680	0.062	0.187	0.149	0.514
FEMSATL16	0.591	0.072	0.192	0.133	0.488
FEMSATL19	0.176	0.034	0.153	0.133	0.024
M2-30	0.173	0.028	0.290	0.109	0.047
Multilocus	0.291	0.057	0.169	0.133	0.141
Significance (*p*)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Significances of the multilocus estimates are computed by permutation tests

stands. "Secondary peaks" were found at K = 30, and a minor one also at K = 65, the actual number of seed mother trees. A "peak" of K = 29 was most pronounced in the runs with the admixture model. Some structure was visible in all these cases from the STRUCTURE bar plots of Q for individuals, in the sense that individuals belonging to the same entity (stand seed lot or mother tree) showed similar Q proportions (for an example, see Figure 1A). When the resulting colour bars were sorted according to the stand seed lots, they indicated distinct patterns for each stand (Figure 1B-C). Within the stands, however, patterns showed also some differentiation (in Figure 1B, the first bars in ASL02 are probably due to many missing data points for the individual seeds). Using the "no admixture" and "location prior" models, the structure was much more apparent, and mostly conformed with seed stand origin (Figure 1C) and single tree lot, respectively.

In order to illustrate the relatedness expressed in these calculations, we present here pie charts of Q values for the seed stands with progressive values for K assumed in STRUTURE (using "no admixture" and "location prior"; Figure 1D-F): for K = 2, the six studied populations separated into two groups that match geographical patterns (Figure 1D). Seed stands ASL27 and ASL98 (blue pies) are situated in the northern and southern outer chains of the Alps, respectively, whereas ASL02, ASL50 and ASL65 (red pies) are stands along the river Danube. The latter ones formed a second group, also comprising ASL 23 in the region of the easternmost edge of the Alps. For K = 4, the three stands along the Danube still share the same (blue) colour, while all others are differentiated from those, and from each other (Figure 1E). At K = 6, only the two closest stands on the Danube retain some relationship (same colour – purple segments in charts; Figure 1E).

In a more formalized way, Euclidian distances based on the Q proportions of the most likely K, calculated between seed stands, gave a similar picture (Table 6). Stand ASL02 and ASL50 along the Danube came out closest in all runs. ASL50 and ASL65 were still reasonably close. The highest distances were obtained for ASL27 and ASL65 (without "location prior") and ASL65 and ASL98 (using "location prior"). Both latter cases involved the same stand on the Danube and one of the Alpine stands.

In order to better evaluate differentiation within the stand seed lots with the STRUCTURE approach, K values between one and 20 were tested in each lot, and values between K = 3 and K = 6 were most meaningful. Distinct colour patterns were visible for the single tree lots of different mother trees, and calculations of Euclidian distances mostly confirmed these patterns (see also further below), although single seeds sometimes did not completely fit to their lot. In some instances, they even seemed to better fit to other single tree lots in the same stand.

Table 6. Euclidian distances between seed stands, averages based on "Q" proportions of ten runs each at K = 6, from STRUCTURE runs using the "no admixture" model

Seed stands	ASL02	ASL23	ASL27	ASL50	ASL65	ASL98
ASL02	-	0.7379	0.8616	0.4030	0.7827	0.7705
ASL23	1.3548	-	0.9429	0.7314	0.8728	0.8196
ASL27	1.3720	1.3825	-	0.8731	0.9901	0.8948
ASL50	1.0908	1.2653	1.2739	-	0.5323	0.7290
ASL65	1.3639	1.3960	1.3996	1.1239	-	0.8874
ASL98	1.3854	1.3945	1.3861	1.2860	1.4115	-

Above diagonal, distances obtained from STRUCTURE runs without "location prior" information; below diagonal, using "location prior"

Reconstructed maternal multilocus genotypes

In addition, multilocus genotypes were reconstructed for the 65 mother trees. On the basis of 3124 successfully assessed single-locus genotypes in 573 seeds (over all loci), a total of 390 single-locus maternal genotypes (65 trees times six loci) was inferred by the method described in Lexer et al. (1999). The majority of single-locus seed genotypes (2863 out of 3124, 91.65 %) fitted the inferred, most likely maternal genotypes. Regarding single seeds, about one third of the seeds in each lot had at least one incompatible single-locus genotype; however, the cases of seeds having two or three such incompatible genotypes were rare (only five cases had three incompatible single-locus genotypes, which is less than 1 %).

The inferred maternal multi-locus genotypes differed from each other at varying degrees. In three out of six seed lots, the results gave some indications that the actual number of genetically distinct seed mothers was lower than

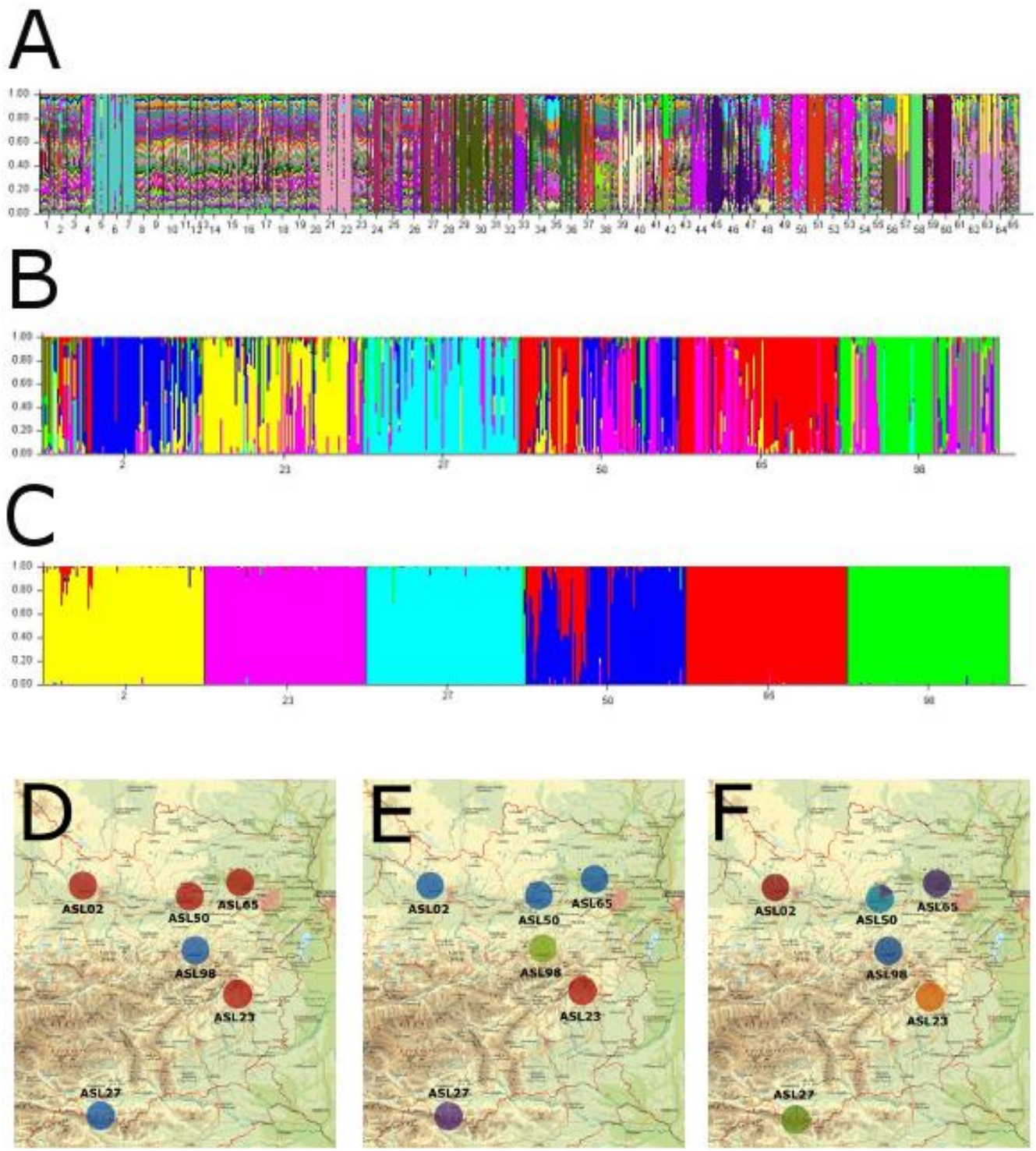

Figure 1. A: Sample bar plot of Q from STRUCTURE runs of the complete data set, at K = 65. Numbers below plot indicate single tree seed lots - ASL02: 1-13; ASL23: 14-23; ASL27: 24-33; ASL50: 34-43; ASL65: 44-53; ASL98: 54-65. Figure 1B-C: Sample bar plots of Q from STRUCTURE runs of the complete data set, sorted according to stand seed lots, at K = 6. Numbers below plot indicate stand seed lots ASL02, ASL23, ASL27, ASL50, ASL65 and ASL98, respectively, at K = 6. 1B, no prior population location info used; 1C, with location prior option (seed stand affiliations as starting points for iterations) Figure 1D-F: Progression of differentiation of stand seed lots at increasing K values assumed in STRUCTURE. 1D, Q proportions in each stand seed lot at K = 2; 1E, at K = 4; 1F, at K = 6

written in the documentation. For example, in stand seed lot ASL23, some inferred maternal multi-loci genotypes were quite similar (Table 7). The degree of allele sharing was higher overall in ASL23 than in the other two seed lots ASL27 and ASL98; 41.3 % versus 26.7-39.4 %, respectively. Given the technical and methodical sources of error, and the greater differentiation among the other reconstructed genotypes, it is fair to assume that the single tree seed lots that arrived in the BFW laboratory may have been derived from identical trees. For the stand seed lot ASL23, this would mean that seed was only harvested possibly from as few as seven individual trees. Additionally, proportions of allele sharing for possibly identical mother trees in the three seed lots ranged from 66.7 to 83.3 %, while other stand seed lots only gave highest POSA at 50.0 to 58.0 %.

Identical mother trees should give fairly identical seed lots (if the seeds are drawn from a well-mixed "bag"). This was tested by comparing Euclidian distances, based on the Q proportions obtained with a likely value for K, among the single tree seed lots. In stand seed lot ASL23, this distance was exceptionally low for single tree lots 6 and 10 (0.01598 +/- 0.003524) which also shared a high proportion of their alleles (Table 7). Lots 8 and 9 had the second lowest distance value (0.05632 +/- 0.009385), but lots 1 and 2, though sharing a lot of alleles in the inferred mothers as well, were much more distant (0.3453 +/- 0.003956). In contrast, in a stand seed lot that did not attract attention for including possibly identical inferred maternal multi-locus genotypes, ASL02, at K = 5, distances between single tree seed lots 5 and 7 were unusually low (0.04233 +/- 0.0245), and this was also the case for trees 9 and 11 (0.07511 +/- 0.01519; while all other 66 pair-wise distances between single tree seed lots were greater than 0.1 in this stand lot). Mother trees 5 and 7 did not share a high proportion of alleles (33.3 %), and 9 and 11 shared only 41.7 %. These values are much lower than for seed stand ASL23, but are among the highest within this seed lot ASL02.

In the two other cases of stand seed lots with possibly identical maternal genotypes, these comparisons appeared as follows: In ASL27, mother trees 1 and 3 shared nine out of 12 alleles, and their Euclidian distance was at 0.1290 +/- 0,01545, the second lowest in this lot. The lowest was between trees 8 and 9 (0.1218 +/- 0.01629), sharing 50 % of the inferred maternal alleles. Another pair, mother trees 6 and 7, shared eight out of 12 alleles (66.6 %). The Euclidian distance between their single tree seed lots was 0.1486 +/- 0.004983). All other distances in this lot were above 0.2. In ASL98, mother trees 10 and 11 shared the highest proportion of alleles (75 %), and also their Euclidian distance was the lowest in the lot (0.1671 +/- 0.003235).

The remaining stand seed lots had single cases of "suspicious" low distances with relatively low allele sharing levels (ASL50: 6 and 8 – 0.1030 +/- 0.02207 – 33.3 % allele sharing; ASL65: 6 and 8 – 0.1064 +/- 0.002228 – 33.3 %, and 7 and 10 – 0.1647 +/- 0.01483 – 50 %).

Discussion

The primers used in this study have a long record of usage (Table 4). Although they are not always "ideal" in a sense that unambiguous allele scoring is easily possible, the data from multiple laboratories (Table 4) show a remarkable synchronicity in allele size ranges, something that is often confounded by the usage of various fluorescent labels, DNA polymerases, and (capillary) electrophoresis apparatus in different laboratories. Sutherland et al. (2010) as well as Beatty et al. (2015) suggested modifications to the primer sequences in order to minimize null allele problems. Although the original primer sequences were used in this study, such null allele problems were not apparent. Sequencing of the *Fraxinus excelsior* genome (see e.g. www.ashgenome.org) will reveal whether the currently used primers may be replaced by more "robust" ones, but sequence data from multiple populations across the range of the species would be necessary for an assessment.

Table 7. Inferred (hypothetical) mother trees of seed lot 23

Individual	FEMSATL4		FEMSATL11		FEMSATL12		FEMSATL16		FEMSATL19		M2-30	
23_1	182	168	196	186	200	184	182	182	201	199	258	212
23_2	182	168	196	186	200	184	182	182	239	201	222	212
23_3	168	166	188	186	200	184	186	182	198	146	218	212
23_4	188	168	200	186	200	184	182	182	198	185	246	198
23_5	206	166	206	196	200	184	182	182	185	183	246	224
23_6	168	166	206	186	200	184	182	182	201	197	220	216
23_7	174	174	200	196	188	184	182	182	195	183	200	216
23_8	184	174	208	196	200	184	182	182	193	183	244	226
23_9	184	174	208	208	200	196	186	182	193	183	226	220
23_10	168	166	206	186	202	200	186	182	219	197	220	216

Identical alleles of possibly identical inferred maternal multi-locus genotypes (mother trees) in identical colour shading

Stand seed lots analysed in this study were within a narrow range of basic genetic parameters (Table 3): only ASL27 (the southernmost, Alpine stand) had somewhat lower allele numbers, but this was not reflected in lower heterozygosities. Although inbreeding coefficients were significantly different from zero in all stands (Table 3), this may either be a remaining effect of a few null alleles present, or of the general tendency of forest trees to tolerate some pollination among relatives in the seed stands. Selection usually removes such effects: adult trees often show the opposite, i.e. heterozygote advantage.

The level of differentiation among seed lots (Table 5) is comparatively high (slightly above 5 %); this is more than could be expected from a typical European forest tree in such a limited area. In studies of ash in regions of comparable sizes, higher values were reported by Heuertz et al. (2001) in Bulgaria (F_{ST} = 0.087), and slightly lower ones for Bavaria (Southern Germany, F_{ST} = 0.046, Fussi and Konnert 2014). Beatty et al. (2015) reported similar low values of differentiation (though they calculated different parameters), in their study of a similar, comparatively small region. In their study, these low values of differentiation were coupled with indications for a strong long-distance gene flow component. Average F_{ST} values were lower in Great Britain (0.025; Sutherland et al. 2010), on an even larger geographical scale than in our study region. It appears that the stands along the Danube river in our study are closer to each other genetically than the rest (Table 6, Figure 1D). There is also some possible relatedness among the Alpine stands (ASL27 and ASL98), although these are quite distant geographically. Moreover, ash distribution in the Alps is more scattered, especially in the inner Alps (Heinze et al. 2017). The remaining genetic connectivity of these two stands, and their differentiation from "floodplain" stands (along the Danube), may hint at a general genetic "divide" among ecotypes in mountain valleys and the lowlands in Austria, but this remains to be investigated in much greater detail. Influences of hybridisation (introgression) with *F. angustifolia*, a sister and neighbour species in the Pannonian basin, could also play a role here. Fussi and Konnert (2014) included two stands from Austria in their analysis of material from Bavaria, one of which was situated very nearby ASL23, and the other somewhat upstream of ASL02. The Austrian stand near ASL23 in their study showed relatively high differentiation from the rest of the populations. As there was a large sampling gap in between, this would hint at typical clinal variation patterns of this species in this wider region.

What is remarkable are the high differentiation values for the single-tree seed lots, within their stands as well as compared to the total (Table 5). This far exceeds usual values obtained in comparison of trees among stands. One possible explanation could be pollen clouds of restricted diversity that fertilize single female flowers. The seeds for our study may have been picked from single clusters of seeds during harvesting.

It could be imagined that colonisation events with seed from just one or a few seed parents may lead to high differentiation in this species. However, ash seed is long-lived. If such colonisation events are repeated over various years, from various parents, differentiation effects may decrease, and this is what we apparently see in this species at the adult stage. For artificial regeneration with nursery-grown plant material, however, it follows that using seed from just a handful of parents may lead to unwanted differentiation effects. Such plants may not represent the entire genetic make-up of the stand they derived from.

Have the persons who harvested the seed for this study taken account of the detrimental effects of limiting the level of genetic diversity? When seeds are harvested by climbers, costs increase significantly with every tree climbed. Also when picking seed from felled trees, it takes much longer to collect a similar amount of seed from many trees, as opposed to just from a few. In Austria, forest seed harvesting regulations require a minimum number of trees to be used, in order to avoid effects of genetic bottlenecks, as outlined above. The data obtained in this study allow an assessment of how this measure was accepted in practice. We have some hints that may indicate issues in this respect. The "suspiciously" similar single tree seed lots could actually stem from identical trees. They showed only a small amount of differentiation among them. This should not be the case for well-mixed seed from the same tree, but seeds picked from different parts of the crown may have been derived from slightly different pollen clouds. We have analysed bigger numbers of seed from a single tree (96 seeds; Heinze et al. 2017 and unpublished data), but in this example, seeds from the whole crown were mixed. A more structured sampling in different parts of a crown may reveal if there is genetic differentiation of pollen clouds in different parts of the crown.

Another possibility for explaining only slightly differentiated seed lots would be clonal trees, e.g. trees grown from sprouts of an identical root system. Ash often grows from woodstocks, but is not known to form large clones of adult trees. It is not so likely that even only two large, seed-bearing trees develop from a single woodstock. Genetically closely related mother trees, e.g. full-sib mothers, may be another explanation for the results obtained.

An alternative method investigated here was the reconstruction of the genotypes of the mother trees. The procedure used has some drawbacks. Technical errors in allele sizing, single mixed-in seeds from other trees, and simple stochasticity effects may blur the determination of the true maternal alleles. It is possible for several tree species to analyse maternal tissue, e.g. seed coats (Ziegenhagen et al. 2003), though this "dead" tissue is more challenging to analyse. The re-constructed maternal genotypes in our

study show reasonable agreement with genetic distances of "questionable" seed lots, so they may come close to the true alleles. Analysing somewhat larger half-sib families (single tree seed lots) may also alleviate the problem. In any case, the methods employed here provide a first possibility of investigating conformity with legal requirements for seed harvests in Austria, and confirm that the suggestions originally made by Lexer et al. (1999) and Heinze and Lexer (2000) are a practicable approach.

The generally great differentiation among single-tree seed lots was also evident from the STURCTURE runs (and the derived Euclidian distances among them). This raises the question of how large a sample of seeds or plants must be in order to "truly" represent the genetic make-up of the stand. This question was answered empirically when the seed regulations were drawn up in Austria. It was decided at the time to demand harvesting from at least ten mother trees for this and other "scattered" hardwood species. The German law for forest reproductive material requires even twenty trees for harvesting seeds in ash. In contrast, in several other European countries no similar regulations for seed harvesting in ash are in place. The data presented here provide evidence that the choice of a minimum of ten trees was reasonable in ash, as the differentiation of whole seed stand lots was in the range of previous genetic studies that have analysed adult trees from different populations, while single tree seed lots showed very large differentiation values. This means that stand seed lots consisting of only few single mother tree seed lots would be atypical for the whole genetic diversity in the stand. This would lead to the effect that seed lots from different years (and different mother trees in each year) would not resemble each other much. Nurseries that would grow plants from such material would have to deal with quite variable traits, at least as far as genetic markers are concerned, but possibly also in growth traits. The reconstructed maternal genotypes were also quite variable (an attempt at inferring genetic clusters among the 65 reconstructed mother trees with STRUCTURE did not show any; data not shown). It should be investigated whether the minimum number of ten harvested trees also holds in similar "scattered hardwood" species like wild cherry (*Prunus avium*) or sycamore maple (*Acer pseudoplatanus*), which are insect-pollinated.

Sustaining a high level of genetic variability will be especially challenging in the wake of the progressing ash dieback disease. Less and less ash stands are utilized for seed harvesting since the onset of the disease (Heinze et al. 2017). If the same stands are utilized in different years, this may lead to a still narrower genetic basis of nursery plants. Contrary to that, it may be desirable to harvest from even more stands, as there may be different levels of disease tolerance in different stands. It is questionable whether this is still feasible, as the disease seems to progress steadily (Heinze et al. 2017). Disease-damaged trees are also dangerous for climbing.

Mixing plants for re-forestation from lots within the same region of provenance (and the same altitudinal zone) may be much desirable. Stored seed may help to overcome any developing bottlenecks in seed supply.

There is still a need to investigate whether trees with varying degrees of disease symptoms pass on any such "tolerance" to their offspring (McKinney et al. 2012, Pliura et al. 2011, Fussi and Konnert 2014), and whether it is durable. Pliura et al. (2014) reported that over time, disease incidence in all trees investigated increased, and that few remained relatively healthy (under 50 % damage) in later observations, but that heritabilities increased with time. Pliura et al. (2016), in a different set of experiments, also stated that none of their (half-sib) families stayed completely symptom-free. It may be concluded from such data that if there is any resistance, it is rather partial or quantitative, and that time series observations over several years, of individual trees, are desirable. "Survival rates" over several years may be better indicators of increased levels of disease tolerance than single "degree of damage" assessments in single years (see also Heinze et al. 2017).

We are currently investigating correlations of health levels between adult trees and their seedlings in forest situations, using microsatellites for establishing the parent-offspring relationships (A. Wohlmuth and B. Heinze, manuscript in preparation).

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Natural Regeneration of Common Ash in Young Stands in Latvia

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Abstract

Due to the dieback caused by ascomycete *Hymenoscyphus fraxineus*, common ash (*Fraxinus excelsior* L.) regeneration currently occurs only naturally and is crucial for existence of the species. Hence, in this study, we assessed the success of the natural regeneration and health condition of common ash in 90 diverse young stands. Additionally, the age structure of ash advance growth (saplings and seedlings) was characterized in four plots, initially dominated by ash. Ash was abundant in the advance growth in the studied plots with the mean density of 4185 ± 401 trees ha^{-1}. Ash advance growth density and health condition decreased with increasing height and age. From the 7533 accounted regeneration ashes, 75% were considered as healthy, 15% damaged and 10% were already dead. Ash regeneration density was the highest and the degree of *H. fraxineus* damage was the lowest in young stands on drained mineral soils. The best ash health condition was found in the densest stands with increased number of advance growth and undergrowth individuals. The highest ash mortality (ca 20%) was found in pure young stands. In the young stands, which were previously formed by ash, regeneration density was relatively low (4319 ± 592 trees ha^{-1}), but the mortality intermediate (ca. 10% of all trees). In contrast, in the stands dominated by black alder and birch, the density of ash advance growth was higher – 7300 ± 6300 and 6933 ± 2711 trees ha^{-1}, respectively, but the number of dead ash was lower (ca. 5%). Ash appeared more susceptible to the disease in the dense and unmanaged stands, as the health condition of ash regeneration was positively related to the number of tendings. A significant correlations between diameter, age and height of ash was observed, yet the analysis of the dimension showed, that the ash regeneration after harvesting and/or dieback has been occurring at different rates.

Keywords: *Fraxinus excelsior*; advance growth; establishment; young stand; ash dieback; natural succession; hemiboreal forest zone

Introduction

Intensive dieback of common ash (*Fraxinus excelsior* L.) caused by the ascomycete fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya, comb. nov. has been observed in Europe since the mid-1990s (Kowalski 2006). The Baltic countries were among the first regions, where the ash dieback was described (Bakys et al. 2009, Stener 2013); yet in Latvia, the disease was confirmed only in 2007 (Kenigsvalde et al. 2010). According to the national inventory conducted in 2009, one third of all ash forest in Latvia has been lost (Kenigsvalde et al. 2010). At present, common ash forms ca. 0.5% (14582 ha) of the total forest area, of which ca. 25% are young stands. Due to the rapid spread of the dieback (Kirisits and Freinschlag 2011, Pliūra et al. 2011), ash planting has been stopped as economically non-sustainable (Kirisits et al. 2011, Bakys 2013). Nevertheless, ash has been regenerating naturally in Latvia (Laiviņš and Mangale 2004) and all Europe (FRAXIGEN 2005, Dobrowolska et al. 2011). In the United Kingdom (Wardle 1961) and Sweden (Dobrowolska et al. 2011), ash has been considered as a pioneer species, while in Denmark it is considered as intermediate between the pioneer and permanent component of forest (Ahlberg 2014). In Central Europe, ash has been associated with invasive species (Wagner 1990, FRAXIGEN 2005), while in northern Europe the term "fraxinisation", which represents the successful self-regeneration of ash, has been used (FRAXIGEN 2005). Still, during the last 10 years, the situation has radically changed, e.g. in Sweden, ash is now a Red listed species (Gärdenfors 2010). Therefore, the assessment of the natural regeneration pathways of ash is necessary to improve the management of existing stands

aiming to maintain existence and better health condition of the species by applying silvicultural activities.

In many European countries, the damaged ash stands are transformed every year and certain part of those territories is left for the natural regeneration, subjecting ash to competition with other species that causes stress. In rich and moist sites, the most common competitors to ash advance growth (saplings and seedlings) are the early successional or pioneer species such as grey alder (*Alnus incana* (L.) Moench), silver birch (*Betula pendula* Roth.) and, in some cases, common aspen (*Populus tremula* L.) (Lygis et al. 2014). Natural regeneration of ash differs amongst site with diverse soil types. Prior to the dieback in Latvia, the best regeneration of ash was observed on mineral and drained mineral soils, especially in stands dominated by ash (Sakss 1958, Laiviņš and Mangale 2004). Due to the different views on the *H. fraxineus* spread and aggressiveness in diverse growing conditions (Kirisits et al. 2011, Stener 2013, Bakys 2013, Bakys et al. 2013), it has been unclear, how the dieback of mature ashes affects its natural regeneration, spread and health condition. At present, there is a large uncertainty about the development of ash forests, as since the onset of the disease, only a few studies dealing with the regeneration of damaged stands have been conducted (Ahlberg 2014, Lygis et al. 2014). Still, the optimistic forecasts suggest that, after a certain period of time, ash should recover from the dieback (Pliūra et al. 2011). The aim of this study was to evaluate the density and health condition of different young stands of common ash in Latvia. We hypothesised that the intensity of the damage was higher in the denser stands on the drained soil types. We also assumed that the susceptibility to damage has been affected by the ash advance growth dimensions.

Material and methods

Study sites, sampling and measurements

Ash regeneration was studied in 90 stands (Figure 1) distributed across Latvia. In Latvia, ash occurs in the mixed forests together with other deciduous trees (e.g., aspen, birch, alder, spruce (*Picea abies* Karst.), etc.); pure stands are rare. Ash is distributed quite frequently, but mainly the stands occur in the central part of Latvia, where soils are fertile and the climate milder (Nikodemus et al. 2009). The climate in Latvia can be classified as transitional maritime to continental, the continentality increases eastwards. Accordingly, the territory can be divided into three regions: the western, central and eastern part of Latvia (Figure 1). In these regions, the mean temperature in January is ca. -1.8, -3.2 and -4.5 °C, but in July ca. 17.4, 18.2 and 17.9 °C, respectively. The mean precipitation in July in these regions is about 748, 619 and 665 mm, respectively.

The young stands of common ash, where in previous rotation ash formed ≥ 40% of standing volume, with the age of 5 to 40 years and the size ≥ 1 ha, were selected from the State Forest Service inventory database. All age groups (distinguished by the step of ten years) were presented by 19–25 plots (Table 1). Stands corresponded to four soils types, mostly dry mineral (46 plots) and drained mineral (26 plots). The studied sites have undergone up to four tending events (mostly once or twice).

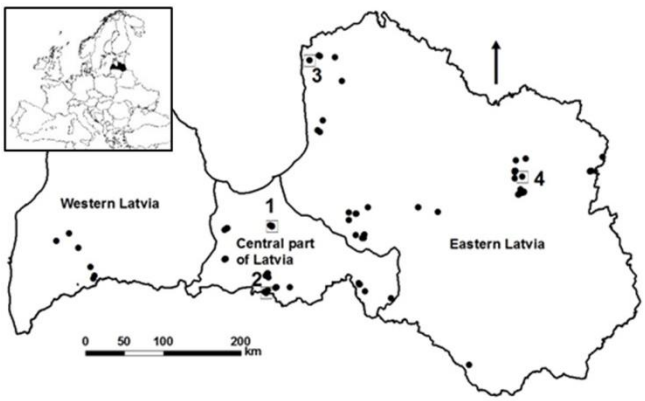

Figure 1. The location of study sites. Dots indicate the studied common ash regeneration plots. Squares denote the plots where stem discs have been collected for the estimates of age (1 – Plakanciems, 2 – Bauska, 3 – Ainaži, 4 – Lejasstradi)

Table 1. Classes of ash height, age and *Hymenoscyphus fraxineus* damage

Class	Age years	Height cm	Degree of ash damage%	Damage visual characteristics
I	< 10	< 50	0 - 10	Tree looks healthy or slightly damaged individual leaves.
II	11 - 20	50 - 100	11 - 25	Damaged several pages, some necrosis of the bark.
III	21 - 30	101 - 150	26 - 60	Fully damaged / dead separate branch; damaged part of the foliage; necrosis of the bark on large areas.
IV	31 - 40	151 - 200	61 - 99	Completely broken up dead part of the crown; partially damaged the entire crown; live separately branches in secondary crown.
V		201 - 250	100	Tree completely dead.
VI		251 - 300		
VII		> 300		

Within each young stand, one 2 × 100 m sampling plot was established along the longest diagonal of the forest district. Within each plot, all trees were accounted and their height was determined with the precision of 0.5 m (Table 1). All species were divided in two groups: advance growth containing all tree species, and undergrowth (shrub species). For each ash, degree of *H. fraxineus* damage was recorded according to five classes described in Pušpure et al. (2015) (Table 1).

To assess the relationship between ash height, diameter, age and the degree of damage, four of the studied young stands (two six-year old and two eight-year old), were selected (Figure 1, indicated by squares). In each stand, one 2 × 100 m sampling plot was established. In the sampling plots, all ashes were sampled, the cutting was done at the soil level and their height was measured with the precision of one cm. From each stem, a sample at its base (stem disc) was taken. The degree of *H. fraxineus* damage was recorded according to the five classes as described above. The number of felled trees per stand ranged from 108 to 160; at least 10 ashes in each height class were sampled. In the laboratory, stem discs were grinded and tree-rings were counted under a microscope.

Growth inventory in the study stands was conducted from mid-June to September 2015 when the damage of *H. fraxineus* was clearly visible and identifiable (Lygis et al. 2014). Ash samples were collected in August 2015. Dominant canopy species in previous rotation were determined according to the national inventory 2015; mostly they were ash (48% of all plots), ash with silver birch (14%) and grey alder (7%) admixture, spruce (8%) ect. The dominance of species in the advance growth was distinguished according to Simson (2006) (the dominant species comprises ≥ 50% of the total number of advance growth individuals and exceeds other species by 20%; the codominant species comprises 25–50% of all individuals). Soil types were distinguished according to data from the National State Forest Service inventory. Peat soils were considered if thickness of peat layer exceeds 30 cm.

Data analysis

To assess the effect of region and species composition on ash regeneration, generalized linear models (GLM) were applied. Differences in ash density according to soils and stand age (classes) were determined by the generalized linear mixed models (GLMM). The region (western, central and, eastern part of Latvia) as well as site was included in the models as the random factors. In both models, Gaussian distribution with "log" function was used. The models were based on the mean values for sampling plots. The GLMM method was also used to determine the factors (ash height and age, soil type, ash density, dominant species in advance growth, number of tending events) affecting ash health condition. For the tested factors, the central part of Latvia, aspen and dry mineral soils were chosen as the reference levels, to which other levels were compared. Such reference levels were chosen as the largest ash forests occur in the central part of Latvia, ash grows best on the mineral soils (Sakss 1958) and its health condition is the best in the stands with admixture of aspen. The significance of the GLMM was evaluated using the Likelihood ratio test (West et al. 2006). The relationships between ash density and height (classes from I to VI, Table 1), between health condition and height, and between the number of undergrowth and advance growth species were quantified by a bootstrapped (Johnson 2001) Pearson correlation analysis. The relationships between the ash diameter, age and height, were evaluated using a linear model. The differences in ash diameter, height and age between the sites and health classes were assessed by one-way ANOVA. The mean values of the gradation classes were compared using the Tukey HSD post-hoc test. The distributions of the dimensions of trees were compared by the chi-square test. All analyses were calculated at the significance level $\alpha = 0.05$ in the program R v. 3.1.2 (R Core Team 2014) using libraries "lme4" (Bates et al. 2014) and "lmerTest" (Kuznetsova et al. 2015).

Results

Species composition and ash regeneration

In total, 11 advance growth and 23 undergrowth species were accounted in the studied young stands, which had the mean density of 18410 ± 1040 trees ha^{-1}. The proportion of the advance growth and undergrowth individuals was 48.4 vs. 51.6. The undergrowth was dominated by two species – bird-cherry (*Padus avium* Mill.) (55% of total number of species, 5163 ± 638 shoots ha^{-1}) and hazel (*Corylus avellana* L.) (15%, 1399 ± 172 shoots ha^{-1}) (Figure 2). The advance growth density differed greatly and ranged from 1050 to 22900, with the mean value of 7150 ± 558 trees ha^{-1}. The highest advance growth density was observed for ash with 4185 ± 401 (ranging from 50 to 17750) trees ha^{-1} followed by grey alder (1620 ± 321 trees ha^{-1}), silver birch (681 ± 114 trees ha^{-1}) and aspen (687 ± 134 trees ha^{-1}) (Figure 2).

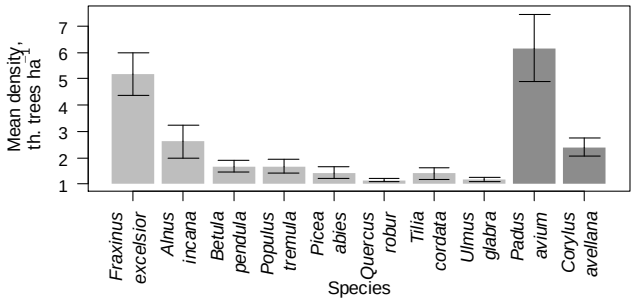

Figure 2. Mean density of the main understorey (advanced- and undergrowth) species

Ash advance growth density was similar amongst the regions (p-value = 0.06), age groups (p-value = 0.29), soil types (p-value = 0.58) and stands with different canopy species in the previous rotation (p-value = 0.06) (Figure 3a-d), yet some tendencies were observed. Ash density decreased with the increasing ash height and age. The density in the age group I was 5405 ± 951 trees ha^{-1}, while in the group IV 3036 ± 486 (Figure 3b). Although the correlation between ash advance growth density and ash height (up to three meters) was not significant (r = -0.08, p-value = 0.30), the highest density occurred in the height group I – 1857 trees ha^{-1}, but in the following groups, it decreased three time.

The highest number of ash was observed in the stands on the drained peat (mean 4584 ± 917 trees ha^{-1}), dry mineral (4275 ± 565 trees ha^{-1}) and drained mineral soils (4269 ± 846 trees ha^{-1}), but the lowest in the stands on wet mineral soil (1880 ± 331 trees ha^{-1}) (Figure 3c). The GLM analysis showed that the ash advance growth density was not significantly (p-value = 0.59) affect by the species composition. Yet the highest density of ash advance growth occurred in the stands where black alder (7300 ± 6 300 trees ha^{-1}) and birch (6933 ± 2711 trees ha^{-1}) were the main species, but the lowest (less than 2000 ash trees ha^{-1}) in stands dominated by lime and aspen (Figure 3d).

Incidence of Hymenoscyphus fraxineus in ash undergrowth

Of the 7533 accounted young ashes, 75% (5644 trees.) were considered as healthy, 15% (1134 trees) were damaged to varying degree, while 10% (755 trees) were dead. The degree of damage differed significantly (p-value < 0.001) among the regions (Figure 3a). The best ash health condition was observed in the central part of Latvia, where 78% of ash was healthy and 8% was dead, but the worst – in the western part of Latvia, where only 49% were healthy and 27% of ashes were already dead.

The degree of damage increased with age and height of ashes that was confirmed by the GLMM analysis (Figure 3b, 4). Significant correlation (r = 0.28, p-value < 0.001) was observed between disease intensity and height of ash. Up to 3 m height, 81% of ashes were healthy, but 4% were dead, while above the height of 3 m, these numbers were 54% and 33%, respectively. The age of young ash also had a significant (p-value < 0.001) effect on the occurrence of the disease. In the age group I, 81% of all ash trees were healthy, but in the group IV, it decreased to 58%, while the amount of dead trees was 3% and 24%, respectively.

The incidence of *H. fraxineus* damage differed significantly (p-value < 0.001) among the stands on different soils (Figure 3c). Similarly to ash density, health condition was the best in stands on the dry mineral and drained soils, e.g. 79% of ashes on drained mineral soils and 76% on dry

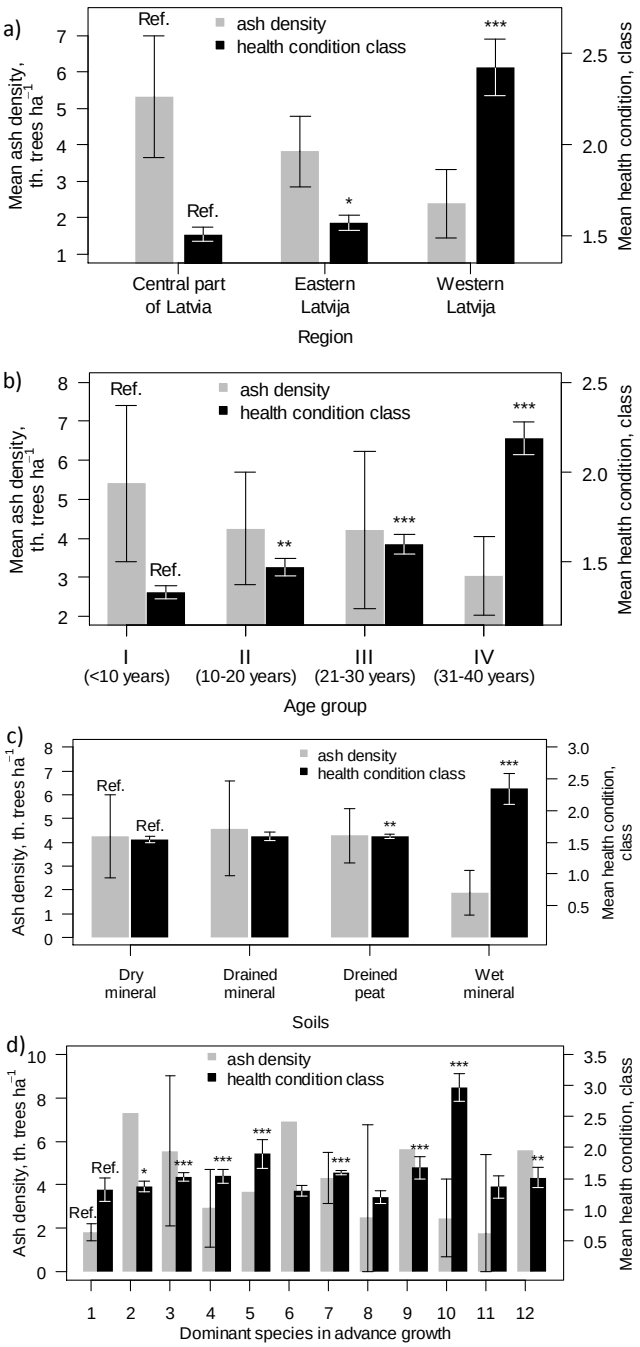

Figure 3. The mean density and health condition of common ash amongst the regions (a), age classes (b), soil type (c) and dominant species in advance growth (d) of Latvia. The asterisks indicate the differences from the central part of Latvia (a), youngest age class (b), dry mineral soil (c) and *Populus tremula* (d) used as the reference level (Ref.). Significance codes: * - p ≤ 0.05, ** < 0.01, *** - p ≤ 0.001. Dominant species in advance growth: 1 – *Populus tremula*, 2 – *Alnus glutinosa*, 3 – *A. incana* ,4 – *A. incana/F. excelsior*, 5 – *Acer platanoides*, 6 – *Betula pendula*, 7 – *F. excelsior*, 8 – *F. excelsior/B. pendula*, 9 – *Picea abies*, 10 – *P. abies/F. excelsior*, 11 – *Tilia cordata*, 12 - *T. cordata/F. excelsior*

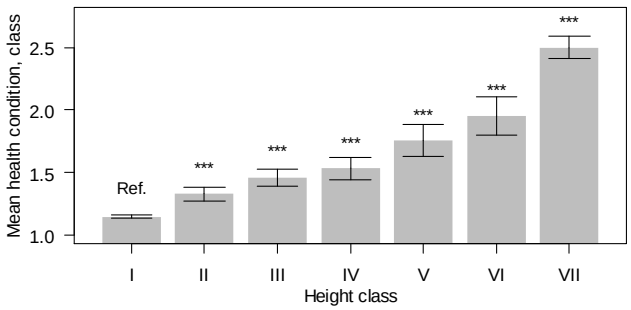

Figure 4. Ash health conditions in he studied young stands depending on tree height compared to the smallest height class, used as the reference level (Ref.). Significance code: * - $p \leq 0.05$, ** < 0.01, *** - $p \leq 0.001$

mineral soils had minimal or no symptoms. The highest degree of the damage of ashes was observed in stands on the wet mineral soils, where only 54% of trees were healthy and 27% were dead.

Although the relationship between ash density and ash health condition differed among sites, a significant (*p*-value < 0.001) negative logarithmic relationship between the mean health condition class and ash density was observed (Figure 5). Ash density was < 2000 tree ha^{-1} when the mean health class exceeded 4.3 (Figure 5). Analogically, negative correlations were observed between the ash density and the density of advance growth and undergrowth density, r = - 0.23 and - 0.24, respectively. The highest ash mortality was observed in the pure stands where 20% of ash was dead. In contrast, in stands where ash was in the admixture, its health condition was better and 82–95% of ash trees were healthy and only 1% was dead.

Health condition of ash significantly differed (*p*-value < 0.001) among the young stands with diverse dominant species (Figure 3d). The main species in the advance growth composition in the plots where ash (53% of the plots), grey alder (10%), grey alder/ash (9%) and spruce/ash (6%), yet the greatest *H. fraxineus* damage was observed in young stands formed by spruce/ash (40% of ash were dead), maple (*Acer platanoides* L.) (14%) and spruce (12%), but the lowest in ash/birch (87% of ash were healthy), birch (87%) and aspen (89%) young stand.

Health condition of the young stands was influenced by management. The intensity of ash dieback differed significantly (*p*-value < 0.001) among the stands with different number of tending events performed. The best ash health condition was in the young stands, which were tended four times, as the mean value of disease class score was 1.12, but it gradually increased with the decreasing number of tending events reaching 1.70 for untended stands. All differences among stands with different number of tending events were strictly significant (*p*-value < 0.001).

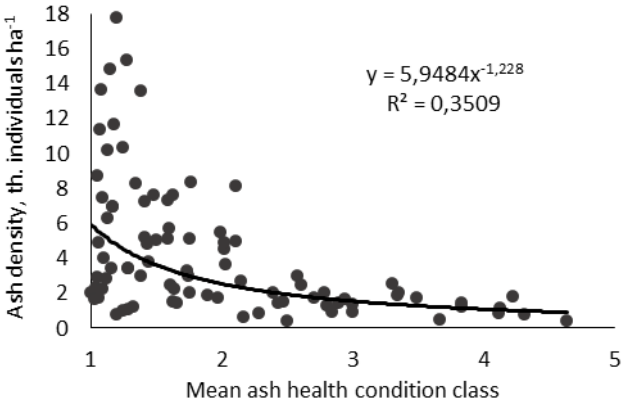

Figure 5. The relationship between the health condition and density of the common ash in the studied plots

Advance growth dimensions

The mean height of ash did not differ significantly (*p*-value = 0.57) among the studied four plots (regions), but the mean diameter (*p*-value = 0.001) and age of ash did (*p*-value < 0.001) (Table 2), although the mean age of ash in the first three height classes (up to 150 cm) was five years. A significant (*p*-value < 0.001) linear relationship between the diameter and height of ash was observed (Figure 6). The relationship between ash age and height was also significant (*p*-value < 0.001), still during the first 5–8 years, the height of ash increased irregularly and individually as rather high variability was present at each age (Figure 6).

Ash height distribution did not differ significantly between the sites (*p*-value = 0.57), but the diameter distribution differed only between Bauska and Lejasstradi sites (*p*-value = 0.02). In contrast, the age distribution was similar only in the same two sites (Figure 7). Ash height was significantly (*p*-value = 0.002) affected by the disease, but significant differences were observed only between the health classes I and II (*p*-value = 0.005).

Table 2. Ash measurement in stem discs collection plots

	Mean density, ash ha^{-1}	Young stand age, years	Mean age, years	Std. Error	Mean height, cm	Std. Error	Mean diameter, mm	Std. Error
Ainaži	4850	8	6.23	0.26	159.77	9.87	264.74	15.81
Bauska	5100	6	7.38	0.32	178.83	11.53	307.83	18.39
Lejasstradi	14850	6	7.23	0.35	166.02	10.32	211.63	13.97
Plakanciems	11650	8	3.77	0.22	175.30	9.50	266.60	16.19

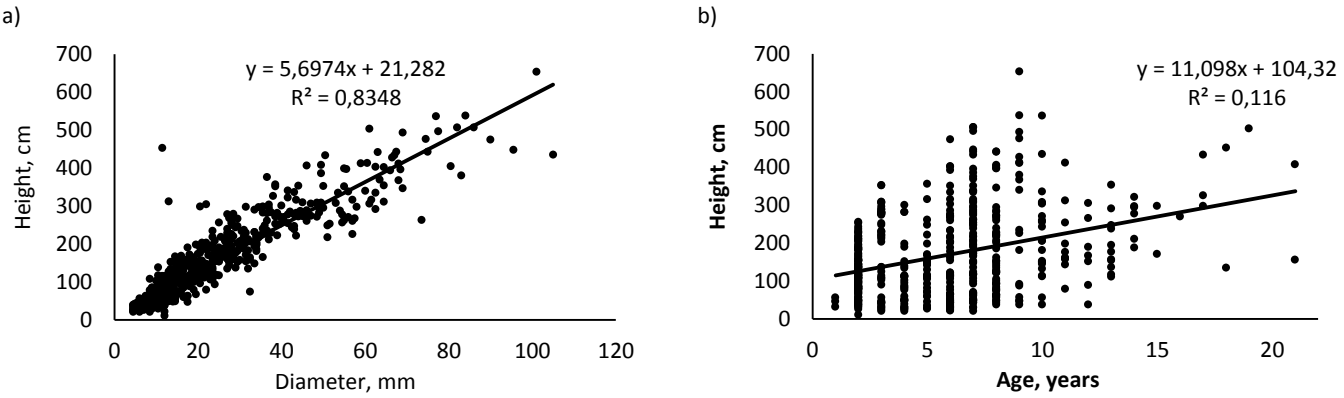

Figure 6. Relationship between the diameter and height (a), and age and height (b) of ash in the studied plots

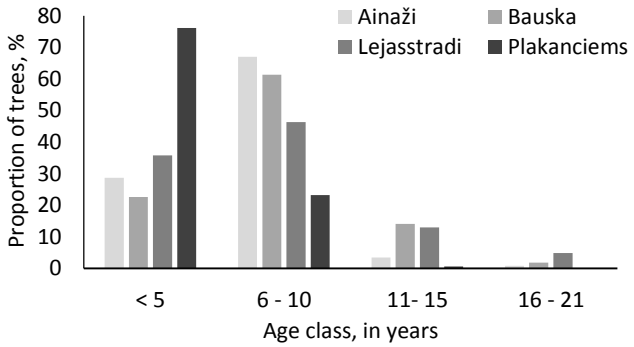

Figure 7. The age distribution of common ash in the understory of the studied plots

The best health condition was observed for the lowest trees. Similarly, ash diameter was affected by the disease (p-value < 0.001); smaller diameter trees were healthier. Ash health condition class differed significantly among the age classes (p-value < 0.001) and decreased with age.

Discussion

This study showed that the density of advance growth and undergrowth (18410 ± 1040 ashes ha^{-1}) was similar as observed before the decline (15 – 30 th. shoots ha^{-1}) (Sakss 1958), though growing conditions might have altered due to changing climate . The floristic composition of regeneration of ash stands, appeared little affected by the dieback as the same species have been observed before (Sakss 1958) and after (Figure 2; Lygis et al. 2014) the outbreak of the disease. Still, the proportions of regenerations species have been altered. In the studies stands, increased proportion of the undergrowth species was observed (Figure 2), suggesting ongoing changes in the stands. Although ash dieback can facilitate development of the undergrowth species, which consumes nutrients and alter light climate stressing ash (Keer 1998, Givnish 2002, Royo and Carson 2006, Skovsgaard et al. 2010), ash advance growth density was higher in stands with dense undergrowth (r = ca. -0.23). In the stands with the densest undergrowth (> 15 th. individuals ha^{-1}), ash advance growth also had the best health condition, likely due to higher species diversity.

Ash advance growth density (Table 2) was considerably higher than recently observed in the neighbouring Lithuania (Lygis et al. 2014) (4185 ± 401 vs. 599 trees ha^{-1}, respectively), suggesting regional differences in intensity of the dieback. Considering that the disease was spreading from south, better ash health condition in Latvia might be related to longer time available for the adaptation (Pliūra et al. 2011). Yet it was lower than before the outbreak of the disease in Europe, when more intense natural regeneration of ash (150000 young ashes ha^{-1}) occurred (Sakss 1958, Tabari and Lust 1999, Lygis et al. 2014). Nevertheless, Ahlberg (2014) suggested that in Denmark optimal ash advance growth density is 1500 individuals ha^{-1}, when the interspecific competition is the lowest. Ash advance growth density was inversely related to the disease intensity (Figure 5) that might be explained by the increased mortality and differences in the resistance of young ash. Similar relationships due to rapid development of the disease was observed by Enderle et al. (2013).

Ash density decreased with increasing age and height (Figures 3b, 4) as the density of the stands younger than 10 years was ca. 5000 trees ha^{-1}, but at the age of 31–40 years, it was only up to 56% of that density, thus following the reverse-J shape distribution of the natural regeneration. Normally, ca. 40–50% of the recruiting young ashes die annually, but under intensive disturbance, e.g. dieback, mortality can reach up to 85% (Harmer et al. 2005). Ash is a gap specialist, which is shade-tolerant at the sapling phase, but is light-demanding when reaches canopy (Petriţan at al. 2009, Kerr and Cahalan 2004), hence insufficient light conditions decreases its competitiveness with other species (Niemelä et al. 1992, Guzman and Dirzo 2001) and resistance against pathogens (Bakys at al. 2013) thus increasing mortality. Probably, the density of young stands was also decreased by the dieback, as a considerable

part of seedlings might be weakened and outcompeted by the herbaceous vegetation (Wardle 1961, de la Cretaz and Kelty 2002), especially in fertile sites (Dobrowolska et al. 2011). Still, the amount of dead seedlings (10% of total number) was lower compared to Lithuania (Lygis et al. 2014), supporting regional differences in health condition of ash. Skovgaard et al. (2010) showed that in a planted stand, small- and medium-sized trees were more susceptible to the disease. However, in this study in the naturally regenerating stands, the opposite was observed as the smaller ashes (height ca. 160 ± 50 cm and diameter ca. 23 ± 7 mm, which comprised 39% of all measured) were the most healthy (Figure 4), but the largest ashes (H > 240 cm, D > 45 mm which comprised 20% of all measured) were the most damaged, suggesting age-related increase in susceptibility to the disease.

Although diverse opinions about the effect of site type on the susceptibility to *H. fraxineus* damage persist in Europe (Bakys et al. 2013), in Latvia, higher susceptibility of ash was observed in the wet sites, as previously shown by Gross et al (2014). Ash is susceptible to prolonged waterlogging (Wardle 1961), hence the most abundant ash regeneration with the best health condition was observed in stands growing in well-drained and dry mineral soils (Figure 3c). The positive effects of drainage system on ash health condition has been emphasized in Denmark (Ahlberg 2014), Germany (Schumacher 2011) and other countries (Dobrowolska et al. 2011) as in the over-moist sites, trees have been more stressed, hence less resistant to disease.

Ash regeneration (mean 8064 trees ha^{-1}) and health condition (82–95% of ash trees were healthy) was better in the mixed stands. In Central Europe, establishment and development of ash seedlings is influenced by the canopy species composition (Götmark et al. 2005). Similarly, lower degree of damaged ash and better increments have been observed in mixed rather than pure stands (Dobrowolska et al. 2011, Schumacher 2011, Stener 2013). The lowest ash sampling mortality was observed in stands where certain satellite species occured (Givnish 2002). Likewise, in this study, the most abundant ash regeneration with the best health condition, was observed in stands formed by black alder and birch 7300 ± 6300 and 6933 ± 2711 trees ha^{-1}, respectively (Figure 3d), as demonstrated previously (FRAXIGEN 2005, Dobrowolska et al. 2011, Ahlberg 2014). Although ash is considered to have the lowest regeneration in sites with acidic humus layer (Tabari et al. 1999, Dufour and Piegay 2008), we found rather high regeneration density also in stands formed by spruce (5650 ± 2650 trees ha^{-1}). This might be related to decreased competition with other broadleaved species likely due to poor light conditions. Yet, ash health condition was considerably lower compared to broadleaved stands (Figure 3d) likely due to stress caused by root competition between ash and spruce (Lei et al. 2012). Although in mixed stands young ash has rapid development (Le Goff and Ottorino 1996, Keer 2004) thus outcompeting others (Rysavy and Roloff 1994, Dobrowolska et al. 2011), the disease might severely decrease its competitiveness. Hence, decreased ash health condition was observed in stands with maple admixture (Figure 3d) pointing to increased competition, as both species have similar growth strategies (Petritan et al. 2009), but the competitiveness of ash (Urbinati and Cillia 1995) has been weakened. In stands where ash was the canopy species, its regeneration density was lower (4319 ± 592 ash trees ha^{-1}) likely due to intraspecific competition.

High site-specificity of the increments of ash has been observed, as ash dimensions had a wide range within each of the four studied sites (Table 2), suggesting the plasticity of the species. The management of young stands had an effect not only on the height and diameter, but also on the health condition of ash advance growth. In the stands that have undergone several tendings, young ashes had a higher stem diameter and best health condition (Table 2, Figure 5), as the highest susceptible to disease has been observed in dense and unmanaged stands (Cech and Hoyer-Tomiczek 2007, Skovsgaard et al. 2010, Bakys et al. 2013). After thinning, the competition amongst ashes is decreased, thus minimizing biotic (competition) and abiotic (increased moisture) stresses (Niemelä et al. 1992, Guzman and Dirzo 2001). Hence, thinning might be recommended as one of the means to improve ash condition (Guzman and Dirzo 2001, Niemelä et al. 1992, FRAXIGEN 2005) also in Latvia. Though, excessive tending can also promote the disease (Bakys et al. 2013).

Conclusions

Our study showed that after 15 years since the initiation of the ash dieback, natural regeneration has been taking place in sufficient quantities. The floristic composition of advance growth and undergrowth species in the declining ash stands have remained similar with pre-dieback stands, yet the proportion of undergrowth species has increased, apparently altering the succession. It is expected that ash regeneration would continue on dry or drained sites, where the species was more abundant and their health condition was the best. Still, at present, 75% of the studied young ash trees are healthy, but mostly they are two to six years old, hence further monitoring is necessary. Considering current health condition and regeneration density, ash will apparently could remain as an admixture species in rich sites.

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Growth Performance of Dense Natural Regeneration of *Fraxinus excelsior* under Attack of the Ash Dieback Agent *Hymenoscyphus fraxineus*

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Abstract

Ash dieback, caused by the ascomycete *Hymenoscyphus fraxineus*, is a tree disease, which currently devastates European ash populations. Only a very small fraction of ash individuals exhibits a high degree of quantitative genetic resistance and is likely to survive the disease. We investigated the growth performance of differentially diseased saplings in order to assess the impact of ash dieback on individual competitiveness in dense natural ash regeneration. The research took place on three sites in south-western Germany. From summer 2013 to winter 2014 / 2015, 20.4% of the monitored ash saplings died. In general, shorter trees were more severely diseased. There were no differences in shoot length between healthy or moderately infected trees, whereas shoot length was significantly reduced in trees with more than 50% of symptomatic shoots ($p \leq 0.006$). These highly impacted trees significantly lost tree height to the disease, whereas only marginal height reduction could be detected for lesser affected trees. Our results indicate that trees resistant enough to maintain at least 50% of their crowns are generally able to survive competition in dense regeneration. Thus, promotion of natural ash regeneration could be an effective measure complementary to breeding for resistance to preserve ash as a tree species in the forests.

Key words: Ash dieback, *Fraxinus excelsior*, *Hymenoscyphus fraxineus*, Competition, Resistance, Tree diseases

Introduction

The fungus *Hymenoscyphus fraxineus* (syn. *H. pseudoalbidus*, anamorph: *Chalara fraxinea*) is the causal agent of ash dieback (Kowalski 2006, Queloz et al. 2011, Baral et al. 2014), a severe and devastating disease of European ash (*Fraxinus excelsior* L.) in Europe. It is suggested that *H. fraxineus* is native to Far East Asia and invasive to Europe (Zhao et al. 2012, Baral and Bemmann 2014, Gross et al. 2014). The disease emerged in the early 1990´s in Poland and has now been recorded in most parts of the natural range of *F. excelsior* (McKinney et al. 2014). In south-western Germany, ash dieback has first been recorded in 2009, but there is evidence that earlier infections occurred in 2006 (Metzler 2010).

Main symptoms are leaf necrosis, dieback of twigs and branches and collar rots that lead to high host mortality after several years of infection. Not only high economic cost resulting from the disease, but also serious ecological consequences following the collapse of populations of this important broadleaf tree species are expected (Jönsson and Thor 2012, Pautasso et al. 2013, Mitchell et al. 2014). However, in a small fraction of ash individuals the presence of high quantitative resistance against the disease has been detected. In several clonal trials and progeny trials, the resistance was demonstrated to be genetically determined and heritable (McKinney et al. 2011, Pliūra et al. 2011,

Kirisits and Freinschlag 2012, Kjær et al. 2012, Stener 2013, Lobo et al. 2014, Pliūra et al. 2014, Enderle et al. 2015, Lobo et al. 2015, Muñoz et al. 2016). Selective tree breeding in seed orchards may enable the establishment of more resistant populations of ash (McKinney et al. 2014). However, for sustainable conservation of the species, adequate genetic diversity in the populations must be retained. Kjær et al. (2012) estimated that approximately 1% of the ash individuals have the potential of producing offspring with less than 10% crown damage. Thus, tree breeding in seed orchards would be a labor-intensive and expensive solution.

In general, *F. excelsior* has a very high potential of regeneration. On suitable sites, young ash trees can grow very rapidly (Wardle 1961, Jaworski 1995). Up to an age of 20 years they are able to survive in stagnant conditions in deep shadow and react to sudden light exposure with good growth (Miegroet and Lust 1972). For example, Wardle (1959) observed a living 14-year-old ash sapling with a total stem length of 29.6 cm in the shadow of a dense layer of dog's mercury (*Mercurialis perennis)*. On suitable sites, these features make ash trees very competitive. In natural ash regeneration, densities of 100,000 plants per ha are not uncommon (Roloff and Pietzarka 1997) and can reach 150,000 individuals per ha (Tabari and Lust 1999). Strong intraspecific competition in such stands results in high evolutionary selection. It is likely that susceptibility to ash dieback diminishes the individual competitiveness of young trees and therefore acts as a decisive factor in early selection. Thus far, observed resistance against ash dieback is partial and quantitative and it is likely that even trees with a high degree of resistance will be moderately affected by the disease during their lifetime. If these trees are able to maintain their competitiveness, stands of dense natural ash regeneration may become a rich source of genetically diverse and comparatively resistant ash trees. Under this condition, silvicultural promotion of ash regeneration could be an effective measure complementary to breeding in seed orchards to conserve the tree species. However, little is known about the influence of ash dieback on the competitiveness of individual trees. From infested natural regeneration stands in Lithuania, ash was reported to have a smaller mean height compared to other tree species on the same sites (Lygis et al. 2014). Moreover, it is known that crown damage due to ash dieback is negatively associated with diameter increment (Skovsgaard et al. 2010, McKinney et al. 2011, Metzler et al. 2012, Stener 2013, Enderle et al. 2013, Lobo et al. 2014). In contrast, in a progeny trial the mean height of young trees increased steadily during two growing seasons, even after temporal height loss when diseased leader shoots had died (Pliūra et al. 2014). It is currently unknown whether moderately diseased individuals will be able to compete with other tree species.

The aim of this study was to investigate the severity and development of ash dieback in dense natural regeneration stands of *F. excelsior* in south-western Germany and its influence on height growth and tree height in order to gain knowledge of individual competitiveness of differently susceptible ash trees. It was intended to create knowledge that enables better assessment of the fraction of ash saplings that is able to survive in the long term in dense natural regeneration stands under attack of ash dieback. Such assessment is necessary when evaluating the prospects of natural selection leading to enhanced resistance in future ash populations. The knowledge can facilitate assessing the silvicultural management options, such as promotion of ash regeneration, that aim to mitigate the future impact of the disease.

Methods

Investigated stands

Data was collected in three stands with dense natural regeneration in south-western Germany, where generally high competitiveness can be expected for ash. Coordinates, elevation above sea level and climate information of the stands are presented in Table 1.

Table 1. Stand characteristics of investigated study sites.

	Stand 1	Stand 2	Stand 3
Latitude	48°19′12′′	48°35′21′′	49°16′23′′
Longitude	9°7′10′′	7°58′3′′	9°53′4′′
Elevation [mamsl]	870	140	480
Mean annual precipitation [mm]	1065	913	929
Mean annual temperature [°C]	6.0	10.2	7.9

Stand 1 was located in the Swabian Jura on shallow, moderately humid but good drained Cambisol on bedrock of Jurassic limestone. It was a 100- to 125-years old beech forest (*Fagus sylvatica*), mixed with about 5% sycamore maple (*Acer pseudoplatanus*), 5% *F. excelsior* and 3% other tree species. In the understory, there was a dense layer of natural regeneration consisting of ash (53%), beech (39%), maple (6%) and some other woody species (2%). Ash regeneration was in general higher than the beech regeneration. The vast majority of beech was smaller than one meter. Advance regeneration in this stand emerged after a winter storm had produced gaps in the canopy in 1990, but the vast majority of the regeneration arose following harvesting operations in 2001, 2004 and 2005.

Stand 2 was located in the upper Rhine valley on nutrient rich, wet soils (flood plain forest). It was established in 1990 on a former agricultural land by planting black alder (*Alnus glutinosa*), sporadically mixed with ash. Dense, understory natural regeneration was

present in an area of about 0.5 ha in the alder plantation, which was located in the vicinity of an adjacent ash-dominated mature stand. The regeneration consisted of 99% ash and 1% other woody plants (*Viburnum-*, *Alnus-* and *Prunus* spp.). There was a dense cover of herbaceous plants, mainly policeman's helmet (*Impatiens glandulifera*), *Mercurialis perennis* and common nettle (*Urtica dioica*).

Stand 3 was located on heavy, calcareous clay on bedrock of lower Keuper in the Swabian-Franconian mountain forest. The former old-growth stand was partially damaged during a storm event in 1999 and clear cut in 2005 after a severe bark beetle outbreak. Subsequently, some parts of the area were re-planted with pedunculate oak (*Quercus robur*), but most of these plants soon were outcompeted by strongly emerging natural regeneration of ash. In 2014, ash was the major tree species in the stand (91%), forming a dense layer that exceeded five meters in height in most parts of the stand. Admixed woody species in the regeneration were *Quercus* spp. (4%), sycamore maple (2%) and some other species (3%).

Data collection

The data collection in stands 1 and 2 occurred in July 2013 and was repeated in January 2015 to follow the development of ash dieback. Hence, the period of investigation included one complete growing season. In both of these stands, 15 circular plots with a radius of 1.5 m were investigated and the centre was permanently marked. In three plots in stand 1, the regeneration was damaged during harvest of mature trees in autumn 2014. Thus, data from only 12 plots were analysed in stand 1. In stand 3, data collection occurred in 14 plots in December 2014, and no repeated assessment was conducted. The locations of the plots were chosen randomly, with the following criteria: a minimum number of 15 trees per plot, which corresponds to a density of about 21,000 trees per ha, a maximum tree height of 5 m and a minimum distance between plot margins of 5 m. In the plots, all ash trees were assessed. For all other woody plant species present in the plots, the number of trees per species was recorded. Ash trees that were broken or obviously affected by factors other than ash dieback (e.g. browsing by game) were counted, but not further investigated.

In order to estimate the degree to which the ash trees were affected by ash dieback, trees were divided into classes of disease intensity. Infections of shoots and branches by *H. fraxineus* are easily recognizable by typical bark necroses, bark discolorations and abnormal branching structures (e.g. Kowalski and Holdenrieder 2008, Kirisits et al. 2009, Skovsgaard et al. 2010). The following classes were used (according to Enderle et al. 2013): class 0: no symptomatic twigs (completely healthy); class 1: 1 – 2 symptomatic twigs; class 2: 3 - 4 symptomatic twigs; class 3: 5 or more symptomatic twigs; and class 4: more than 50% of the twigs are symptomatic. The division of trees into classes was conducted regardless of the location of the infected twigs in the tree crown. As infection of the apical shoot (the leader) may have a special influence on tree growth and is crucial for the development of high quality timber, infections of apical shoots were noted additionally. Completely dead ash trees were counted and their height was measured.

As indicators for the individual competitiveness, the total tree height and the living tree height were recorded, such that a loss of height due to the disease could be calculated. The total height of all ashes was measured with a levelling staff. When the highest part of the tree was dead, the height of the highest living tip of the tree was also measured. As an additional indicator for competitiveness, the shoot growth of the present year of the highest living shoot was measured as the distance from the tip of the shoot to the first bud scar. In Figure 1, the method of measurement is sketched.

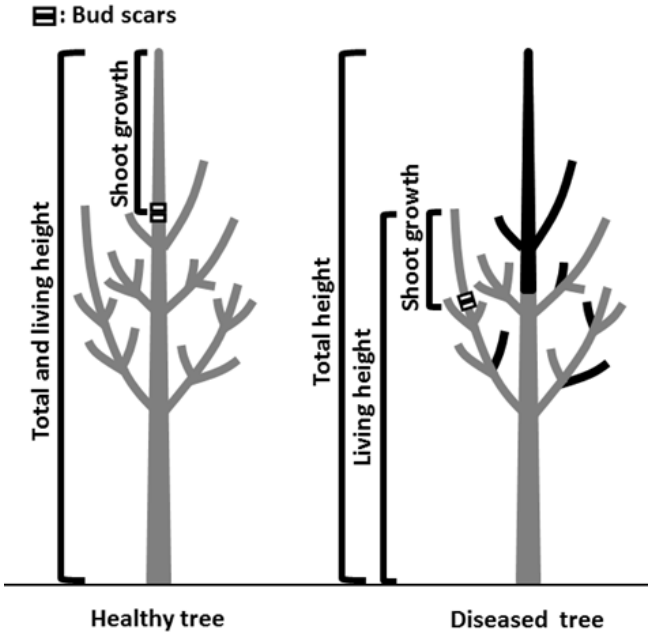

Figure 1. Sketch of the measurement method used to determine total height, living height and shoot growth on a healthy and a diseased tree. Black colour represents diseased or dead parts of the trees; grey colour represents healthy parts

Statistical analysis

Of special interest in this study was the situation of ash trees that had a chance to overcome other competitors. A main factor influencing competitiveness of young ash is their tree height in relation to their neighbours; smaller trees are more likely to be over-topped and hence to be outcompeted. Thus, analyses were conducted additionally for the stratum of trees of above-average height, to which belonged all ashes, including dead trees, with a height

higher than the overall mean of total height of the respective plot.

The height growth of young ash trees depends mainly on the light regime (Petritan et al. 2007) and is also influenced by other local micro conditions such as density and water and nutrient supply (e.g. Kerr and Cahalan 2004 and references therein). The mean shoot growth varied considerably between the plots and stands. Assuming relatively homogeneous micro-local conditions within the small plots, we eliminated the plot effect in order to compare the impact of ash dieback on individual shoot growth regardless of local conditions. For this purpose, we chose a ranking approach, because shoot growth was not normally distributed in the plots. The relative rank of shoot growth was determined for each investigated ash tree as follows:

$$[1]\ Rr_i = \frac{Rp_{ij}*100}{n_j} - \frac{100}{n_j*2}$$

where Rr_i is the relative rank of the shoot growth of tree i with the plot effect eliminated, Rp_{ij} is the rank of shoot growth of ash tree i within plot j and n_j is the number of ash trees in plot j. Rr can have values between 0 and 100. The range of Rr is wider in plots with a higher number of trees than in plots that have only few trees. In other words, the tree with the highest shoot growth of a plot with a high number of ashes was assigned a higher relative rank than the highest tree of a plot with a rather small number of ashes. The arithmetic mean of Rr is 50 (per plot and in total). For each tree, the loss of height was calculated as the difference between total height and living height as a percentage of the total height. None of the variables were normally distributed, so Mann-Whitney tests were used to investigate the significance of differences of variables between classes of disease intensity. For these tests, the classes of disease intensity 0 to 2 were combined, because of the small number of trees in these classes. The tests were performed using SPSS 21 (IBM, Chicago, USA).

Results

In 2013, there were 1,145 ash trees within the 27 plots of stand 1 and 2. A fraction of these ashes (1,037 trees) were examined in more detail (Table 2). Over the period of investigation, the total number of trees remained unchanged in the stands with repeated measurement, while the number of living ash trees decreased from 867 trees in 2013 to 690 in 2015 (Table 2). This corresponds to a mortality of 20.4% during the period of investigation. The reduction was especially evident in stand 1. The proportion of dead ashes increased from 14.8% to 23.7% over this period of time.

In total, in stand 1 and 2, 5.9% of the living ashes (51 ash trees, Table 2) showed no ash dieback symptoms (disease intensity class 0) in 2013. This fraction decreased to 4.6% in 2015 (32 ash trees, Table 2). Meanwhile, the proportion of ashes with more than half of the crown affected (disease intensity class 4) increased from 37.0% to 50.0% during the period of investigation. The disease in stand 2 was less severe than in stand 1 for both years ($p < 0.001$; Mann-Whitney test) (Figure 2). Moreover, decreased disease intensity was observed in the stratum of the higher trees (in 2013 $p = 0.001$; in 2015 $p < 0.001$; Mann-Whitney test). Especially, the portion of trees in disease intensity classes 4 was smaller in the stratum of above-average height.

In the 14 plots of stand 3, there were 341 ash trees, of which 338 trees were examined in more detail (Table 2). Here, 48.5% of the ash trees were already dead. Of the living trees, 76.4% were assigned to disease intensity class 4 and 21.3% to class 3. Only one tree was assigned to class 2 and three trees to class 1. No tree was considered completely healthy. Similar to trees in stand 1 and 2, trees of above-average height were healthier than trees belonging to the stratum of below-average height in stand 3 (Figure 2).

Table 2. Number of trees and ash trees in the plots separated by year and stand

Year	2013			2014 / 2015			
Stand	Stand 1	Stand 2	Both stands	Stand 1	Stand 2	Stand 3	All stands
Total number of:							
ash trees in the plots	542	603	1145	455	570	341	1366
ash trees examined	497	540	1037	423	510	338	1271
ash trees dead	83	87	170	169	74	164	407
living ash trees with symptoms of dieback	414	453	867	254	436	174	864
ash trees healthy	12	39	51	3	29	0	32
trees (all species) in the plots	1026	610	1636	1063	582	374	2019
Mean tree number per plot (all species)	85.5	40.7	60.6	88.6	38.8	26.7	49.2
Mean ash tree number per plot	45.2	40.2	42.4	37.9	38.0	24.4	33.3
Mean percentage composition of ash [%]	52.8	98.9	70.0	42.8	97.9	91.2	67.7

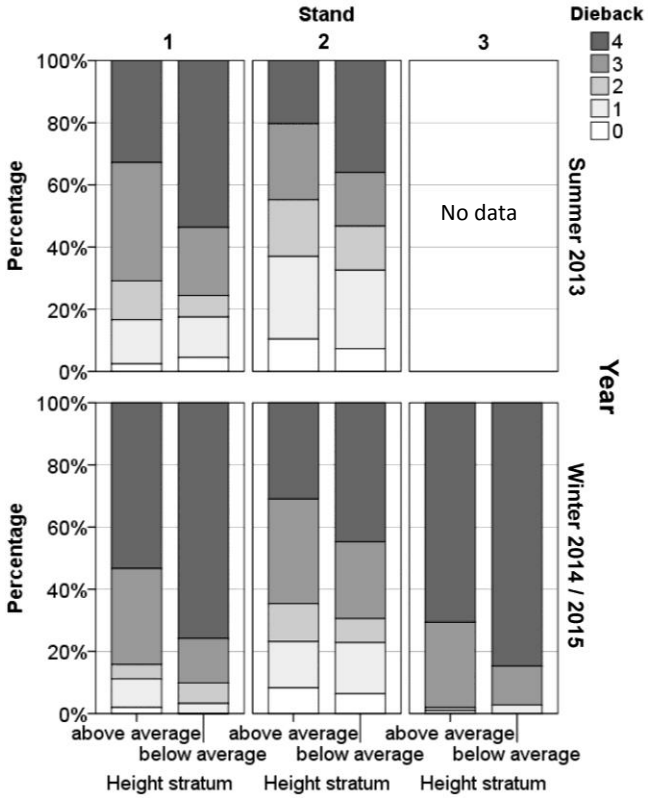

Figure 2. Percentage of ash trees in classes of disease intensity (dieback), differentiating for stand, year and stratum of relative tree height

The mean total height of living ash in stands 1 and 2 increased by 14.0% from 163.4 cm to 186.2 cm during the period of investigation. The trees were shortest in stand 2 and tallest in stand 3 (Figure 3).

The mean height of dead trees was smaller, but increased by 12.4% from 126.3 cm in 2013 to 141.9 cm in 2015. The total height decreased with increasing disease intensity (Figure 3). The differences in total height between classes of disease intensity were not that evident when considering only the stratum of the above-average relative height. The living height was significantly reduced for trees of disease intensity class 4 compared to all other classes, regardless of if all trees or only trees of above-average height were considered. Consequently, a considerable loss of height was characteristic to these ashes (Figure 3). Mean loss of height for trees of disease intensity class 4 was smallest in stand 2 in 2015 and highest in stand 1 in 2013 (Figure 3).

The annual shoot growth was highly variable in all stands and ranged from less than 1 cm to 134 cm (the highest shoot growth was detected on a tree of disease intensity class 4). The mean shoot growth was smallest in stand 1 and largest in stand 3 (Figure 4). The mean shoot growth of ashes of disease intensity class 4 was smaller than that of the other disease intensity classes. These differences between the classes were significant for stand 1 and 2, but not for stand 3, where the smallest number of ash trees were present. Shoot growth did not differ significantly between classes 0 to 2 and 3. Similar results were found when the relative rank of shoot growth (*Rr*) was considered (Figure 4). For trees of above-average height, the difference between class 4 and the other classes was not always significant. Moreover, the difference between the disease intensity classes was not significant for the trees in stand 3, where relatively small numbers of trees were present in this stratum and in the classes of lower disease intensity. By testing the data of shoot growth and *Rr* combined for all stands, class 4 was distinguished clearly from all other classes, which did not differ significantly from each other (Table 3).

In the stands 1 and 2, the proportion of living ashes with an infected stem leader remained constant (79.5% in 2013 and 80.3% in 2015) and was slightly reduced for the stratum of above-average height (70.3% in 2013 and 70.9% in 2015). In stand 3, 94.1% of the stem leaders were diseased. Approximately half of the trees of disease intensity class 1 had infected stem leaders. This proportion increased with increasing disease intensity, up to 100% for trees of class 4 (Figure 5). Within the classes 1 to 3, infection of stem leaders was connected to a significant reduction of shoot growth, indicating that shoot growth was more influenced by diseased stem leaders than by other infected twigs.

Table 3. P-values of comparisons of classes of disease intensity for shoot growth and the relative rank of shoot growth (*Rr*) combined for all stands according to Mann-Whitney tests. Above diagonal: summer 2013. Below diagonal: winter 2014 / 2015. Significant differences are labelled by * (Bonferroni correction: $p \leq 0.005$)

	Shoot growth					*Rr*				
Class	0	1	2	3	4	0	1	2	3	4
0	1	0.084	0.263	0.131	< 0.001*	1	0.187	0.567	0.151	< 0.001*
1	0.663	1	0.438	0.627	< 0.001*	0.653	1	0.293	0.861	< 0.001*
2	0.680	0.918	1	0.761	< 0.001*	0.956	0.597	1	0.328	< 0.001*
3	0.994	0.411	0.425	1	< 0.001*	0.708	0.199	0.513	1	< 0.001*
4	0.004*	< 0.001*	0.004*	< 0.001*	1	0.006	0.001*	0.001*	< 0.001*	1

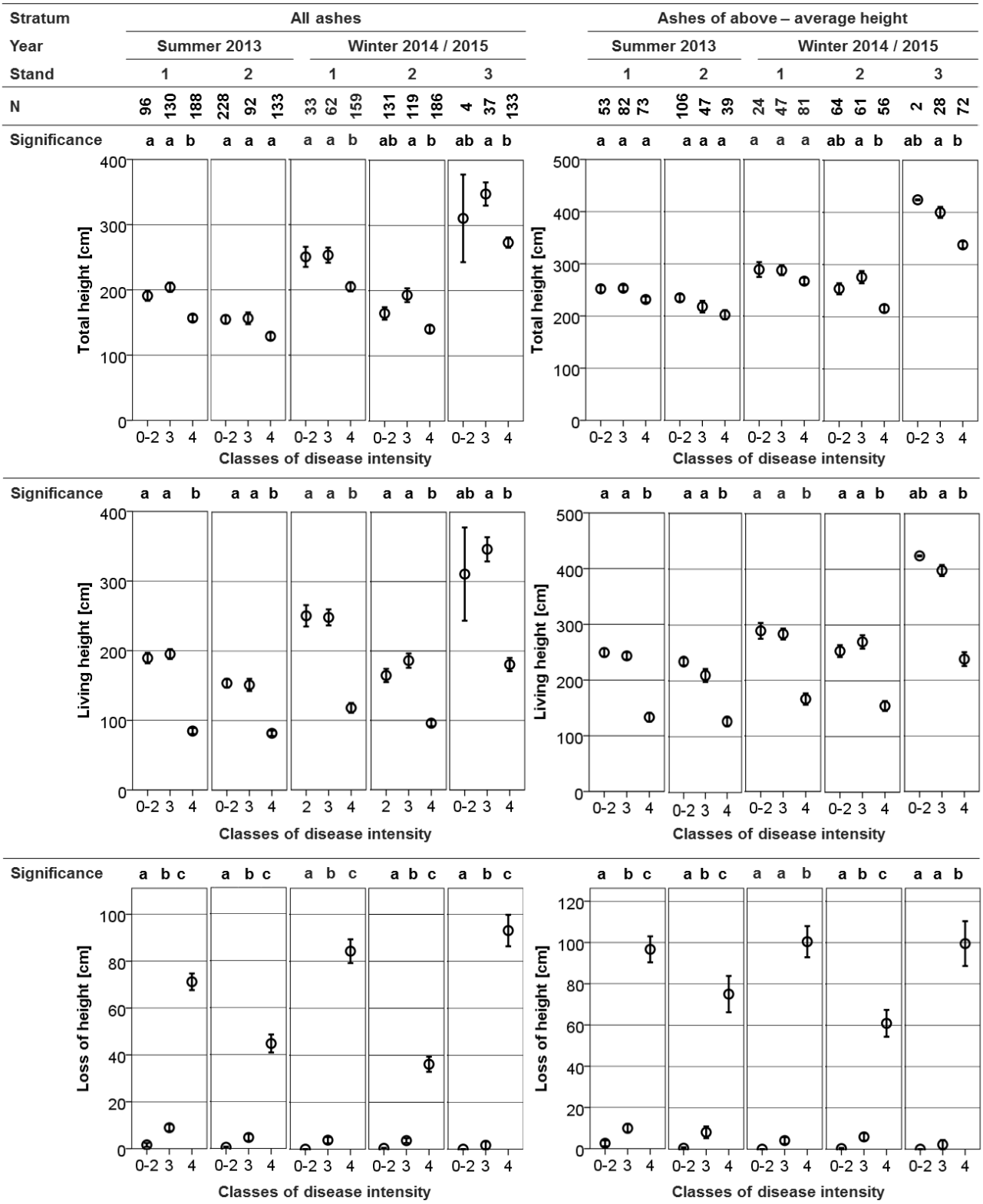

Figure 3. Mean ± standard error of tree height variables separated by classes of disease intensity for all ashes and for the ashes of above-average height in the different stands and years of investigation. The classes of disease intensity 0 to 2 are combined because of the small number of trees in these classes. Groups of trees with differing letters differ significantly according to Mann – Whitney tests with Bonferroni alpha correction ($p < 0.017$)

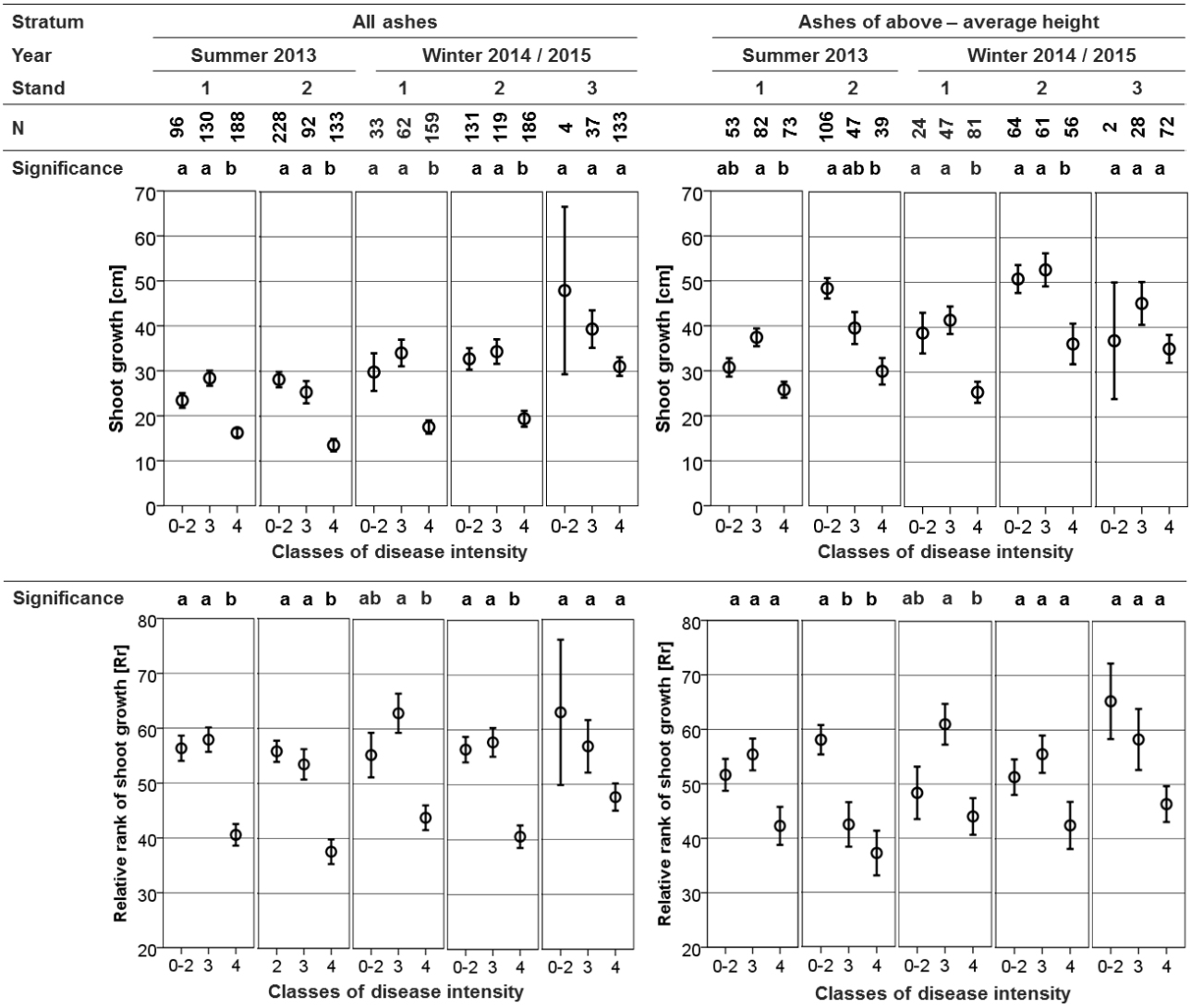

Figure 4. Means ± standard errors of shoot growth variables separated by classes of disease intensity for all ashes and for the ashes of above-average height in the different stands and years of investigation. The classes of disease intensity 0 to 2 are combined because of the small number of trees in these classes. Groups of trees with differing letters differ significantly according to Mann – Whitney tests with Bonferroni alpha correction ($p < 0.017$)

Discussion

Overall, the disease severity in the natural regeneration stands was similar to the disease intensity in a provenance trial located in south-western Germany (Enderle et al. 2013). As in the provenance trial, disease intensity increased with time.

The worst health condition was found in stand 3, which is located in the region where the oldest evidence for ash dieback, a necrosis occurring in 2006, in south-western Germany was found (Metzler 2010). The high disease severity in stand 3 may be due to its relatively long disease history. Furthermore, a criterion for the selection of the plot locations was a maximum tree height of five meters. In most areas of stand 3, regeneration was taller, so the investigated plots cannot be considered as a random sample of this stand. Dieback was more severe in smaller ash trees, and thus a bias towards an overestimation of ash dieback severity is likely in stand 3.

The proportion of asymptomatic trees was much smaller than in a Lithuanian study, where 43.6% of ash seedlings (not sprouts) in natural regeneration stands were asymptomatic (Lygis et al. 2014). This could be connected to the longer disease history in Lithuania, where processes of natural selection in favour of more resistant trees might already have commenced, as was indicated by results of a study of a Lithuanian progeny trial with different European provenances (Pliūra et al. 2014). But a smaller infection

pressure must be assumed for Lithuanian stands, given the decreased number of remaining ash trees. On the other hand, the proportion of dead trees is comparable between the Lithuanian and the present study. However, the amount of dead trees is difficult to interpret, as the time of death is unknown, and trees which died a longer time ago may not have been found anymore. The reduction of living ash of 20.4% in the stands with repeated measurements allows a better understanding of the mortality rate, which was considerably higher in stand 1 than in stand 2 (Table 2). The competition due to the high density of woody plants in stand 1 might have led to this result. Natural self-thinning depends on the maximum number of living stems, the mean stem diameter and the species (Reineke 1933) and must be considered thoroughly when interpreting the influence of ash dieback on the mortality.

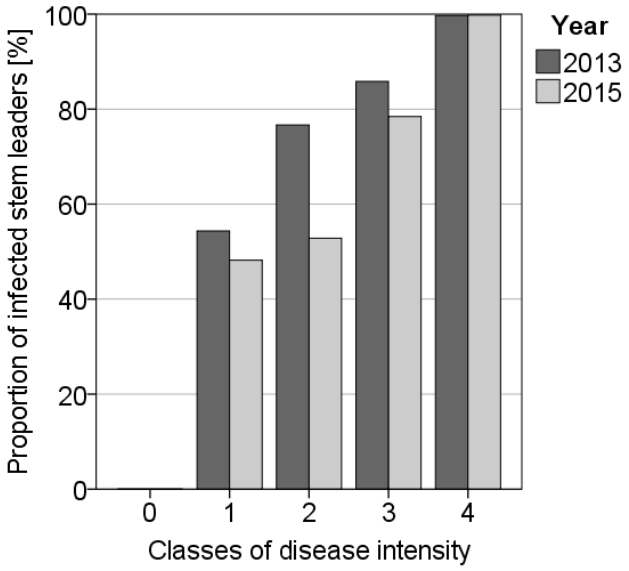

Figure 5. Percentage of living trees with infected stem leader in stands 1 and 2 by classes of disease intensity, differentiating for year

The shoot growth differed between the years of investigation, which is probably connected to the season of data collection. In 2013, fieldwork was conducted in July, when the growing season was not yet over and the yearly shoot growth might not have been completed in all individuals. In 2015, data collection took place in January. This has to be considered when interpreting the presented data, as there is high variation of disease activity between seasons (Bengtsson et al. 2014). However, the repeated measurements clearly demonstrate that patterns of height and height growth in differently diseased trees remained stable for more than one complete growing season.

The health condition of the stratum of the taller trees was significantly better than for shorter trees (Figure 2). On average, a smaller total tree height was recorded for trees of disease intensity class 4 (Figure 3). This could be either due to higher infection pressure (Chandelier et al. 2014) or better infection conditions (i.e. humidity) near the ground. Lobo et al. (2014) found strong negative correlations between the severity of ash dieback and diameter and height in an ash field trial established in 2001 and proposed that these correlations were due to the reduced crown area and the allocation of substantial amount of resources for the formation of epicormic shoots in more susceptible trees. In contrast, better health condition of smaller trees in natural ash regeneration was demonstrated in a recent study from Latvia (Pušpure et al. 2017). Apparently, the relationship between tree height and disease severity depends strongly on the development stage and the disease history of the stand.

The living height of trees of disease intensity class 4 was significantly lower than that of trees of the other classes of disease intensity, which did not differ noteworthy in this criterion (Figure 3). Moreover, marginal loss of height for trees up to disease intensity class 3, but a considerable loss of height for trees of disease intensity class 4 was observed (Figure 3). This result is remarkable, as infections were detected particularly often on the stem leaders (Figure 5). Trees with less than half of the crown affected by dieback can obviously compensate the loss of the upper part of the stem leader easily by enhancing growth of overtaking twigs.

A similar pattern was demonstrated for the shoot growth, where, on average, only trees of disease intensity class 4 showed reduced lengths of shoots (Figure 4). This result was further confirmed by the relative rank of shoot growth (*Rr*). However, for the stratum of trees of above-average height, the shoot growth difference was less pronounced. Also in stand 3, reduced shoot growth of the stratum of trees of above-average height was not significant. This might be due to the comparatively small number of trees in the classes of disease intensity 0 to 2. When the data of all three stands were combined, class 4 was clearly distinguished from all other classes (Table 3).

The largest observed annual shoot growth of 134 cm was recorded on a tree of disease intensity class 4, providing evidence that highly diseased trees can have extraordinary shoot growth, too. It can be hypothesized that this phenomenon may occur when the size of the crown is diminished rapidly by the disease and the few remaining twigs receive a bulk of resources from the still comparatively large root system. The loss of side twigs may even support the growth of the leading shoot. However, we think that these trees will not be able to compete with other trees in the long term, because of new and recurring infections every year linked with the loss of height and energy.

Tree height and height growth both are factors most relevant for inter- and intraspecific competitiveness in dense natural regeneration. Our results indicate that young ash trees maintain their height and height growth, as long as less than half of their crown is affected by the disease. Further research is required to confirm our results, which are based on observations in only three stands and one complete growing season, but our findings provide viable management options that aim to save the species for timber production.

Reports from areas with longer disease history demonstrate successful regeneration of ash under natural infection pressure. According to Lygis et al. (2014), average density of natural ash regeneration in former ash dominated and clear cut stands in Lithuania was only 599 plants per ha, which they traced back to relatively low numbers and the poor condition of seed trees in the clear cuts. Another study reports a sharp increase of advance ash regeneration from 2005 to 2015 in the understory of former ash dominated stands in Latvia (Pušpure et al. 2016). This indicates good prospects for prolific ash regeneration, if mature stands are not clear cut.

In conjunction with breeding programs in clonal seed orchards, stands with generally good site conditions for ash and with dense ash regeneration could become a simple, inexpensive and rich future source for genetically diverse and highly resistant ash propagation material. It is likely that in such stands, several hundreds of trees per ha are resistant enough to sustain in the long-term more than half of their crown. As our results indicate, these trees would have the prospective ability to survive the disease and the competition. However, an element of uncertainty is the serious symptom of collar rot (Bakys et al. 2011, Husson et al. 2012, Enderle et al. 2013, Muñoz et al. 2016). Most surviving trees will probably not be suitable for high quality timber production, as dieback affection in young plants, especially at the stem leader, is connected with a deterioration of stem quality (Enderle et al. 2013).

The symptoms of ash dieback are easily recognizable (e.g. Kowalski and Holdenrieder 2008, Kirisits et al. 2009, Skovsgaard et al. 2010), but it cannot be excluded that individual twigs that died due to other causes, such as shading, were incorrectly assessed as disease symptoms. This might have led to slightly inaccurate assignments of trees to the classes 0, 1 and 2. However, as these classes were combined in analyses, we are sure that this did not have a noteworthy influence on our results.

Conclusion

In this study, the severity of ash dieback and its effect on individual tree growth of *F. excelsior* saplings in natural regeneration was analysed. The influence of the disease severity on the individual competitiveness could thus be clarified. Severe damage due to ash dieback was found in the investigated natural ash regeneration. Repeated measurements showed a drastic increase of disease intensity, and experiences from earlier infested areas suggest further deterioration of the regeneration. However, our results indicate that young ash trees maintain their height and height growth, as long as less than half of their crown is affected by the disease. As these factors are very relevant for competitiveness in dense natural regeneration, it can be assumed that trees with a high degree of resistance are generally able to survive ash dieback and competition in dense natural regeneration in the long term. On suitable sites, silvicultural promotion of ash regeneration thus may be an effective measure complementary to breeding for resistance to maintain a participation of ash in European forests, to mitigate the impacts of ash dieback and to increase the degree of resistance in future ash populations.

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Host Phenological Stage Potentially Affects Dieback Severity after *Hymenoscyphus fraxineus* Infection in *Fraxinus excelsior* Seedlings

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Abstract

Ash dieback is a serious forest health problem throughout Europe attributed to the emerging, infectious pathogen *Hymenoscyphus fraxineus*. Preceding studies have revealed large genetic variation among *Fraxinus excelsior* trees in disease susceptibility, but the underlying mode of tolerance is still unknown. Previous research has revealed a genetic correlation between susceptibility and phenology, where the more tolerant genotypes were characterized by early flushing in the spring and early senescence (leaf yellowing) in the autumn. The main objective of the present study was to explore the influence of host phenological stage on symptom development after controlled inoculation with *H. fraxineus*. We induced early budburst in a set of seedlings, and compared the symptom development after artificial inoculation, with a control group where flushing had not been induced prior to infection. We observed that severe dieback symptoms were more frequent in seedlings infected prior to budburst compared to seedling inoculated after budburst. We speculate that resistance mechanisms may be more effective in the ash trees while in their growing seasons, and this can contribute to the observed genetic correlation between early flushing and reduced susceptibility.

Keywords: *Fraxinus excelsior*, *Hymenoscyphus fraxineus*, Inoculation, Phenology, Susceptibility, Tolerance

Introduction

Common ash, *Fraxinus excelsior* L., is currently under threat by the invasive pathogen *Hymenoscyphus fraxineus* (Bakys et al. 2009; Kowalski and Holdenrieder 2009). The pathogen has spread rapidly across Europe since it was first observed in Poland and the Baltic region in the 1990s, and ash forests in several countries are currently severely affected by the disease (Pautasso et al. 2013; McKinney et al. 2014). Several studies have revealed significant genetic variation in disease susceptibility among ash trees exposed to natural infections (McKinney et al. 2011; Kirisits and Freinschlag 2012; Stener 2012; Lobo et al. 2014; Enderle et al. 2015). The partial resistance is inherited from parents to their offspring (Lobo et al. 2015; Pliura et al. 2011; Kjær et al. 2012) giving hope for the future conservation and breeding of ash.

The genetic mechanisms behind disease tolerance among ash clones are not yet understood. Danish and Swedish studies have found significant genetic correlations between phenological traits and crown damage due to *H. fraxineus* infection (McKinney et al. 2011; Stener 2012). The genetic correlation between spring phenology (bud burst) and health of clones was significant but moderate in the Danish study, and showed that earlier flushing clones on average were less susceptible to the disease. Stronger genetic correlation was observed both in Denmark and Sweden for leaf senescence (measured as autumn colouring of leaves) and crown damage where less-affected clones showed earlier leaf yellowing interpreted as indicative of early senescence (McKinney et al. 2011; Stener 2012). As *H. fraxineus* is commonly believed mainly to infect the trees through their leaves during late summer (Gross et al. 2012) it has been speculated that an asynchrony between the phenology of the host (i.e. early senescence) and the

pathogen life cycle could assist the early-senescing trees to escape the disease. However, a controlled inoculation study showed that trees with a high level of resistance towards natural infections also developed shorter lesions when the leaf infection pathway was circumvented by controlled inoculation directly into the cambium with infected wood plugs (McKinney et al. 2012). The lesions continuously increased in size both while trees were foliated from late spring to early autumn and while trees were without leaves from autumn to spring (McKinney et al. 2012), but the lesions were shorter in the tolerant clones. This result points towards the presence of an active defence mechanism that can suppress the development of disease symptoms once the pathogen has invaded the branches, and that the observed partial resistance therefore cannot solely be attributed to phenological disease escape.

The few tested clones that performed well in the controlled inoculation study from 2012 (McKinney et al. 2012) were also among those that performed well under natural infection i.e. those with early bud burst and early leaf yellowing (McKinney et al. 2011). We therefore speculate that the phenological stage of the trees plays a role in an active host defence towards the pathogen. In the present paper we test whether ash seedlings are less affected by ash dieback if infected during their active growing season compared to being infected before flushing.

Materials and methods

Plant material

A total of 58 seedlings (offspring from a clonal seed orchard FP202 (Nielsen et al. 2009)) were used in the study. On January 20th, 2011, 28 two years old seedlings were placed in a greenhouse with the following light and temperature settings: 16 hours of daylight (from 06:00) at 19ºC (suitable for *H. fraxineus* (Kowalski and Bartnik 2010; Bengtsson et al. 2014)), 8 hours of darkness at 17ºC. During the testing period (March 21st to May 5-6th) the average midday temperature was around 20ºC in the greenhouse except for one week (21/4-30/4) where the temperate was between 24º and 27ºC. The remaining 30 seedlings (controls) were kept under semi outdoor conditions in a cold green house where temperatures followed outdoor conditions, although without frost.

Inoculation

Two isolates of *H. fraxineus* (3.2.1/1 and 3.5.1/2) were tested on the seedlings. The isolates were obtained from single spore cultures derived from two apothecia collected in August 2010 from a forest stand, Nørreskov, close to the city Aabenraa (GPS coordinates 55º03.531, 009º 24.825). The inoculum was grown on malt extract agar at room temperature and two weeks prior to inoculation sterile ash wood plugs (~1cm long) were added to the medium allowing the hyphae to invade the wood plugs. The stem of the seedlings were inoculated on March 21st, 2011 by cutting into the bark (1.5 cm in length) and placing an infected wood plug into the wound. Parafilm was wrapped around the inoculated stem. From the heated greenhouse 14 seedlings were inoculated with isolate 3.2.1/1 (Isolate 1) and another 14 were infected with isolate 3.5.1/2 (Isolate 2), while from the cold house 15 plants were inoculated with the two isolates, respectively. Of the 28 plants from the heated greenhouse all except one had flushed at the time of inoculation while the 30 plants from the cold greenhouse were all without signs of budburst.

Assessment

Plants were assessed on 5-6/5 2011, approximately 6 weeks after inoculation. The longitudinal spread of the necrosis was assessed by measuring the sum of the upwards and downwards visible length of the necrosis (*i.e.* discoloration of the outer bark). Crown damage of the plants was scored in categories where 0 was no visible symptoms, 1: few visible symptoms, 2: clear visible symptoms with main shoot beginning to wilt 3: shoot above inoculation spot dying or dead, 4: entire seedling dead. Three plants never flushed (1 from the warm house and 2 from the cold house), hence, we could not score crown damage of these plants.

Data analysis

Analysis of variance (ANOVA) was performed for necrosis length with stage (before flushing versus after flushing) and isolate (3.2.1/1 versus 3.5.1/2) as treatments. Necrosis length was square root transformed before analysis to meet the assumptions behind the analysis of variance model. For crown damage, significance of differences between stage and isolate were analysed by Fishers exact test of the 2 x 4 frequency tables.

Results

Around 6 weeks after inoculation the lesions were approximately 4 cm long for the non-flushed cold house seedlings (mean ± SE for isolate 3.2.1/1: 5.23 ± 0.75 cm and isolate 3.5.1/2: 3.65 cm ± 0.99 cm) while around 5 cm for the flushed seedlings inoculated in the heated greenhouse (isolate 3.2.1/1: 5.36 cm ± 0.54 cm and isolate 3.5.1/2: 5.56 cm ± 1.45 cm. These differences were neither significant between the stage of the plant when inoculated ($P < 0.42$) or between the two isolates ($P < 0.34$). However, the distribution of crown dieback symptoms was significantly different between seedlings inoculated before and after flushing, ($P = 0.04$; based on Fishers exact test, Table 1). The frequency of seedlings with severe symptoms (class 3) was thus 25% (7/28) in seedlings inoculated prior to flushing compared to 4% (1/27) in seedlings inoculated

after flushing (Table 1). Differences between isolates were not significant (P = 0.27) based on Fishers exact test.

Table 1. Distribution of seedlings to ash dieback damage classes six weeks after controlled inoculation. Inoculations were done March 21 2011. Approximately half of the seedlings had been forced to flush in a heated greenhouse prior to inoculation (inoculated after flushing) while the other half was kept in a cold house and had not flushed (inoculated before flushing)

Inoculation	Class 0 No symptoms	Class 1 Few symptoms	Class 2 Severe symptoms	Class 3 Very severe
Before flushing	17	2	2	7
After flushing	23	3	0	1

Discussion and Conclusions

Our results suggested that the phenological stage of the tested ash trees (seedlings) influenced the symptom severity when challenged by *H. fraxineus* through controlled stem infection. The growth (lesion lengths) of the pathogen was slightly higher in plants inoculated in the heated greenhouse, probably because the temperature was more suitable for the growth of *H. fraxineus* (Bengtsson et al. 2014; Kowalski and Bartnik 2010). Similar to field observations by Bengtsson et al. (2014) lesions did also develop under cold conditions and the difference in lesion length was not significant, and fairly fast necrosis development was observed in some seedlings kept in the cold greenhouse. The significantly stronger crown dieback symptoms in seedlings from the cold house compared to seedlings that had already flushed before inoculation may indicate a less effective defence mechanism in the seedlings while partly in winter dormancy. The presented dataset is small and additional studies are required to verify this hypothesis.

A relation between the severity of infections and host tree phenology has been observed in other tree pathosystems. Sudden oak death threatens the coast live oak (*Quercus agrifolia*) in central and northern California, where the pathogen (*Phytophthora ramorum*) is known to sporulate from December to May (Davidson et al. 2005). An annual cycle of inoculation experiments showed that the lesion size after infection was highly correlated with the cambial activity measured (Dodd et al. 2008). Trees with an early onset of cambial activity would therefore be more susceptible to infections because the activity coincides with the sporulation of the fungus. Similarly, in elm, it has been shown that budburst and susceptibility to Dutch elm disease was highly correlated in *Ulmus minor*, suggesting that early flushing clones were less affected by inoculations (Santini et al. 2005). The time of maximum susceptibility to *Ophiostoma novo-ulmi* happens simultaneously with the maximum growth rate and formation of large size vessels, indicating that infection is highly dependent on host cycle and phenology (Solla et al. 2005).

In the above cases, the timing of host susceptibility must match with a limited window for infection and proliferation of the fungus. Asynchrony of these events may therefore contribute to lower disease susceptibility in these species in the form of disease escape. It has been questioned if the strong correlation between crown damage and senescence in *F. excelsior* (early senescing ash clones being the most healthy ones) could likewise reflect disease escape (McKinney et al. 2011). It is known from Norway that pathogen sporulation peaks from mid-July to mid-August with corresponding fungal DNA concentrations in living ash leaves reaching a high plateau around mid-August, several weeks before leaf shed (Hietala et al. 2013). These results contradict the explanation of lower susceptibility by disease escape, but pre-dormancy changes (biochemical or physiological) taking place already in the early autumn could perhaps dis-favour the spread of *H. fraxineus* from infected leaves into the stem.

Our suggestion, that host phenology at the time point for infection plays a role in disease development, supplements earlier results showing that crown damage correlates negatively at the genetic level to early spring flushing (McKinney et al. 2011). If an active defence response depends on the host being in its active growing season, this could give the early flushing ash trees an advantage in supressing early infections before the temperatures become more suitable for *H. fraxineus* during late spring and summer.

The physiological, anatomical and/or chemical mechanisms involved in the observed correlation between phenology and partial disease resistance in *F. excelsior* remain unknown. Recent studies have revealed a strong association between disease susceptibility and MADS box transcription factors (Harper et al. 2016; Sollars et al. 2016) that may function as regulators of plant developmental processes (Pařenicová et al. 2003). These MADS box genes may be involved in the observed patterns.

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Susceptibility of *Fraxinus angustifolia* Clones to *Hymenoscyphus fraxineus* in Lowland Croatia

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Abstract

In this study on the narrow-leaved ash's resistance to *Hymenoscyphus fraxineus*, significant differences were obtained between the nine tested clones, among which the clones BJ25, BJ38, NG03 and NG31 turned out to be the least susceptible to the pathogen. A significant difference was obtained between genotypes in clonal seed orchards for the leaf unfolding parameter as well. Earlier leaf unfolding was found in clone NG03, which revealed smaller necrosis development in the inoculation experiment, while the clone NG55 revealed later leaf unfolding and longer necrosis lengths, leading to the conclusion that phenology could play an important role in narrow-leaved ash clones' resistance to *H. fraxineus.* Preliminary results on three types of agar medium with leaf extracts from native Croatian *Fraxinus* species revealed that the nutrition status of the ash host could also play a role in resistance to the pathogen. The experiment revealed the fastest growth of the pathogen isolates on the agar enriched with *F. excelsior* leaf extract, followed by the agar with *F. ornus* leaf extract, while the slowest growth was obtained on the agar with *F. angustifolia* leaf extracts.

Keywords: narrow-leaved ash, clones, fungal pathogen, resistance

Introduction

Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz, Hosoya (basionym: *Chalara fraxinea*) a new invasive pathogen, has shown its presence to be the cause of severe dieback of ash (*Fraxinus* spp.) throughout Europe since the 1990s. In Croatia, it was first recorded on the common ash (*Fraxinus excelsior* L.) in 2009 in Gorski Kotar, a mountainous area close to Slovenia (Barić and Diminić 2010). Typical symptoms of the disease: discolouration and wilting of leaves and petioles, formation of necrotic tissue progressing to elongated necrotic lesions and subsequently forming open cankers on the stem, and inner wood discolouration have been observed and described by many authors (Lygis et al. 2006, R. Bakys et al. 2009a, Kowalski and Holdenrieder 2009, Kirisits 2012, Gross et al. 2014). Two main infection pathways of the pathogenic fungus have been proposed, one via ascospores that infect leaves and petioles and then progress to the ligneous tissue (Kirisits et al. 2009, Kräutler and Kirisits 2012), and the other through lenticels (Husson et al. 2012).

Since 2009, *H. fraxineus* in Croatia has been found and confirmed in natural forest stands of the two native ash species, the common and the narrow-leaved ash (*Fraxinus angustifolia* Vahl) (Barić et al. 2012). Attempts to isolate the pathogen from the third native Croatian ash species, *Fraxinus ornus* L., have not been successful in natural conditions to date.

The narrow-leaved ash in central Europe and the Pannonian Basin in the Balkans occurs mainly in riparian and floodplain forests (Fraxigen 2005). In Croatia, the narrow-leaved ash is distributed in the Pannonian lowland area in mixed stands with the pedunculate oak (*Quercus robur* L.). The largest complexes of *F. angustifolia* in Croatia

(80%) and the majority of its genetic variabilities are located in the area of 30 000 ha along the Sava river, hence two clonal seed orchards of the narrow-leaved ash were established. The first established clonal seed orchard of *F. angustifolia* (Nova Gradiška) represents the eastern Sava river seed provenance, and the second clonal seed orchard (Čazma) represents the western Sava river seed provenance. The implementation of feasible conservation measures in order to preserve the ash in its natural environment is of great importance as a part of sustainable forest management policies in Croatia.

In affected ash stands, the number of trees which are capable of producing seeds is reduced to a few individuals per hectare, meaning that the effective population size (N_e) has decreased substantially, thus compromising the genetic diversity of *F. excelsior* in mature stands and in regenerating offspring (Pliûra et al. 2011). The current genetic diversity might be insufficient for further physiological and genetic adaptation and sustainability of ash populations, thus predisposing damaged populations to collapse (Pliûra et al. 2014). Several European authors have implied that among the limited disease control measures, the most promising potential option would be to take advantage of the naturally occurring individuals' resistance to the fungus (Bakys et al. 2009b, Kirisits et al. 2009, McKinney et al. 2011, McKinney et al. 2012b, Kirisits and Freinschlag 2012). So far, the work carried out on *F. excelsior* has revealed that there is a significant difference among individuals, populations and families regarding their tolerance to *H. fraxineus* (Kirisits et al. 2009, Pliūra et al. 2011, McKinney et al. 2011, McKinney et al. 2012b, Stener 2013). However, there is very little work published regarding the individual resistance of *F. angustifolia* to ash dieback (Hauptman et al. 2016), Schwanda & Kirisits 2016). Temporal dynamics of the disease and genetic characteristics of the affected populations of ash stands are also crucial for the evaluation of genetic variability, sustainability and adaptation potential of ash species.

A substantial variation has been observed in the degree of damage and symptoms occurrence in natural populations of ash stands in Croatia. Similar reports have been published all across Europe on *F. excelsior* natural stands, but as detected from the various progeny trials, only 2-5 % of trees remained symptom-free (McKinney et al. 2011, Pliûra et al. 2011).

A large number of European countries, through research on natural stands, progeny trials and clonal seed orchards, provide evidence that susceptibility of *F. excelsior* to *H. fraxineus* varies considerably between individuals, populations and families, and that there is a significant genetic heritability in disease resistance/tolerance (Pliūra and Baliuckas 2007, Pliûra et al. 2011, McKinney et al. 2011, Kjær et al. 2012, Husson et al. 2012, Kirisits and Freinschlag 2012, Stener 2013).

The aim of this study was to provide initial results about possible differences in susceptibility to *H. fraxineus* among the studied clones of *F. angustifolia* from two clonal seed orchards in Croatia. As part of the research, an analysis was conducted of the leaf unfolding phase in the clonal seed orchard of the narrow-leaved ash to determine intrapopulation and interpopulation variability and the existence of ecotypic forms in relation to the beginning of leaf unfolding. A correlation among the phenology and the degree of damage on *F. excelsior* has been reported (McKinney et al. 2011, Stener 2013, Bakys et al. 2013), but the data on *F. angustifolia* is still insufficient. An additional preliminary test was done on *H. fraxineus* isolates growing on agar with leaf extracts of the three native Croatian *Fraxinus* species to complete the data on possible differences in resistance or tolerance in ash.

Materials and methods

According to the aim of the research of investigating possible differences in susceptibility of *F. angustifolia* to *H. fraxineus*, an inoculation experiment was performed on seedlings selected from the two clonal seed orchards of Nova Gradiška and Čazma. Isolates of *H. fraxineus* used in the experiment were obtained from *F. excelsior* from the site where the disease was recorded for the first time in Croatia in 2009. Phenology of the narrow-leaved ash clones was included in the study and focused on the phase of the beginning of leaf unfolding as one of the possible factors which could influence the host's susceptibility to the pathogen. The host-pathogen relationship was tested in a growth experiment on the *H. fraxineus* isolates growing on different media containing leaf extracts of the three native *Fraxinus* species of Croatia. The obtained data were statistically analysed.

Clonal material

In the study, nine narrow-leaved ash clones originating from two clonal seed orchards were preselected for susceptibility trials against the invasive pathogenic fungus *H. fraxineus*. Narrow-leaved ash clones originated from the two clonal seed orchards of Nova Gradiška and Čazma, each representing one of the two most important provenance seed regions of *F. angustifolia* in Croatia. The first clonal seed orchard, Nova Gradiška, was established in 2005 on an area of 3.5 ha with a total of 56 clones from the eastern Sava river seed provenance. The grafts were planted with a 4 × 4 m spacing. The second clonal seed orchard, Čazma, was established in 2007 on an area of 7.3 ha. The grafts were planted with a 5 × 5 m spacing, and the orchard contains a total of 50 clones representing the western Sava river seed provenance. Nine clones in total were used in the research, four of which represented the eastern Sava river seed provenance clones from the orchard in Nova Gradiška:

clones NG03, NG31, NG41 and NG55; and five of which represented the western Sava river seed provenance clones from the orchard in Čazma: clones BJ25, BJ28, BJ32, BJ35 and BJ38.

Fungal isolates

The two isolates of *H. fraxineus* used for inoculations were obtained in spring 2014 from the Educational and Research Centre Zalesina of the Faculty of Forestry, University of Zagreb, located in the Gorski Kotar region, where the disease was first detected in Croatia (Barić et al. 2010). Isolate no. 1 was obtained from the micro-location with the coordinates: 45.383858°N, 14.873822°E; from a symptomatic solitaire tree of *F. excelsior*, 20 cm in diameter and 18 m in height, from the wood tissue sample taken as a cross-section of the trunk, approximately 1 m from the ground level. Isolate no. 2 was obtained from the micro-location with the coordinates: 45.385710°N, 14.872510°E; from a symptomatic young tree of *F. excelsior*, 4 cm in diameter and 3 m in height, from the shoots of the crown with visible necrosis, and the tissue sample for isolation was taken from the wood tissue just beneath the bark. Shoots were collected at approximately 2 m from the ground level.

Samples were processed according to the EPPO diagnostic standard PM 7/117 (1) (EPPO 2013) for *H. fraxineus*. Pieces of plant tissues (woody material without bark) were disinfected in 10 times diluted commercial bleach (20 s), rinsed three times in sterile water, cut into pieces of approximately 5 mm^2 in size, and placed on 2% malt extract agar (MEA Oxoid™ CM0059) supplemented with 100 mg/l streptomycin sulphate (Sigma-Aldrich GmbH). Petri dishes were incubated at room temperature in the dark for 4–5 weeks. Isolates after purification were subcultured on 2% MEA.

Inoculation of grafts of Fraxinus angustifolia

Narrow-leaved ash clones were produced by grafting one-year-old scions onto one-year-old rootstocks in the nursery. One year before the beginning of the experiment, clone seedlings were replanted into plastic containers of 7.5 l to ensure their proper establishment and adaptation. Seedlings were placed in the nursery of the Faculty of Forestry in order to be exposed to natural climatic conditions. Selected clones were inoculated by two *H. fraxineus* isolates in order to monitor the development of induced necroses in plant stems during the specified period of time. The five clones from the clonal seed orchard of Čazma were represented with 15 ramets each, while the four clones from the clonal seed orchard of Nova Gradiška were represented with nine ramets each, resulting in a total of 111 inoculated plants. Each *H. fraxineus* isolate was used for inoculation of five ramets of every clone originating from the Čazma orchard, leaving the remaining five ramets of each clone as a control group. The procedure was repeated for the clones originating from the Nova Gradiška orchard, with the difference of inoculating three ramets of every clone with each isolate and leaving three ramets per clone as a control group.

Prior to the wound formation (inoculation spot), each plant stem was sterilized with 96% ethanol in the area 10 cm above and below the planned inoculation spot, approximately 20 cm above the root collar. Inoculation wounds were made with a special circular sharp tool of 0.5 cm in diameter, and potential bark remains were removed with a sterile scalpel. Mycelium plugs (0.5 cm in diameter) were cut from the margin of 4-week-old *H. fraxineus* cultures grown on the PDA (Difco™ No. 213400) at 20 °C and placed into the wound. Inoculation spots were covered with a Parafilm™ sealing tape and additionally covered with aluminium foil. All the tools and equipment were sterilized with ethanol (96%) and flame prior to each use. Control plants were inoculated with sterile PDA (Difco™ No. 213400) plugs (0.5 cm in diameter), following the same procedure as for inoculations with isolates.

Seedlings were inoculated on 30th September 2014, and stem diameter at the inoculation spot of each ramet was measured. Plants were monitored and necroses measured every two weeks in the period from 14th October 2014 to 17th February 2015. Plants were inspected once more one year after the inoculation, on 22nd September 2015, to observe the one-year progression of the disease and to establish the survival rate of the experimental plants.

Phenology observation in clonal seed orchard

The Nova Gradiška clonal seed orchard of the narrow-leaved ash contains 56 clones (plus trees) selected in natural stands. The clonal seed orchard has been regularly maintained by pruning and with other agrotechnical and pomotechnical treatments from the moment of establishment (Kajba et al. 2008).

Through a period of three years (2012, 2014, 2015), phenological clonal differences of flushing phases to determine interclonal and intraclonal variability were observed and studied. Analysis was focused exclusively on the phase of the beginning of leaf unfolding. The average number of days from 1st January and the start date of the leaf unfolding stage were defined as the point at which the entire leaf blade and leaf stalk were visible. Each year, observation began before any buds began to break (10th March), and was performed every seven days until 1st June. Monitoring included 42 clones originating from three populations (Jasenovac, Novska and Stara Gradiška), with the ramets randomly selected across the entire area of the orchard. Each clone was represented with four ramets (168 plants in total).

Fungal growth on leaf extracts

Due to a complex life cycle of *H. fraxineus* and susceptibility of the plant which may be expressed in the leaf or/and wood (Kirisits and Freinschlag 2012), the conducted experiment was based on the growth rate of the selected two isolates on four different growth media for two weeks at 20 °C. The experiment was designed as a preliminary test which could reveal possible differences in the growth of the same isolate on a different agar medium with added leaf extracts of the three native *Fraxinus* species in Croatia.

Three growth media referred to potato dextrose agar (PDA Oxoid™ CM0139) enriched with ash leaf extracts from *F. excelsior*, *F. angustifolia* and *F. ornus* respectively, and the fourth one used as control contained only PDA, all without antibiotics. Leaves for the experiment were collected from individual trees of *F. excelsior* and *F. ornus.* Leaves of *F. angustifolia* were collected from the individuals of five clones used in the inoculation experiment belonging to the west seed provenance of Čazma, and mixed together to form a unified sample.

Exactly 50 g of fresh leaf fragments of each species of ash was added to one litre of prepared PDA respectively, prior to autoclaving (similar as described by Kirisits et al. 2013, Carrari et al. 2015). Before fragmentation with a sterile scalpel, the leaves were superficially scrubbed and the surface sterilized with 70 % ethanol on cotton pads and rinsed with sterile water three times. PDA containing leaf fragments was then autoclaved at 121 °C for 15 min. Liquid medium was poured through a sterile sieve, so the leaf fragments were separated and removed before it was poured into the Petri dishes. Finally, 20 ml of each media was poured into 90 mm Petri dishes in a laminar flow chamber. For each isolate, a total of 40 replicates (cultures) were made (10 replicates of each of the medium leaf extract contained and 10 replicates for control without the leaf extract). Growth of the cultures was measured every two days in four opposite directions (cross) previously marked on the Petri dish. Average growth, calculated as the average of differences between growths measured every two days, was taken into account for the statistical analysis.

Statistical analysis

Regarding the final measured lengths of necroses, coefficient of variation (CV) was calculated for each clone as an indicator of the intraclonal variability in order to determine whether susceptibility is on the clonal level or an individual feature. Assumptions of normality were checked using the Shapiro-Wilk W test, and the assumption of homogeneity of variance using Levene's and Brown-Forsythe tests. Due to non-normal variable distribution, unequal variances among observed groups and small sample size in each group (n=3 or 5), non-parametric statistical tests were used, Mann-Whitney U Test for the comparison of two independent groups (difference in final necroses length between the two isolates used), and Kruskal-Wallis ANOVA by Ranks for the comparison of multiple independent groups (difference in final necroses length among the observed clones). Post-hoc comparisons of mean ranks of all pairs of clones analysed with Kruskal-Wallis ANOVA by Ranks were also computed to determine which clones differ significantly.

The data on leaf unfolding were analysed and shown by standard descriptive statistical parameters (arithmetic mean, standard error of the mean, standard deviation, and coefficient of variation). The significance of difference for the given property among the studied genotypes and populations was tested using the analysis of variance. The affiliation of clones to ecotypic forms regarding the beginning of leaf unfolding (early and late flushing) was established using the algorithm for the classification of objects into clusters, i.e. k-means cluster analysis.

The calculated average growth of isolates on different growth media was shown by standard descriptive statistical parameters: arithmetic mean, median, maximum, minimum, standard deviation, and coefficient of variation. The significance of difference in the growth rate between the two *H. fraxineus* isolates, regardless of the growth media used, was tested with non-parametric Mann-Whitney U Test for two independent samples, and the significance of difference in the growth rate among the four used media for each of the two isolates was tested with non-parametric Kruskal-Wallis ANOVA by Ranks for multiple independent samples. Non-parametric tests were used because of the non-normal distribution of the analysed variable (growth rate of isolates) and inhomogeneous variances among observed groups (growth media) for the given variable. These were tested with the Shapiro-Wilk W test for normality, and Levene's and Brown-Forsythe tests for homogeneity of variances.

All data were statistically analysed in the StaSoft. Inc. (2011) STATISTICA version 10 software package.

Results

Inoculation of grafts of Fraxinus angustifolia

The inoculation experiment revealed a high capability of *H. fraxineus* isolates to cause and develop tissue necroses in the tested clones of *F. angustifolia*. In four months, necroses developed in 72 ramets (97.3%), and only 2 ramets (2.7%) of clones BJ38 and BJ25 (inoculated with isolate no. 1) did not develop necroses. All the control plants remained symptomless during the whole monitoring period.

The coefficient of variation (CV) was calculated in order to analyse intraclonal variability regarding the final measured lengths of necroses in plant stems (Figure 1) for each *H. fraxineus* isolate used. The results revealed a very high intraclonal variability, except within the clone NG55,

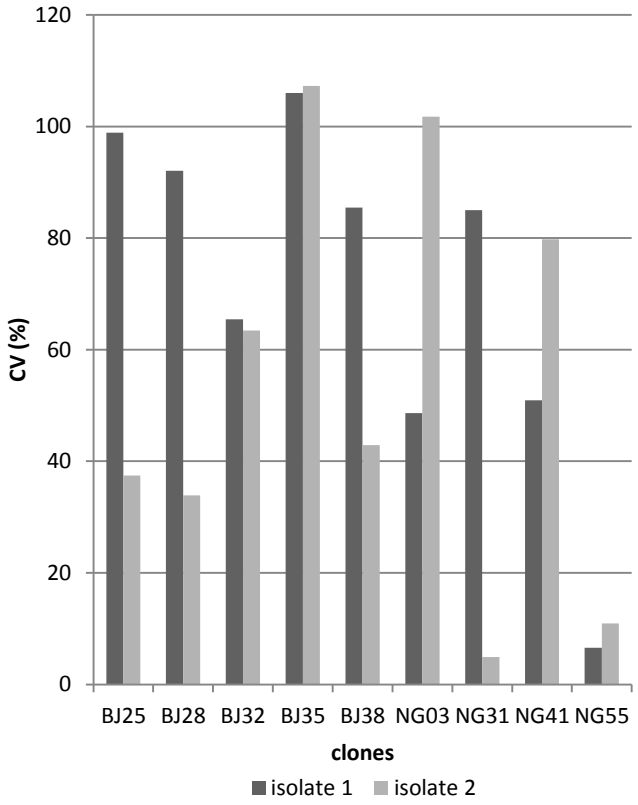

Figure 1. Coefficient of variation (CV) within clones (%) regarding the final measured lengths of necroses for *H. fraxineus* isolate

where intraclonal variability was the lowest for both isolates. With isolate no. 1, the rate of intraclonal variability in the clones from the Čazma orchard remained very high (above 60%), but lowest intraclonal variability was shown in three of the four clones from the Nova Gradiška orchard (clones NG03, NG41, and NG55). With isolate no. 2, the lowest intraclonal variability was shown in three of the five clones from the Čazma orchard (clones BJ25, BJ28 and BJ38), and also in two of the four clones from the Nova Gradiška orchard (clones NG31 and NG55).

Analysis of the data on developed necroses demonstrates that isolate no. 1 caused the longest necroses in clones BJ32, BJ28 and BJ25, NG41 and NG55, and the shortest necroses in clones NG03 and NG31. Isolate no. 2 caused the longest necroses in clones BJ35, BJ28, NG41 and NG55, and the shortest in clones NG03 and NG31. This correlates with the results of inoculation with isolate no. 1, revealing clones NG03 and NG31 as the least susceptible or with the most pronounced tolerance to *H. fraxineus* among the tested clones (Figure 2).

Kruskal-Wallis ANOVA by Ranks was used to determine if there was a significant difference between clones in the final length of the developed necroses (Figure 3). Analyses revealed a highly significant difference ($p = 0.0249$) between the clones regardless of their origin and the *H. fraxineus* isolates used in the test. Post-hoc comparisons of mean ranks of all pairs of clones revealed a significant difference between clones NG03 and BJ28 (p value with Bonferroni adjustment = 0.032272) (Figure 3).

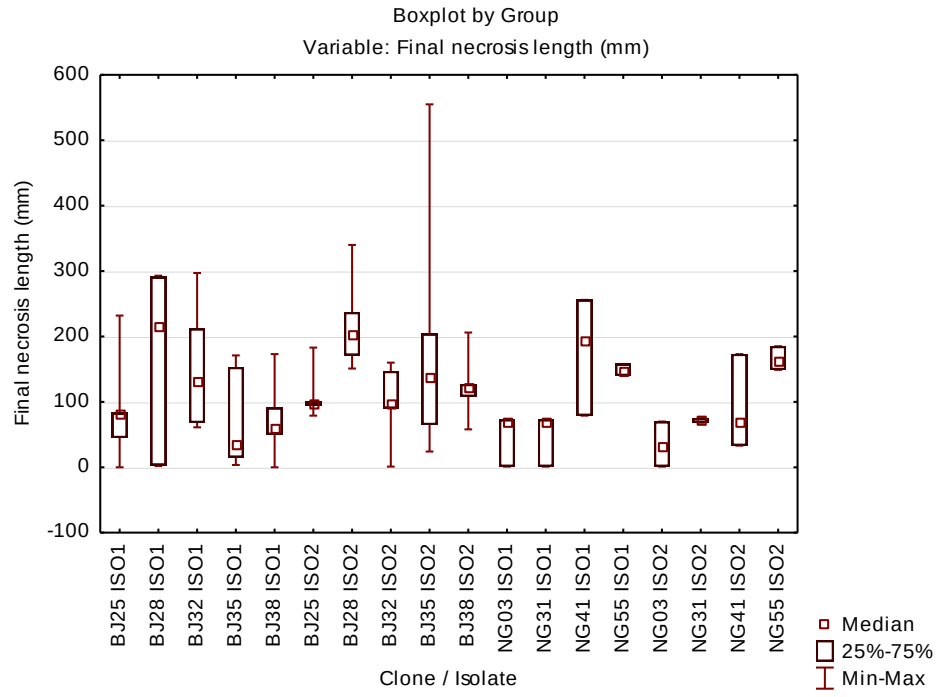

Figure 2. Final necrosis lengths for the clones inoculated with isolate no. 1 (ISO1) and no. 2 (ISO2)

According to the performed phenology study in Nova Gradiška (Figure 4), Kruskal-Wallis ANOVA by Ranks was repeated to determine if there was a significant difference in the final length of necroses between the tested clones NG03 and NG55 originating from the same clonal seed orchard. A significant difference was obtained (p=0.0063) when both fungal isolates were taken into consideration. Post-hoc comparisons of mean ranks of all pairs of clones for the Nova Gradiška clonal group revealed a significant difference between clones NG03 and NG55 as well (p value with Bonferroni adjustment = 0.013198) (Figure 3).

Average necrosis growth rate was calculated as the mean value of differences between necrosis lengths measured every two weeks for a period of the first four weeks of monitoring. Mann-Whitney U Test showed no statistically significant difference between the growth rates of the two isolates used in the experiment (p = 0.321938). Kruskal-Wallis ANOVA by Ranks revealed a significant difference between clones (p = 0.0094) in the development rate of the necroses.

One year after the inoculation, when the plants were examined, it was determined that 36 (48.6%) of a total of 74 inoculated seedlings were completely dead. Additional 4 seedlings (5.4%) revealed no live tissues above the inoculation point (including the crown), although resprouting beneath the inoculation point was observed.

In total, 54% of the inoculated plants had dieback or were in critical condition and expected to die in a very short period of time. From the remaining 46% of the inoculated plants, the majority were heavily affected, revealing severe symptoms of the disease and degradation of vitality.

Phenology observation in clonal seed orchard

The collected data on phenology - leaf unfolding and the performed analysis of variance revealed a statistically significant difference between the studied genotypes in the clonal seed orchard for each year of investigation (F = 5.95, F = 7.57, F = 5.66, Pr <.0001). No statistically significant differences were found between the three studied populations (Jasenovac F=0.43, Novska F=2.04, Stara Gradiška F=0.27). The average number of days from 1st January to the beginning of leaf unfolding was 98 days in 2012, 93 days in 2014, and 103 days in 2015. The results of k-means clustering of the clones according to their leaf unfolding clearly classified genotypes into two ecotypic forms: early and late flushing. Statistically significant differences were found for intrapopulation variability for the beginning of leaf unfolding; however, no statistically significant differences were found between the studied populations (Figure 4).

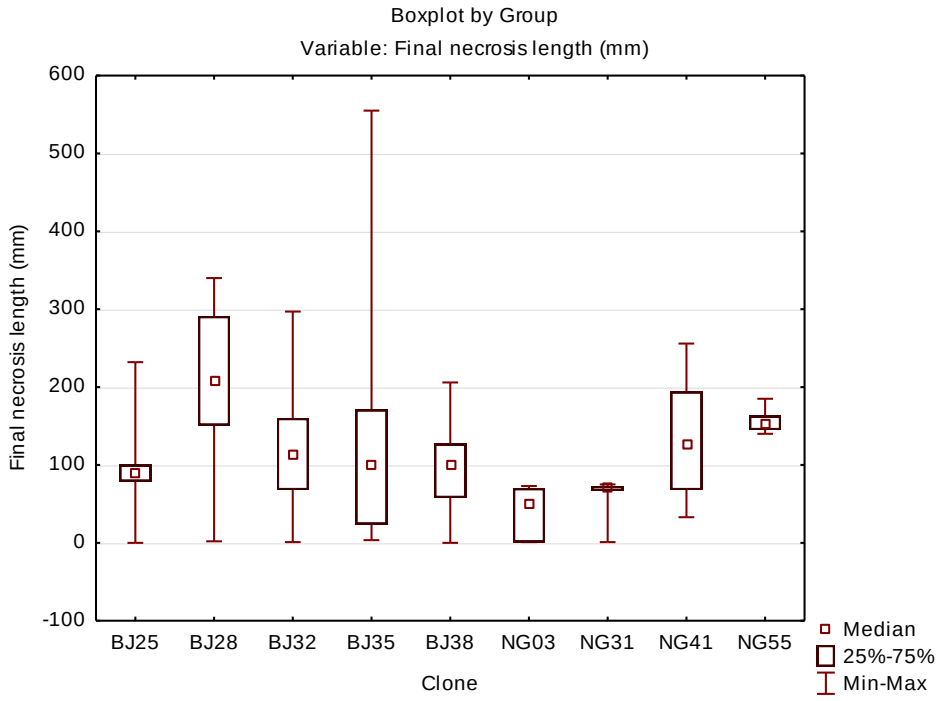

Figure 3. Final necrosis lengths for the clones regardless of the isolate

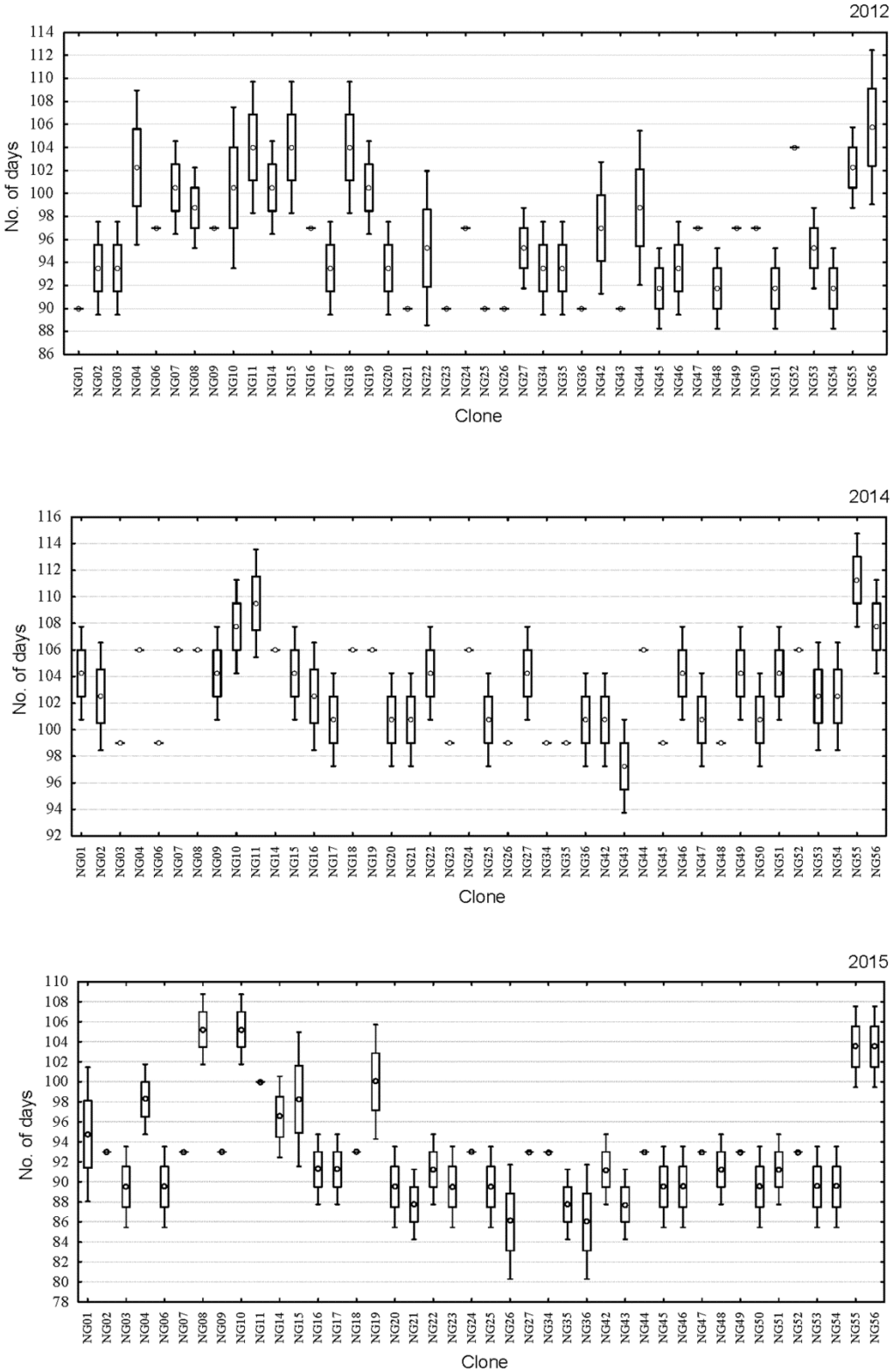

Figure 4. Average number of days per clone required for leaf unfolding for all three investigation years (2012, 2014, 2015) of the Nova Gradiška clonal seed orchard. The horizontal hyphen represents the aritmethic mean of the clone in a certain year, the box represents the standard error of the mean, and whiskers represent the standard deviation

The affiliation of the clones to ecotypic forms did not coincide with their geographic origin, which additionally confirmed important intrapopulation variability of the narrow-leaved ash. Intraclonal values of the coefficient of variability (C.V. %) for the property of leaf unfolding decreased with the age of the plantation and on average amounted to 15.22% at age 2 + 8 years, 13.46% at age 2 + 10 years, and 7.8% at age of 2 + 11 years, indicating higher stability and uniformity of phenological characteristics among the ramets as their age increased. In this period, the clonal seed orchard was also continuously monitored and checked for the presence of *H. fraxineus*. The presence of the pathogen was not confirmed, and clones did not reveal symptoms of the disease.

Fungal growth on leaf extracts

H. fraxineus isolates achieved growth on all tested media with and without the leaf extracts. Morphology of the mycelium varied according to different media (Kirisits et al. 2013), but corresponded well with that described by Kowalski (2006). Leaf extracts influenced growth variations between each medium. Variability of isolates growth within each media was expressed with the coefficient of variation (CV), which was very or relatively weak for both of the isolates and all of the growth media used, except for isolate no. 2 replicates grown on agar enriched with *F. angustifolia* leaf extract (Table 1).

Regarding the growth rates of both isolates, the fastest growth was observed on agar enriched with *F. excelsior* leaf extract, followed by agar with *F. ornus* leaf extract, and the slowest growth was observed on agar with *F. angustifolia* leaf extract as shown in Table 1. Mann-Whitney U Test revealed statistically significant differences between growth rates of the two isolates ($p < 0.0000001$). Isolate no. 2 grew faster on all four media when comparing mean and maximum average growth rates. In order to determine if differences among growth rates on the different media used were statistically significant, Kruskal-Wallis ANOVA by Ranks test was conducted and showed high statistical significance (Table 2, Figure 5). As shown in Figure 5, the growth of both isolates revealed the highest range (minimum and maximum values) on PDA with *F. angustifolia* leaf extracts when compared to other media used in the experiment.

Discussion and conclusions

In order to supplement the body of knowledge for the future forestry management policy on preserving the narrow-leaved ash in lowland forests of Croatia, in view of the new disease threatening its existence, the susceptibility of *F. angustifolia* clones to *H. fraxineus* has been studied.

The inoculation experiment revealed a considerable increase in the disease progression already in the first four months after the inoculation. Development of symptoms, damage severity, and development of necroses were observed in 97.3% of all the ramets tested in the study. High susceptibility of *F. angustifolia* clones to the pathogen was observed, thus confirming similar reported studies on natural susceptibility and resistance to *H. fraxineus*, especially in the first progeny trials with resistance abilities showing great variation (McKinney et al. 2011, Pliûra et al. 2011, Enderle et al. 2013). 54% of the plants had dieback or were in critical condition after the first year of disease progression monitoring, revealing continuous breakage of resistance in the majority of the tested clones. Many authors have shown similar results even after the first selection of resistant clones and progeny trials. Lithuanian clones were selected for resistance in progeny trials in 2005 and revealed inconsistency in resistance abilities in subsequent trials (Pliûra et al. 2014). In Germany, the rate of disease spread in a provenance trial established in 2005, increased from 13% in 2007 up to 94% in 2012 (Enderle et al. 2013).

Table 1. Descriptive statistics for all the growth media and isolates used. Mean, median, minimum and maximum correspond to the values (mm) for a two-day period

Average growth at 20 °C	Mean	Median	Minimum	Maximum	St. Dev.	Coeff. of variation (%)
Isolate 1 Medium 1	1.89	1.87	1.69	2.15	0.18	9.37
Isolate 1 Medium 2	1.57	1.55	0.67	2.27	0.42	27.02
Isolate 1 Medium 3	1.84	1.83	1.71	2.02	0.10	5.65
Isolate 1 Medium 4	1.07	1.02	0.77	1.56	0.27	25.29
Isolate 2 Medium 1	4.68	4.81	3.29	6.06	0.94	20.11
Isolate 2 Medium 2	2.50	1.85	0.42	5.58	1.73	69.53
Isolate 2 Medium 3	3.16	3.15	2.79	3.88	0.35	11.04
Isolate 2 Medium 4	3.37	3.31	2.85	4.27	0.40	11.84

Medium 1 – PDA (Oxoid™ CM0139) with *F. excelsior* leaf extracts
Medium 2 – PDA (Oxoid™ CM0139) with *F. angustifolia* leaf extracts
Medium 3 – PDA (Oxoid™ CM0139) with *F. ornus* leaf extracts
Medium 4 – PDA (Oxoid™ CM0139) without leaf extracts

Table 2. Kruskal-Wallis ANOVA by Ranks p values

	Both isolates	Isolate 1	Isolate 2
p value according to the Kruskal Wallis ANOVA by Ranks	0.0069	< 0.00001	p = 0.0014

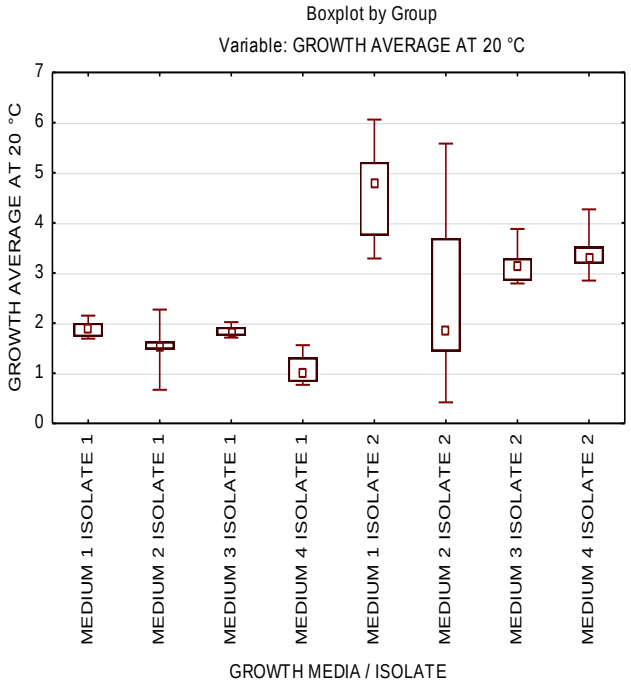

Figure 5. Growth rates of isolates no. 1 and no. 2 on four media. Medium 1 – PDA (Oxoid^{TM} CM0139) with *F. excelsior* leaf extracts, Medium 2 – PDA (Oxoid^{TM} CM0139) with *F. angustifolia* leaf extracts, Medium 3 – PDA (Oxoid^{TM} CM0139) with *F. ornus* leaf extracts, Medium 4 – PDA (Oxoid^{TM} CM0139) without leaf extracts

In a clonal study in Denmark, on two sites disease symptoms increased over three years, from 32 to 55% (McKinney et al. 2011). Only a few studies reported a decrease in ash damage, such as the study performed in Sweden, but the study revealed only a one-year decrease in damage, while in the five-year temporal trend there were some periods of increased damage (Stener 2013).

In a clonal study in Austria, mean ash dieback intensity reached only 18.1% in 2009, and 17.6% in 2010 (Kirisits and Freinschlag 2012). The explanation for the results obtained was that affected trees often responded intensively with the formation of epicormic shoots to compensate for the loss of dieback ones, which resulted in a temporary appearance of decreased dieback symptoms. The authors also assumed that climatic factors might contribute to the phenomenon. One-year monitoring of the disease rate in clones may vary substantially, and every progeny trial revealed a very high disease incidence rate and more uniform results after three or more years of monitoring.

In our study, regardless of the high disease incidence rate, mortality of trees reached almost 49% in one year, while in Lithuanian clone resistance trials mortality increased very little and reached only 1.2% (Pliûra et al. 2014). This could be expected and explained by the long temporal exposure to the pathogen in Lithuania, and as a consequence of the intense natural selection process in Lithuanian *F. excelsior* stands (Pliûra et al. 2014). In Croatia, plus trees in clonal seed orchards were collected from healthy stands of *F. angustifolia* before the outbreak of the disease, where susceptibility-driven natural selection according to the pathogen had not yet started. Also, the age of the seedlings used in our trials may have been the reason for the high mortality rate, as young trees are very susceptible to the disease.

On the other hand, a clonal study in Sweden revealed a 33% mortality range during a five-year period (Stener 2013). If we consider the results from the first Lithuanian progeny trials which are far more similar to our first susceptibility experiment regarding the first trials of selecting possibly resistant clones, there was an almost 90% mortality rate in the five-year period (Pliûra et al. 2011). Second-generation progeny trials always show a significantly lower mortality rate because of greater resistance and/or tolerance inside the group of clones that has previously been selected for exhibiting resistant genetic attributes.

In our study, none of the tested clones revealed total resistance to ash dieback, but a few exhibited reduced susceptibility, which is also in accordance with similar studies performed on resistance of *F. excelsior* genotypes (McKinney et al. 2011, Stener 2013, Pliûra et al. 2014). The study showed a great variety, with an average degree of damage among genotypes consistent with research conducted in Denmark, ranging from 1 to 69% (McKinney et al. 2011), and in Austria, where the degree of damage between clones varied from 0 to 80 % (Kirisits and Freinschlag 2012).

The results of the experiment revealed clones less susceptible or more tolerant to *H. fraxineus*, BJ25, BJ38, NG03 and NG31. These were the clones that exhibited some sort of reduced susceptibility to the pathogen, which should be tested in future experiments. If the resistance remains solid, vegetative propagation could be a very efficient option for breeding, as resistance/tolerance of ash is generally observed more at the individual genotype level than on the population or family levels (Douglas et al. 2013). The coefficient of variation on necrosis lengths revealed that only a small percentage of the tested clones have a small coefficient of variation which correlates to a uniform result in necrosis length. Only the result with small necrotic lengths and a small coefficient of variation could be considered for second-generation progeny trials.

Based on the results of phenological monitoring, we were able to classify narrow-leaved ash clones into early and late flushing groups. Some authors reported a correlation between early leaf shedding and susceptibility to *H. fraxineus* (McKinney et al. 2011, Bakys et al. 2013, Stener 2013, Hauptman et al. 2016). According to our preliminary results, they correlate with the results of McKinney et al. (2011) and Bakys et al. (2013), who claim that clones with earlier leaf unfolding and leaf shedding are less susceptible to the disease. Significantly smaller necrosis lengths were obtained in clone NG03, and longer necrosis lengths in NG55 (Figure 3). Clone NG03 revealed earlier leaf unfolding, approximately 8 to 14 days before clone NG55 in study years 2012, 2014, and 2015 (Figure 4). Earlier leaf unfolding could be one of the important characteristics in the narrow-leaved ash clones' resistance to *H. fraxineus*, as stated in the research done on *F. excelsior* (McKinney et al. 2011, Bakys et al. 2013, Stener 2013). However, according to the study by Hauptman et al. (2016), this correlation was not confirmed in *F. angustifolia*. Due to the data obtained only on two clones, NG03 and NG55, for the length of necroses and leaf unfolding parameter, a conclusion cannot be made. To test the hypothesis on the role of the leaf unfolding parameter as one of the possible phenological characteristics important in *F. angustifolia* resistance or tolerance to the disease, future experiments should be performed and include a larger number of clones and *H. fraxineus* isolates.

Intraclonal variability during the investigated years indicated a higher stability and uniformity of phenological characteristics among the ramets as their age increased. The proportion of symptomatic ramets in a clone for ash dieback disease was highly variable during the studied years (Pliūra et al. 2014). The results of investigation by Stener (2013) suggest that resistant trees in natural ash stands are quite rare, implying that it will be necessary to select a large number of candidate individuals. In the context of global climate change, the composition and structure of genetic variability of the narrow-leaved ash, particularly in terms of adaptive potential such as growth, survival and leaf phenology, should be considered.

Statistically significant differences in our study were obtained between genotypes in clonal seed orchards for the leaf unfolding parameter, but no statistically significant differences were found between the studied populations. Intraclonal values of the coefficient of variability (C.V. %) for the property of leaf unfolding parameter decreased with the age of the plantation, indicating a higher stability and uniformity of phenological characteristics among the ramets as their age increased.

A preliminary experiment carried out on three types of agar with leaf extracts from native *Fraxinus* species in Croatia revealed that the fastest growth of *H. fraxineus* isolates was present on agar enriched with *F. excelsior* leaf extract, followed by agar with *F. ornus* leaf extract, and the slowest growth was obtained on agar with *F. angustifolia* leaf extracts. According to Carrari et al. (2015), growth rates on the media containing extracts of susceptible hosts are higher than those on the media containing extracts of resistant hosts. The results obtained from our experiment cannot be explained at this stage, but could serve as a good basis for further research on *H. fraxineus* isolates. In new tests, isolates should be obtained from different ash host species and host tissues, and the tests should be more focused on the nutrition status of the hosts as a possible susceptibility / resistance characteristic.

The presented preliminary study on the narrow-leaved ash revealed clones less susceptible or more tolerant to *H. fraxineus*: BJ25, BJ38, NG03 and NG31. These were the clones that exhibited some sort of reduced susceptibility to the pathogen, which should be tested in future experiments. If the resistance remains solid, vegetative propagation could be a very efficient option for breeding. The data obtained on clones less susceptible to *H. fraxineus* can be a good basis for future research targeting host genotypes more resistant to the disease, and these studies should provide better insight for the future forestry management policy on preserving the narrow-leaved ash in lowland forest ecosystems of Croatia.

Acknowledgement

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Tree-ring Width of European Ash Differing by Crown Condition and its Relationship with Climatic Factors in Latvia

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Abstract

The spreading dieback of European ash (*Fraxinus excelsior* L.) that is a serious threat to the existence of the species in Europe, has been related to climatic changes. Still, not all trees in stands are damaged equally, suggesting that sensitivity to weather conditions might have affected the susceptibility to the disease. Climate-growth sensitivity of ash with visually healthy and damaged crowns growing in four stands in the central and eastern part of Latvia was assessed by dendrochronological techniques. The patterns of tree-ring width variation showed high diversity amongst trees, stands and regions; differences were observed between the damaged and healthy trees. Tree-ring patterns showed higher diversity amongst the healthy trees in the central part of Latvia, but, in the eastern part of Latvia, amongst the damaged ones. Mainly, the damaged trees were ca. 10–15 years older than the healthy ones suggesting age related differences in susceptibility, which might be related to vigour. The damaged and healthy trees differed also by growth trends, suggesting affiliation to different crown class, particularly at younger age. The sets of the significant climatic factors differed between the central and eastern part of Latvia. In the central part of Latvia, ash was mainly affected by the precipitation and daily temperature difference in the summer preceding formation of the tree-ring. Although the damaged trees were more sensitive to daily temperature difference and precipitation in the preceding August, the healthy trees were also additionally affected by maximum temperature in the preceding August. In the eastern part of Latvia, the sets of the significant factors were site specific, however, trees were mainly affected by temperature in the preceding autumn and current spring. In one site, the damaged ashes were more sensitive to temperature in July and September, while in other site the damaged trees were more affected by precipitation in July; the healthy trees were additionally affected by precipitation in September and temperature in April. Hence, the susceptibility to the disease appears partially related to the climatic sensitivity of trees.

Keywords: *Fraxinus excelsior*; ash dieback; dendroclimatology; climate-growth relationships; tree-ring width; radial growth patterns.

Introduction

The dieback of ash, which has been spreading across the Europe (Kowalski et al. 2010, Timmermann et al. 2011), is considered as a serious threat to the existence of the species (Kowalski 2006, Bakys et al. 2009a). The dieback is a rapid process, as the infected tree might die within a few years after the first symptoms, such as reduction of crown, appear or, in some cases, symptoms might not be even visible (Bakys et al. 2009b, Timmermann et al. 2011, Enderle et al. 2013). Although the mechanisms of ash dieback are not completely understood, it is considered to be caused by a complex of factors including climate and pathogens (Pautasso et al. 2010, Skovsgaard et al. 2010). The fungus *Hymenoscyphus fraxineus*, has been considered as a initiator of the dieback process (Kowalski 2006), which promotes further infestation and damage by the secondary agents (Kowalski et al. 2010, Skovsgaard et al. 2010, Bakys et al. 2011). The fungus, which attacks root system of ash (Bakys et al. 2008) affecting physical stability (susceptibility to uprooting) and water relations (increasing risk of water deficit) (Tulik et al. 2010), is considered to infest stressed trees, e.g. by unfavourable weather conditions (Thomsen and Skovsgaard 2006, Pautasso et al. 2010). Still, not all trees within a stand are damaged equally (Kirisits and Freinschlag 2011, Pliūra et al. 2011), suggest-

ing different resistance to the disease (McKinney et al. 2011, Stener 2012). Hence, the sensitivity to climate might be one of the factors affecting the susceptibility of ash to the dieback. Similar has been observed for the declining pedunculate oak (*Quercus robur*) in Southern Finland, which showed different climate-growth sensitivity also before the decline (Helama et al. 2009).

Climate is one of the main factors affecting vigour and growth of trees, which are archived in the variation of wood increment (Fritts 2001). Hence, detailed information about the sensitivity of tree growth to climatic factors can be obtained from a retrospective analysis of the variation of tree-ring width (TRW) (Speer 2010). As tree growth has an explicit biological i.e., age trends (Fritts 2001), the effect of climatic factors is commonly assessed from the high-frequency variation of TRW (Cook et al. 1992). Considering that a tree-ring forms during a certain period of the vegetation period, combined effects of several factors might be recorded in TRW (Cook 1992, Schweingruber 1996).

The aim of this study was to assess the variation of TRW of ash with different crown condition and its relationship to climatic factors at the inter-annual scale. We hypothesized that the damaged trees had different growth patterns and were more sensitive to climatic factors than the healthy ones, and that the sets of the significant factors differed.

Material and methods

Studied sites, sampling and measurements

Four mature stands dominated or co-dominated by ash with different crown condition located in the central and eastern part of Latvia near Ukri (UKR), Rundāle (RND), Gulbene (GBN) and Barkava (BAR) (Figure 1) were studied. Sites in these regions were selected, as differences in growth have been observed for other species (Matisons et al. 2012, Baumanis et al. 2001). All of the stands were situated on a flat terrain in a normal moisture conditions on loamy soil. According to the national classification by Bušs (1976), site type in all stands was *Aegopodiosa*. The elevation of stands was ca. 35 and 110 m above the mean sea level in sites in the central (UKR and RND) and eastern (GNB and BAR) part of Latvia, respectively. The maximum age of ash in the BAR, GBN, RND and UKR sites, as determined from the obtained wood samples, was ca. 70, 190, 110 and 100 years, respectively. Advanced regeneration occurred in all stands.

The climate in the studied sites is determined by the dominant western winds, which bring cool and moist air masses from the Baltic Sea and the Atlantic. The weather conditions are harsher in the eastern part of Latvia. The mean annual temperature is ca. +6.4 and +5.5 °C; the mean monthly temperature ranges from -4.3 to +17.5 °C and from -6.2 to +17.4 °C in January and July in the central and eastern part of Latvia, respectively. The vegetation period, when the mean diurnal temperature is above +5°C, extends from mid-April to mid-October; it is usually 10–15 days longer in the central part of Latvia. The mean annual precipitation is about 610 mm in all sites. The highest monthly precipitation sums occur in the summer months, usually resulting in a positive water balance (Klavins and Rodinov 2010). Climatic changes are reflected as an increase of temperature in the autumn-spring period, which is extending the vegetation period (Lizuma et al. 2007). In the same time, summer precipitation regime is becoming more variable (Avotniece et al. 2010).

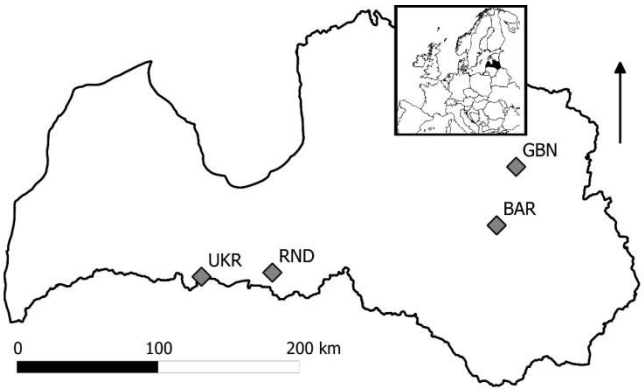

Figure 1. Location of studied sites near Barkava (BAR), Gulbene (GBN), Rundāle (RND) and Ukri (UKR)

In each stand, 10 dominant ashes with visually healthy crowns (crown reduction, i.e. dry branches, ≤ 15%) and 10 ashes with damaged crowns (crown reduction 30–60%) were selected. From each tree, two increment cores from the opposite sides of stem were collected with a Pressler increment corer at ca. 1.4 m height, avoiding reaction wood if trees were tilt. The sampling was done at the end of May 2015, when leaves had fully flushed. In the laboratory, increment cores were air dried and mounted on fixation planks for grinding. Sandpaper of four roughness grits (120, 140, 320 and 400 grains per inch) was applied, using hand sanding machine. The polished surface of samples was rubber with chalk to increase the contrast between early and latewood and to aid the recognition of tree-rings. The TRW was measured by a Lintab 5 measurement system (RinnTECH, Heidelberg, Germany) with the precision of 0.01 mm.

Data analysis

All of the measured chronologically ordered series of TRW were crossdated (i.e. their dating and synchronicity compared against each other) and their quality was checked by graphical inspection and statistically, by the program COFECHA (Grissino-Mayer 2001). Series showing low agreement with the rest of the dataset ($r < 0.35$) were omit-

ted from further analysis. The TRW series of the healthy trees were used as a reference for crossdating of the damaged trees. The crossdated series were then averaged for trees and their quality was verified. For description of the datasets, expressed population signal, signal to noise ratio (Wigley et al. 1984), Gleichläufigkeit, interseries correlation (based on detrended series) and the first order autocorrelation coefficients were calculated.

For assessment of high-frequency variation of TRW, residual chronologies based on the crossdated datasets were produced by the program ARSTAN (Cook and Holmes 1986) for each site and group of trees (damaged and healthy). Double detrending, firstly by a negative exponential curve and secondly by the cubic spline with rigidity of 64 years and 50% frequency cut-off level, was applied. The relationships between climatic factors and high-frequency variation of TRW was assessed by a bootstrapped Pearson correlation analysis (Johnson 2001) conducted for the common period from 1934 (1948 for the healthy trees in the RND site) to 2010. The significance of correlations was determined at $\alpha = 0.05$, performing 10000 iterations. The climatic factors showing significant correlations with TRW were tested for collinearity. The tested climatic factors were the minimum, maximum and mean temperature, potential evapotranspiration (PET), precipitation sums and mean daily temperature difference for months. The climatic window from January in the year preceding formation of tree-ring to September in the year of tree-ring formation (21 months) was used. Climatic data were obtained from the high-resolution gridded datasets provided by the Climatic Research Unit of UEA for the closest to the sites grid entries (Harris et al. 2014). The statistical analysis were conducted in the program R (R Core Team 2014) using the library "dplR" (Bunn 2008).

Results

After the crossdating and quality checking, from 75 to 100% of the series per group/site were maintained for further analysis. The crossdated datasets covered the periods beginning from 1824 to 1948 in GBN and RND sites, respectively (Table 1). Generally, TRW series showed better agreement in the central part of Latvia, as shown by higher values of interseries correlation and expressed population signal. The agreement of TRW of the healthy trees was better in sites in the eastern part of Latvia, where values of interseries correlation (0.35 vs. 0.15), Gleichläufigkeit (0.62 vs. 0.58) and expressed population signal (0.75 vs. 0.55) were higher. The opposite was observed in sites in the central part of Latvia, where the environmental signal was stronger, as shown by a higher signal to noise ratio (4.8 and 2.3, respectively). The agreement of TRW was considerably weaker, hence the noise was considerably stronger for the damaged trees in the BAR site, compared to the rest of the datasets. The value of expressed population signal exceeded 0.85 only for the damaged trees in the UKR site. Nevertheless, common tendencies, such as the decreased TRW in 1940, 1963, 1984, 1990 and 2006 were observed in all chronologies (Figure 2).

Table 1. Statistics of the crossdated datasets of tree-ring width (TRW) of ask with the damaged and healthy crowns in sites near Barkava, Gulbene, Rundāle and Ukri. A – stand age, D– mean diameter of trees, S – mean sensitivity, N – number of crossdated trees, IC – mean interseries correlation, AC – autocorrelation, GLK – Gleichläufigkeit, EPS – expressed population signal (Wigley et al. 1984), SNR – signal to noise ratio

	N	Period	A, years	D, cm	Min. TRW, mm	Max. TRW, mm	Mean TRW, mm	St. dev. TRW, mm	S	IC	AC	GLK	EPS	SNR
Barkava (BAR)														
Healthy	8	1933–2014	91	32.9	0.68	5.69	2.53	0.90	0.20	0.39	0.70	0.63	0.79	3.67
Damaged	10	1928–2014	91	28.9	0.10	10.00	1.99	0.99	0.22	0.09	0.80	0.58	0.42	0.71
Gulbene (GBN)														
Healthy	10	1824–2014	195	48.6	0.17	5.39	1.48	0.61	0.18	0.31	0.77	0.60	0.72	2.53
Damaged	10	1831–2014	195	43.9	0.12	4.77	1.38	0.56	0.19	0.23	0.79	0.58	0.69	2.20
Rundāle (RND)*														
Healthy	9	1948–2014	68	27.2	0.46	6.24	2.05	0.81	0.21	0.36	0.73	0.60	0.82	4.49
Damaged	10	1916–2014	112	34.2	0.16	6.35	1.79	0.88	0.19	0.36	0.83	0.60	0.83	4.90
Ukri (UKR)														
Healthy	7	1933–2014	106	33.2	0.53	5.28	1.99	0.99	0.19	0.40	0.86	0.60	0.81	4.32
Damaged	8	1925–2014	106	29.5	0.20	5.80	1.65	0.89	0.17	0.44	0.87	0.64	0.86	5.73

* – uneven aged stand.

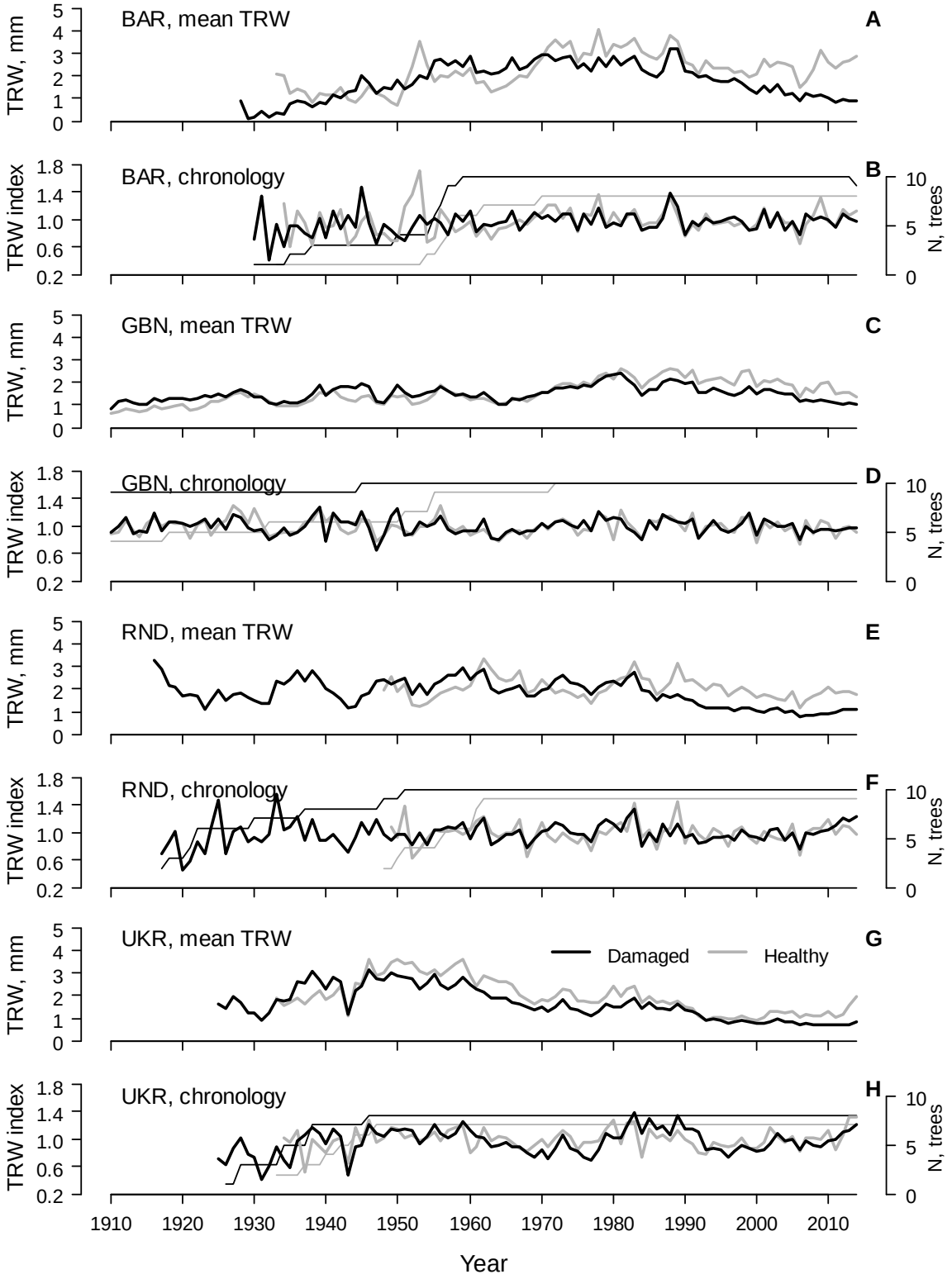

Figure 2. Mean series (A, C, E, G), residual chronologies (thick lines) and sample depth (thin line) of tree-ring width (TRW) (B, D, F, H) of ash with damaged (black lines) and healthy (grey lines) crowns for sites near Barkava (BAR), Gulbene (GBN), Rundāle (RND) and Ukri (UKR), respectively. Curves are based on crossdated datasets. For GBN site, only data for period from 1910 to 2014 are shown

In most of the cases, the healthy trees were younger, had higher mean TRW and contained less autocorrelation (0.77 vs. 0.82) than the damaged ones (Table 1). The damaged trees showed growth suppression during a few recent decades, although in some sites, they have been growing faster than the healthy trees at younger age (Figure 2).

The indices of chronologies generally ranged from ca. 0.60 to ca. 1.40, but the range tended to become narrower during a few recent decades (Figure 2). The agreement among the chronologies was weak, as the mean values of correlation calculated between them was 0.26, although Gleichläufigkeit was 0.63. The correlation between chronologies of the healthy and damaged trees ranged from 0.36 to 0.70 in the BAR and GBN sites, respectively. Nevertheless, the agreement between the chronologies of trees with different crown condition increased with age, particularly during the recent decades, as shown by the mean values of correlation coefficients of 0.50 and 0.65 at the beginning and at the end of the 20th century, respectively (not shown).

From the tested 132 climatic factors, 18 significantly correlation with TRW of ash (Figure 3). The number of significant correlations was higher in sites in the central than in the eastern part of Latvia (23 and 13, respectively). The values of correlation coefficients were generally low and did not exceed 0.32, except for precipitation in March (r = 0.48). The climate-growth relationships were quite individual, particularly in the eastern part of Latvia, as the sets of the significant correlations differed amongst the sites. In the central part of Latvia, TRW was affected by climatic factors (precipitation and daily temperature difference) related to the previous vegetation season (May–August), as well as some correlations with precipitation in February and March of the current year were observed. In the eastern part of Latvia, TRW of ash appeared mainly sensitive to weather conditions (maximum and mean monthly temperature) in the previous autumn (September and October) and in the current vegetation season (April–September). Generally, temperature and its mean daily difference had a negative effect on TRW, as shown by the prevailing negative correlations, while precipitation had a positive effect.

Only a few pronounced and systematic differences in the sets of the significant climatic factors were observed between the healthy and damaged trees (Figure 3). The damaged trees in the central part of Latvia displayed stronger correlation to the mean daily temperature difference and precipitation in August of the preceding year. The healthy trees showed additional sensitivity to the maximum temperature in previous August. In the RND site, TRW of the healthy trees showed the strongest of the observed correlations with precipitation in March. In the UKR site, the healthy trees showed stronger correlation with PET in May of the preceding year. In the eastern part of Latvia in the BAR site, the damaged trees were more affected by temperature in July and September of the current year, while the healthy trees were additionally affected by temperature in September and October of the preceding year. In the GNB site, the healthy trees were affected by precipitation in previous September and temperature in April of the current year, but they were less sensitive to precipitation in previous July.

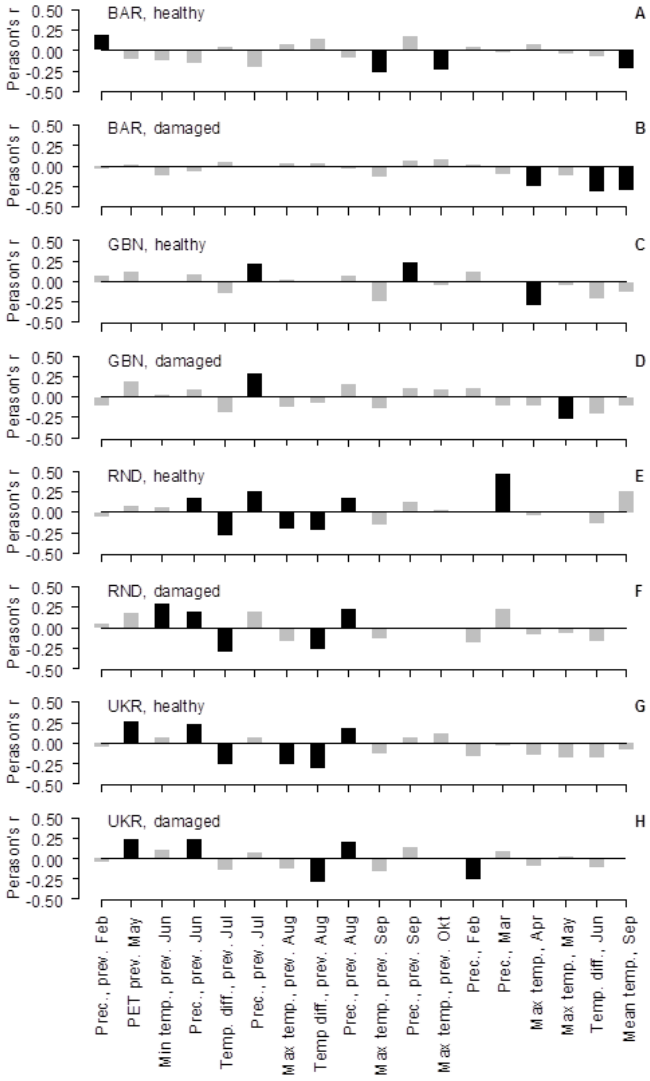

Figure 3. Bootstrapped Pearson's correlation coefficients calculated between climatic factors and residual chronologies of tree-ring width of ash with healthy (A, C, E, G) and damaged crowns (B, D, F, H) for sites near Barkava (BAR), Gulbene (GBN), Rundāle (RND) and Ukri (UKR), respectively. The period from 1934 (1948 for healthy trees in RND site) to 2010 was analysed. The significant correlations (at $\alpha = 0.05$) are shown in black. Only the significant factors are plotted. PET – potential evapotranspiration

Discussion and conclusions

Most of the TRW series were sufficiently crossdated, as they had common signature years (narrow tree-rings in certain years). Yet the individuality of growth was rather pronounced (Table 1), as the values of expressed population signal were mainly below 0.85 (Wigley et al. 1984). Such individual growth patterns apparently caused the noise in the datasets, hence the values of signal to noise ratio were below 5.8 (Table 1). Pronounced site-specifics in the growth patterns were also evidenced by weak correlation amongst the established chronologies (Figure 2), despite the fact that, in Latvia, ash occurs near its northern distribution limit (EUFORGEN 2009), where distinct effect of a common climatic factor(s) is expected (Fritts 2001). The observed regional differences in agreement of TRW series (Table 1) matched with the current knowledge on the diversity of tree growth patterns between the central (western) and eastern part of Latvia (Baumanis et al. 2001, Matisons et al. 2012). Nevertheless, weaker agreement of the TRW series was observed in the eastern part of Latvia that might be explained by stressed growth under harsher climate, when the effect of competition diversifies growth patterns (Speer 2010). In contrary, individuality of growth has been observed also for trees growing in optimum conditions, where a strict limiting factor is lacking (Fritts 2001). In the central part of Latvia, where climate is milder, ash, apparently, was less suppressed, hence the common signatures in TRW were clearer (Table 1). This is supported by the differences in agreement between the healthy and damaged trees. In the central part of Latvia, trees, which had stronger crown damage, showed better agreement of TRW likely due to higher sensitivity to environmental variability, suggesting the role of climatic factors in the dieback process (Thomsen and Skovsgaard 2006, La Porta et al. 2008). In the eastern part, crown damage, apparently, additionally stressed the trees, diversifying their growth patterns (Speer 2010).

The susceptibility of ash to fungal infection (Enderle et al. 2013) appeared to be age-related, as the healthy trees were mainly younger than the damaged ones (Table 1). With age, maintenance costs increases (Ryan 1990) and less resources might be allocated for production of defence substances (Pallardy 2008). This is supported by higher autocorrelation in TRW of the damaged trees (Table 1), suggesting stronger dependence on nutrient reserves (Fritts 2001). Nevertheless, the differences in growth rates between the tree groups (Figure 2) suggested that the susceptibility to disease might be also related to the social status (i.e., crown classes) of trees (Martin-Bento et al. 2008). The damaged trees, apparently, had a higher social status at the young age, as shown by faster growth (wider tree-rings) during a few earliest decades of their life. In contrast, trees, which grew slower, were less susceptible to crown damage at the older age. In all sites, the damaged trees showed reduction of TRW for ca. three recent decades compared to the healthy ones (Figure 2) suggesting that previously suppressed trees have been infected (Timmermann et al. 2011). Similarly, stronger symptoms of decline has been observed for the suppressed oaks (Helama et al. 2009). Simultaneous reduction of TRW in all sites during the recent decades (Figure 2) might be explained by the effect of weather conditions, such as the extreme drop of temperature in the winter of 1978/1979, striking the insufficient hardened trees and causing shifts in their growth trends (Matisons et al. 2013). The reduction of TRW decreased its annual variation particularly for the damaged trees (Figure 2) due to the suppression of growth (Speer 2010), but the patterns became more similar for both tree groups (Figure 2) suggesting clearer effect of common limiting factor(s).

High-frequency variation of TRW was significantly affected by the tested climatic factors (Figure 3). Still, the rather low values of correlation coefficients might be explained by the individuality of TRW variation within a site due to stresses, when the common response is reduced (Speer 2010). Similarly, the diversity of the significant factors amongst the sites and tree groups, particularly in the eastern part of Latvia (Figure 3), might be explained by the individuality of growth rhythms (Table 1). This apparently explains the number of observed significant correlations. In the sites in the central part of Latvia, the sets of significant climatic factors were similar (Figure 3), suggesting that trees have been able to show clearer reaction to weather conditions. The TRW mainly correlated with climatic conditions in the preceding summer, suggesting effect of nutrient reserves on wood increment (Barbaroux and Breda 2002). In the ring porous species, including ash (Carlquist 2001), nutrient reserves are mainly deployed for early growth (Barbaroux and Breda 2002) that affects water relations of tree in the following vegetation period (Tyree and Zimmermann 2002) and hence the increment. The amount of precipitation in summer showed positive correlation with TRW for all groups (Figure 3), suggesting that in the central part of Latvia ash has suffered water deficit. Shifting temperature conditions can burden assimilation and physiological processes (Pallardy 2008), as certain time is needed for the adjustment of photosynthetic apparatus to current conditions (Berry and Downton 1982), explaining the observed negative correlations between mean difference in daily temperature and TRW (Figure 3). The effect of precipitation and temperature in the preceding August was significant in all sites (Figure 3), as it is the time when the formation of nutrient reserves initiates (Barbaroux and Breda 2002).

In the sites in the eastern part of Latvia, TRW of ash was mainly negatively affected by temperature (Figure 3), but the mechanisms of influence shifted during the season. In autumn, raised temperature can increase respiration,

causing losses of stored nutrients (Ögren et al. 1997). In September, increased temperature, apparently, might also increase evapotranspiration (Traykovic 2005) causing water deficient, as positive correlation with precipitation was observed (Figure 3). This applies to current and previous September temperature. The negative effect of temperature in current spring might be explained by earlier onset of the active period or earlier leaf flush, subjecting trees to damage from late frosts (Gu et al. 2008), which are quite common in the eastern part of Latvia (Avotniece et al. 2010).

In the context of regional and local diversity, the sets of the significant factors between the healthy and damaged trees differed slightly (Figure 3), suggesting that climatic sensitivity had non-drastic effect on susceptibility to the disease and crown reduction (Figure 2), although the effect of weather extremes might not be visible in the residual chronology (Schweingruber 1992). Still, some regional or stand differences were observed (Figure 3). In the central part of Latvia, the damaged trees were more affected by water deficit and temperature regime in the preceding August, as shown by higher values of correlation (Figure 3). Stronger relationships with climatic factors suggested that under unfavourable conditions, i.e. warm and dry summers, which are becoming more frequent (Avotniece et al. 2010), trees are more stressed hence predisposed to the infection and damage. Nevertheless, negative effect of the maximum temperature in the preceding August was observed for the healthy trees (Figure 3), which apparently have been able to react to additional factor, probably due lower stress (Speer 2010). On the other hand, the negative effect of maximum temperature in August, which is the second warmest month (Lizuma et al. 2007), might be related to slower growth of the healthy trees before 1970s (Figure 2). In the RND site, only the healthy trees showed positive relationship with precipitation in March, which had the highest value of correlation coefficients (Figure 3). Precipitation in March is usually in the form of snow, and its effect might be explained by the insulating properties of snow layer, which influences soil temperature, decreases soil freeze (Hardy et al. 2001) and hence winter mortality of fine roots (Tierney et al. 2001), affecting water relations of a tree later in spring (Tyree and Zimmermann 2002). The absence of such relationship might suggest that the damaged trees have had less sensitive root system before the infection. The effect of potential evapotranspiration in the previous May in the UKR site (Figure 3) might be related to excess of soil water at the beginning of vegetation season, which influence root respiration (Pallardy 2008). Still, the differences in reaction to this factor between the healthy and damaged trees is difficult to explain. Probably, it might be related to slower growth of the health trees at younger age, when they have been more influenced by this factor. In the eastern part of Latvia in the GBN site, where trees were the oldest, TRW was influenced by precipitation (Figure 3) that might be explained by the age-related sensitivity to water deficit (Carrer and Urbinati 2004). The damaged trees showed stronger correlation to precipitation (Figure 3), suggesting increased susceptibility to water deficit, hence they were more stressed in the dry years that, presumably, led to the infection by fungus and reduction of crown (Kowalski et al. 2010). In the BAR site, the differences in sets of the significant factors are difficult to explain and might be caused by the district individuality of growth patterns (Table 1).

Although located near its northern distribution limit, in Latvia, ash showed site-specific growth patterns and climatic sensitivity, probably due to the stresses caused by the pathogen(s). This also approves the complexity of ash dieback process. In all sites, ash of different crown conditions was affected by the climatic factors related to water deficit in summer, suggesting that under the changing climate, growth of ash might become even more stressed. The sensitivity of growth was higher under more continental conditions. Trees with different crown condition, apparently differed by social status, as the damaged trees grew faster at young age, but the TRW of the healthy trees was the highest at medium age. Still, the damaged trees showed growth reduction during recent three decades. The susceptibility to disease also appeared age-related, as the damaged trees were older than the healthy ones, likely due to the increasing maintenance costs. Still, some differences in climatic sensitivity were observed. In general, trees with the damaged crowns were more affected by the climatic factors, suggesting that climatic stresses have been at least partially involved in the development of the disease. Nevertheless, the healthy trees also showed effect of some climatic factors not observed for the damaged ones, suggesting influence of e.g. microclimate and microtopography on the susceptibility to the disease. For better understanding of the role of tree water relations in ash dieback, analysis of wood vessels might be useful.

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Introduction Part 3: Other Invasive Tree Pathogens

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The outbreak of ash dieback, caused by the spread of *Hymenoscyphus fraxineus* in Europe has caused much excitement in the public and press, rightly so, as yet another invasive pathogen spreads across the continent threatening the stability of one of our iconic trees and the forest ecosystems it inhabits, from Russia to Northern Spain, from Greece to Finland. As interest focuses to a great extent on this particular problem, however, we must not lose sight of the many other invasive pathogens that are currently spreading in Europe (Santini et al. 2013), some of which have long been known, others recognised rather recently. Arguably, we can trace invasions by *Phytophthora cinnamomi* and *P. cambivora* back to the early 19th Century, although it is difficult to be precise over this time span, particularly as spontaneous generation remained the perceived 'cause' of plant problems, amongst others, at that time. After the debunking of spontaneous generation, however, it eventually became clearer that microorganisms cause disease in plants and animals. At the same time, it was recognised that pathogens could be transported around the world by various means, not least of which was through human activities.

We now assume, with considerable justification that the arrival of *H. fraxineus* in Europe – probably in the 1980s – resulted from an inadvertent co-transport of the pathogen on ash tissues, although the precise cause remains unclear. Sadly, this mode of arrival of invasive pathogens in regions and continents distant from their evolutionary origins is all too common (Fisher et al. 2012; Santini et al. 2013).

Highlights of this issue

The following set of papers is focused on several other invasive pests and pathogens that have entered Europe, some recently, one over 50 years ago. For example, the pathogen causing the current epidemic of Dutch elm disease, *Ophiostoma novo-ulmi*, first entered Europe though British ports in the early-mid 1960s on consignments of elm logs from North America. A previous epidemic of elm wilt, caused by *Ophiostoma ulmi*, had swept through Europe in the early-mid 20th Century, but many trees either recovered from the infection, or proved of low susceptibility. The appearance of a second pathogen causing Dutch elm disease, however, led to the deaths of millions of elms, and threatens the genus with extinction in Europe. The disease is still spreading in the northern-most areas of Europe. Pecori et al. (2017), however, highlight the use of selection and breeding in the fight against Dutch elm disease in Italy, where there have been some successes in crossing Asian elms against European *Ulmus* species to produce hybrids of suitable form and growth rates to utilize in southern Europe. Dutch elm disease is still causing much destruction of trees in northern Europe, however, as illustrated in the paper by Menkis et al. (2017), in which attempts were made to slow disease spread by applying decay fungi in an attempt to kill stumps of felled elms, thereby preventing resprouting and perpetuation of the presence of host tissues susceptible to beetle attack and, subsequently, infection by *O. novo-ulmi*. This method was not of particular practical value, although it is clear that choice of fungus to kill the stumps must be considered carefully.

Two pathogens causing leaf blights of *Buxus sempervirens* entered Europe (Henricot et al. 2000), probably in the 1990s, and have spread very rapidly through natural and cultivated populations of this valuable understory shrub since then. *Buxus sempervirens* is the main understory woody species in the forests of north-east Turkey, but is being very badly damaged by *Calonectria pseudonaviculata* and *C. henricotiae* (Lehtijarvi et al. 2017) in that region. As with many invasive pathogens, the two *Calonectria* species are proving extremely difficult to manage, and boxwood is now considered endangered. The same problem is also wreaking havoc in the forests of Georgia and Iran.

Pitch canker of pine poses a massive threat to the future of pine forests in Europe (Bezos et al. 2017). It also attacks *Pseudotsuga menziesii.* Transmitted via seed, the pathogen is proving extremely difficult to detect in pathways of introduction, but appears to colonize plants in nurseries, often asymptomatically. The pathogen is then transported to forest sites on the young plants. Although established only in Spain and Portugal (solely in forest nurseries so far in Portugal), given the massive area of various pine species in Europe, the disease is clearly a major threat, particularly to coastal pine forests, where relative humidity is conducive to disease development. Again, this disease is proving rather recalcitrant to mitigation measures.

Over the past 20-25 years, Dothistroma needle blight (DNB), caused by *Dothistroma septosporum* and, in more limited locations, *D. pini*, have increased massively in overall importance, also threatening the integrity of pine forests and plantations throughout Europe (and beyond). It appears to infect most pines (if not all), along with several *Picea* spp. and *Cedrus.* The paper herein, by Lazarevic et al. (2017), extends the number of known host species varieties on which DNB occurs, and increases the knowledge of the pines affected in Montenegro.

Pests discussed in this collection of papers include the ash black sawfly, *Tomostethus nigritus* (Meshkova et al. 2017) and the emerald ash borer, *Agrilus planipennis*. *Tomostethus nigritus* has spread greatly in Europe recently, although populations fluctuate over years. Although not usually a lethal pest to European ash, the sawfly adds another layer to the woes faced in Europe by *Fraxinus excelsior*. In contrast, the emerald ash borer (Selikhovin et al. 2017, Musolin et al. 2017) is highly destructive, as witnessed in invaded regions in North America and around Moscow. As with *H. fraxineus, A. planipennis* is native to Far East Asia, including the far east of Russia itself. It is, nevertheless, not native in the European parts of Russia, from where the problem is now spreading westwards. Already recorded on the borders of Belarus and Ukraine, it is highly likely that the insect will continue to migrate westwards, where it will decimate the populations of *Fraxinus excelsior* remaining after ash dieback. It is essential, therefore, that any attempts to develop ash resistant to *H. fraxineus* also take the emerald ash borer into account.

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Lights and Shadows of a Possible Strategy to Cope with Alien and Destructive Forest Pathogens: the Example of Breeding for Resistance to Dutch Elm Disease in Italy

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Abstract

Since the onset of the 20th century, two pandemics of Dutch elm disease (DED) destroyed native elms throughout Europe and North America. The disease is caused by two invasive fungi, *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier, which appeared one after another and were new to science. From the late 1920s, in Europe and in the US, researchers strove to find natural resistance to DED in native elms, but their efforts yielded ephemeral success. The resistant cultivars obtained in the 1930s by hybridizing European elm genotypes, were defeated by the second pandemic. The inclusion of Asian resistant species in breeding programs finally produced resistant second-generation hybrids. In Italy a program to breed resistant clones for the Mediterranean climate, was started in the mid 1970s. The successful use of Asian species in the country encouraged an in-depth assessment of their adaptability to local climates for broadening the genetic base of breeding. Selection of superior genotypes reduces genetic variation. However, when breeding is designed to obtain multiple genotypes for diverse conditions and uses, variation can be maintained. The case of elm is paradigmatic. Since elms have many uses, and an important one is as ornamentals, breeding included the selection of genotypes with fast growth, and attractive crown shape and foliage. To meet all needs and provide genetically variable cultivars to deal with climate change and new diseases, genetic resources were broadened. Native elms with good aesthetic qualities were crossed with DED-resistant and adaptable Asian genotypes. The program produced resistant clones adapted to summer drought and winter floods, yet endowed with notable ornamental features. Five of these clones were patented. A similar strategy including both the crossing of European *Fraxinus* species and of native with non-native resistant genotypes, may be successful against *Hymenoscyphus fraxineus*, the invasive agent of the European ash dieback epidemic.

Keywords: Breeding for disease resistance, invasive alien pathogens, Dutch elm disease, *Ophiostoma novo-ulmi*, biodiversity conservation, ash dieback, *Hymenoscyphus fraxineus*

Introduction

Since the beginning of the 20th century, elms throughout Europe and North America have been devastated by two pandemics of Dutch Elm Disease (DED), caused by the introduction of two alien and invasive fungal pathogens with different aggressiveness*: Ophiostoma ulmi* (Buisman) Nannf., probably introduced in the early 1910s, followed in the 1970s by *O. novo-ulmi* Brasier, a three times more deadly pathogen of elms (Brasier 2000). In

Europe the current DED epidemic is caused by two subspecies of *O. novo-ulmi*, the ssp. novo-ulmi, which was introduced to Europe from the Moldova-Ukraine basin and moved westward, and the ssp. *americana*, which was introduced, through infected rock elm wood, in the UK, whence it reached continental Europe spreading both west and eastward. *O. novo-ulmi* subspecies differ in many morphological and physiological characters, such as for instance colony morphology, growth rate, and pathogenicity (Brasier 1986, Brasier and Kirk 2001). Since the 1980s, hybrid individuals of the two subspecies have been found in several European countries, and in some areas their distribution ranges seem to have overlapped for quite a long time (Brasier 1979).

The elm was at the time an important and characteristic component of the cities' tree-lined roads and of the rural landscape in several European and North American countries. The epidemic had so widespread, severe and impressive effects that it stirred up the interest of researchers and public opinion, such as to necessitate a solution to the problem.

Research efforts to find a source of DED resistance in native elm species and to accumulate it in hybrid clones through breeding were initiated in 1928 at the Willie Commelin Scholten Phytopathological Laboratorium in Baarn (The Netherlands). The etiology of the disease was studied and the causal agent was finally isolated by Dina Spierenburg (Spierenburg 1921, 1922) and short afterwards described and named by Marie B. Schwarz (Schwarz 1922). Christine Buisman developed a reliable inoculation method (Westerdijk et al. 1931). The studies by Spierenburg and Buisman laid down the foundations for building a breeding program (Holmes 1993).

At first, researchers tried to select DED-resistant individuals within native species. Two *U. minor* clones were indeed selected and named 'Christine Buisman' (1936) and 'Bea Schwarz' (1947). These genotypes however turned out to have slow growth and poor shape, and to be in addition susceptible to branch canker by *Nectria cinnabarina* (Tode) Fr.. In order to possibly combine different resistance mechanisms and improve growth, Dutch scientists started to cross genotypes from different elm species. In addition to DED-resistance, the breeding program aimed to select clones resistant to coral spot by *N. cinnabarina*, frost, and wind. Fast growth, good crown shape, decorative leaves, and valuable timber were also sought. The first two clones launched onto the market, 'Commelin' (1960) and 'Groeneveld' (1963), were first-generation hybrids between individuals belonging to European elm species, and seemed to be a successful completion of research efforts. Unfortunately, in the late 1960s the new and more aggressive pathogen *O. novo- ulmi* was introduced to which 'Commelin' was especially susceptible. Decades of breeding have shown that, although slowly, it is possible to accumulate resistance through subsequent crossings and back crossings in second or third generation clones of purely European elms (Heybroek, personal communication). Complete resistance to DED has never been found in native European or American elms, but highly resistant individuals have nevertheless been identified (Townsend et al. 2005).

When the second DED epidemic was spreading in Europe, it was noticed that the clones still surviving the new pathogen were second-generation hybrids with a grandparent of Asian origin. Since then, DED-resistant Asian elms have been crossed to native elms to accelerate selection of resistant trees. A group of genotypes belonging to native elm species and bearing desirable morphological features was bred with genotypes of Asian elms species that were fairly resistant to DED and had shown the ability to adapt to a range of climatic conditions and environments. This way the genetic resources involved in selection were artificially broaden, a process so called "incorporation" (Simmonds 1993), in order to obtain DED-resistant clones suitable at the same time for all traditional uses of elms, showing also fast growth, nice tree silhouette, decorative leaf and bark colour, and leaf shape.

The risk inherent in selecting superior genotypes is to reduce genetic variation and move toward a genetic bottleneck (Simmonds 1993, Tanksley and McCouch 1997). In the case of elm breeding, the involved risks were, however, contained by designing breeding as such to obtain numerous genotypes with different genetic background and suitable to different environments and uses, possibly resulting even in increased variability (Cox and Wood 1999).

The Italian Program for breeding DED resistant elms

In Europe the second program for breeding DED resistant elms was started in Florence by the Institute of Sustainable Plant Protection of the Italian National Research Council (IPSP-C.N.R.) in the late 1970s, when the arrival of *O. novo-ulmi* was causing a new DED epidemics in Italy. The goal of this program was the selection of DED-resistant elm cultivars adapted to the Mediterranean climate. A group of clones of European origin and bearing desirable morphological and physiological traits were hybridized with individuals of DED-resistant Asian species (Smalley and Guries 1993), which had shown good adaptation to the Mediterranean climate, broadening that way the genetic resources involved in the program (Simmonds 1993).

The Italian program owes to Dutch researchers the breeding strategy (Fig. 1) and many of the clones used in crossings. Additional plants were collected from wild populations of native elm species and from plantations of Siberian elm, or received from American colleagues and

research Institutes worldwide (Tab. 1). The techniques used in Florence for inoculation and crossing were also borrowed from the breeding program carried out in The Netherlands, with few technical improvements, such as for instance pollination by blowing the pollen without the need of lifting the isolation sack (Mittempergher and La Porta 1991).

Materials and Methods

Collection of plant material

In the late 1970s, individuals of elm species and provenances from all around the world were collected with a preference for Asian species, which are generally more resistant to DED (Smalley and Guries 1993), and established in a clone collection in order to check the ability of these plants to adapt to environmental conditions in the Mediterranean area, including biotic and abiotic damage agents that might affect introduced species, and their hybrid progenies, and to use them for crossing.

Hybridization Studies

A large survey was carried out under Mediterranean climatic conditions to assess the crossability among elm species, which included European elms and several Asian

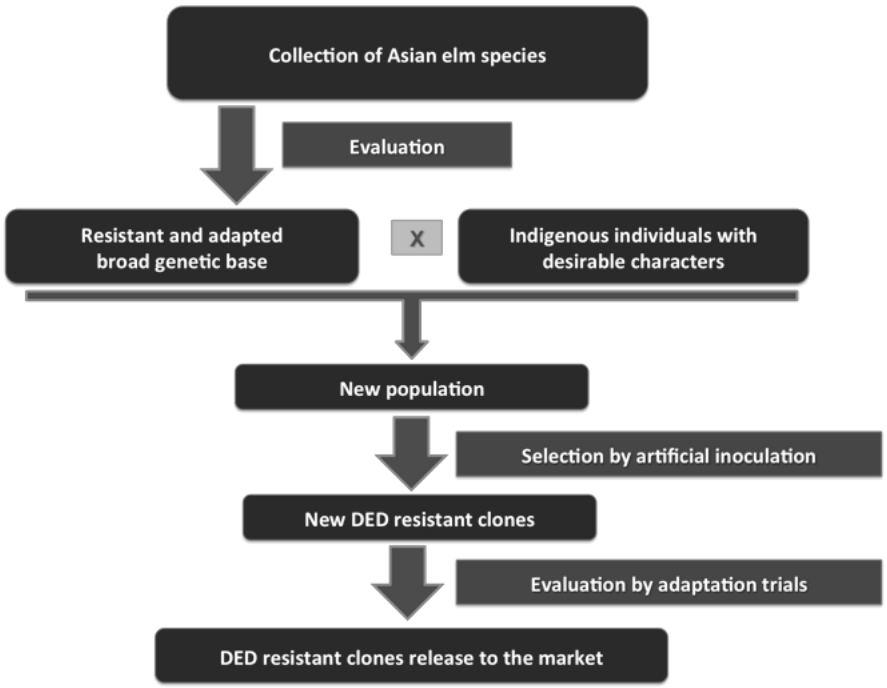

Figure 1. Scheme of the strategy applied in the Italian program for elm breeding

Table 1. Genotypes most frequently used in the Italian breeding program for selection of DED-resistant elms. DED resistance scores: - = non resistant; + = resistant; ++ highly resistant

Species	Common name	Origin of parent trees	DED Resistance
U. laevis Pall.	European white elm	France	-
U. minor Mill.	European field elm	Italy	-
U. glabra Huds.	Wych elm	Italy	-
U. pumila L.	Siberian elm	Turkestan, W Siberia	++
U. japonica Sarg.	Japan elm	Japan	+
U. wilsoniana Schn.	Wilson elm	China	++
U. elliptica Koch.	Armeniam elm	Caucasus	-
U. x hollandica Mill.	Dutch elm	The Netherlands	-
U. parvifolia Jacq.	Lacebark elm	Korea, Japan	++
U. chenmoui Cheng	Chenmoui elm	NE China	+
U. villosa Brandis	Cherrybark elm	Himachal Pradesh	+

* - = non resistant; + = resistant; ++ highly resistant

species belonging to different taxonomic sections (Mittempergher and La Porta 1991). Pollen was obtained from cut branchlets held in vases with water during the pollen dispersal phase. Different species and individuals were kept in separate rooms of a greenhouse to avoid contamination. The pollen was dehydrated to 10 percent relative humidity (RH) and conserved at 3° to 4°C for use within few days to weeks. Dehydrated pollen was conserved at -20 °C when it had to be stored for 6 months (Asian species that flower in autumn), or for about 1 year (to cross the later pollen donor with the earlier spring flowering species). Pollen vitality was checked before pollination by using the Fluoro-chromatic Reaction technique (Heslop-Harrison and Heslop-Harrison 1970, Heslop-Harrison et al. 1984).

Flower pollination was carried out in triplicates by injecting pollen into pollination bags with forced air. In order to test for occurrence of self-pollination, foreign pollen was not injected in at least three control bags on each mother tree. Matured seeds were sown in open-air nursery beds where germination was monitored. Morphological traits of taxonomical relevance were assessed in the seedlings for two years in order to ascertain their hybrid nature. Viability at the end of the first growing season was recorded.

Screening Disease Resistance

DED resistance was assessed through mass inoculation, which was followed by selection of resistant genotypes

Three-year-old elm seedlings from controlled crosses were grown in the nursery and planted in the field. In the third week of May of the following year, at the time of peak of flight activity of the beetles species (Scolytus spp.) that vector the disease in the area of study, seedlings were inoculated. Inoculation was performed with a single wound per plant, using a knife blade carrying two 0.2 ml drops of a $1x10^6$ ml^{-1} suspension of yeast-phase cells of *O. novo-ulmi* so that the inoculum got sucked into the vertical sap flux (Santini et al. 2008). A blend of two tester isolates, one of subsp. *novo-ulmi* and the other of subsp. *americana*, was used to account in the selection process also for differences in virulence and other relevant traits between subspecies of the fungus (Brasier 1986). Resistant elm genotypes were selected according to standard protocols, which set inoculum type and quantity, inoculation method, and assessment of disease symptoms guaranteeing efficacy and reproducibility of the process.

Symptoms of disease (percent defoliation and percent dieback) were assessed 4 weeks, 3 months and 8 months after inoculation by three independent observers. Seedlings showing less than 10 percent dieback were propagated by hardwood cuttings and after a year were planted in the field using a randomized complete block design. Twelve rooted cuttings per genotype and three blocks were used. Seedlings showing less than 25 percent dieback were considered resistant, and were evaluated for additional traits. Two years after planting, inoculation and disease symptoms assessment were repeated as described above, and symptoms were compared with those expressed by reference clones with known response to DED, i.e. the Dutch clones ‘Commelin’ and ‘Lobel’, which are defined as ‘highly susceptible’ and ‘intermediately resistant’, respectively.

Adaptation trials

In order to assess phenotypic plasticity and to determine the optimal environmental condition for growth of each selected genotype, a phenotypic assessment of the clones at several traits including DED-resistance was repeated in field trials at multiple sites under different ecological conditions. Clones were planted in a randomized complete block design with three blocks and four ramets per block and clone. Traits were measured once per year at all sites and a final assessment was done at the end of the trial, 6 to 10 years after planting.

Clones defined as DED-resistant on the basis of defoliation and dieback after repeated artificial inoculations were evaluated for the following additional traits: 1) Leaf shape, including length, breadth, and slenderness. The shape defined as preferable was that of *U. minor* leaves, which are generally rounder than the leaves of Asian elm species. 2) Leaf colour. Dark green, which is the colour of the leaves of the native field elm, was considered preferable. 3) Shape of the crown. The favourite shape was columnar with a monocormic straight trunk and slender branches. 4) Tree growth in height and diameter.

Three independent observers attributed to each genotype a score on a 5-step scale that synthesises the phenotype at morphological traits, growth and DED-resistance. The scale goes from ‘no marks = not an eligible clone’, to ‘four marks = clone that accomplishes all the requested characteristics: resistance, adaptation (growth), and leaf colour, trunk and crown shape’.

Results and Discussion

More than 50,000 hybrid seedlings were grown and tested, about 80 of which received a very high score. A quite numerous group of resistant elm clones obtained by crossing very diverse parents and showing valuable traits are in the process of being placed on the market. Availability of a large number of resistant genotypes with different genetic background and adapted to different environmental conditions should reduce the risk of being defeated by new and possibly more aggressive strains of the pathogen, as it occurred in the 1970s when the *O. novo-ulmi* appeared in Europe, or by other unforecasted

environmental changes (Santini et al. 2008).

Five DED-resistant elm clones have been patented and released to the market. 'San Zanobi' (Pat. n. RM97NV0006) and 'Plinio' (Pat. n. RM97NV0005) (Santini et al. 2002), significantly more resistant than 'Lobel' and other reference clones, were obtained by crossing the Dutch hybrid 'Plantijn' with two genotypes of *U. pumila* and were launched on the market in 1997. In 2006, 'Arno' (Community Plant Variety Right n. 27598) and 'Fiorente' (Community Plant Variety Right n. 27599) (Santini et al. 2007) were released. The first is a full sib of 'Plinio,' while the second is a first generation hybrid between *U. pumila* and a genotype of *U. minor* native to Italy. The DED-resistance of these clones is similar to that of 'Lobel.' In 2010, the Italian elm breeding program produced a new variety obtained by crossing a specimen of *U. chenmoui* W. C. Cheng with the Dutch hybrid clone '405.' This new release, named 'Morfeo' (Community Plant Variety n. 2011/0223) (Santini et al. 2012), is extremely resistant to DED, has attractive crown shape and foliage, is fast-growing, able to stand without a support stick at a very early age, and tolerates drought and soil waterlog in winter (fig. 2). 'Morfeo' seems therefore able to adapt to maritime climates with wet and mild winters, such as those found in north-western Europe and in some parts of the Mediterranean region. The results of growth trials performed in England indicate that 'Morfeo' might help in the conservation of several invertebrates endangered as a consequence of elm disappearance due to DED.

Evaluating the risk of damage by other pests

Introduction of elm species from other continents was one of the key-points of many programs for breeding DED-resistant elm clones. Introduction of non-native species involves the risk that local parasites, which cause minor damage to native species, might seriously attack new and naive introduced species. For instance, a disease named 'Elm Yellows' (EY), caused by phytoplasma (*Candidatus Phytoplasma ulmi*, Lee et al. 2004) was found to be harmful and even deadly for a number of Asian elms resistant to DED and for their hybrids (Mittempergher 2000). This disease was known in North America since the 1930s (Sinclair 2000) where it commonly kills the American elm (*U. americana* L.). In Europe instead EY was well tolerated by the populations of native elms, with only a few individuals showing typical symptoms, such as witches' brooms, growth retardation and general decline (Mittempergher 2000). The disease is commonly vectored and locally spread by some species of phloem-feeding Hemiptera (Carraro et al. 2004). Nowadays, EY has become common because of the co-occurrence of several factors: large spreading of leafhopper vectors, presence of host-plants that act as a reservoir for phytoplasma, and an increasing number of susceptible elm clones. The result is that even *U. minor* is seriously attacked by EY, especially in mixed plantations such as clonal banks (Pecori et al., 2013).

Numerous insects are also known to damage European elms, including the elm leaf beetle (*Xanthogaleruca luteola* Müller) and the goat moth (*Cossus cossus* L.). Asian elm species used in breeding programs show variable susceptibility to these insects. For example, the Chinese species *U. laciniata* (Trautv.) Mayr is susceptible to the elm leaf beetle to such a high degree that the tree can be hardly grown in central Italy without chemical control. *U. parvifolia* Jacq. and *U. wilsoniana* Schneid. are instead scarcely damaged by the insect. The Institute for Sustainable Plant Protection (IPSP-CNR) in Italy has thus established a research program to assess the susceptibility to Elm Yellows and to the elm leaf beetle of the most commonly used elm species of Asian origin. Resistance to multiple pests is scored and evaluated in adaptation trials planted in field conditions by scoring the susceptibility to natural infection or infestation.

Figure 2. The Dutch elm disease-resistant clone 'Fiorente'

Environmental risks associated to the introduction of non-native species

In Italy, the native field elm (*U. minor* Mill.) has been commonly employed for various uses since ancient times, for instance as living support for grapevine, as fodder for cattle, timber for construction, and as firewood. Field elm was also important as shadow tree in pastures and as an ornamental tree along city avenues and in parks (Goidanich 1936). Given its widespread use, the disappearance of field elm owing to the first DED epidemic was disastrous, and stimulated private nurseries and academic researchers to try to find suitable tree species for substituting dead elms (Sibilia 1932, Ansaloni 1934, Passavalli 1935). The introduction of *U. pumila* as a barrier against DED was strongly encouraged by local authorities during the 1930s (Passavalli 1935). The effects of the DED epidemic on field elm populations were obviously disastrous, while the impact of hybridization with *U. pumila* is more difficult to evaluate. Recent studies indicate that hybridization and introgression between field elm and Siberian elm are causing irreversible changes in the genetic structure of the European species (Brunet et al. 2013, Zalapa et al. 2009).

A possible advantage of introgression from *U. pumila* toward *U. minor* would be the transmission of DED resistance genes, which would most probably increase *U. minor* survival in Italy. On the other hand, introgression toward *U. pumila* could facilitate the acquisition of useful genes from the native *U. minor* that would enhance the ability of *U. pumila* to invade the habitats originally occupied by *U. minor* and enhance its capacity to spread (Brunet et al. 2013).

Conservation of native species

One of the most successful strategies for conservation of European elms was *ex situ* conservation. In the EU RESGEN 78 project, which was devoted to characterization and conservation of the genetic resources of European elm species, hundreds of elms were collected in many European countries (Belgium, France, Germany, Greece, Italy, Spain and Sweden) and planted outdoors in clone collections at different sites.

In these collections, studies on morphological and phenological traits and genetic characterization of elms from different parts of Europe have been carried out, which has facilitated the selection of native genotypes displaying resistance to DED and desirable phenotypes at adaptive and ornamental traits (Collin et al. 2000).

In the past few years, several *U. minor* clones were selected for their resistance to DED in susceptibility tests in Spain and recommended also as reproductive material for employment in forestry (Martín et al. 2015).

An interesting result from studies on bud burst phenology and inoculation trials performed on elms in clone collections is that early flushing *U. minor* clones, generally originating from southern regions of Europe, are less susceptible to DED than late flushing clones, when inoculated at the same date (Santini et al. 2005, Ghelardini et al. 2006). The hypothesis that DED susceptibility is related to spring phenology was investigated observing the relation between disease susceptibility and date of bud burst in European and hybrid elm clones. This result might be explained by a different physiological response of early flushing elm that early in the growing season, when the flight of beetles that vector the disease reaches a peak, are allocating carbohydrates to secondary metabolism, ensuring a better defence against DED (Herms and Mattson 1992, Ghelardini 2007, Ghelardini and Santini 2009). Moreover, at the time of beetle flight, early flushing elms are already producing summerwood with small and scattered vessels with thick cell walls, which may hinder the diffusion of fungal spores and hyphae in the vascular system (Solla et al. 2005).

The studies on phenotypic plasticity have shown that some DED-resistant elm clones have superior growth at all experimental sites, while other clones have stronger genotype x environment interaction and have superior growth only under specific environmental conditions (Santini et al. 2010). The clones that proved to be DED-resistant and adaptable to various sites may be recommended for use in a wide range of environmental conditions.

May breeding for resistance be a suitable strategy against Hymenoscyphus fraxineus?

Dutch elm disease was the first, with chestnut blight (*Cryphonectria parasitica* (Murrill) Barr.), and one of the most impressive tree diseases caused by introduction and spread of alien fungi, which have almost wiped out the populations of native host species from Europe and North America. The strategies adopted to conserve native elms and to cope with such an implacable disease might serve as an example for developing prompt and effective responses against recently introduced invasive fungi, which are currently menacing native tree species. Collection and screening of native populations for finding resistance genes, accumulation of resistance through recurrent breeding and selection, and breeding with non-native and resistant species as a last resort, may be suggested for protecting European ash species against *Hymenoscyphus fraxineus*, the fearsome agent of European ash dieback (Kowalski and Holdenrieder 2009, Baral et al. 2014).

Recent studies have shown that susceptibility to the ash dieback pathogen varies within European ash populations (Cleary et al. 2014) and potentially resistant genotypes are found in stands of European ash trees under high infection pressure by *H. fraxineus* (Lenz et al. 2016, Enderle et al. 2015, Lobo et al 2014, McKinney et al. 2011). Studies also suggest that healthier clones are able to limit the growth and spread of the fungus thereby

minimizing the occurrence of symptoms, and emphasize that high susceptibility is associated with low fitness (McKinney et al. 2012; Lobo et al 2014). In Europe, besides the European ash *Fraxinus excelsior* L., the narrow-leafed ash has also been found susceptible (Kirisits et al. 2009) but nothing is known about variation in susceptibility in this ash species. The flowering ash (*Fraxinus ornus* L.) can be naturally infected by the pathogen when exposed to heavy disease pressure (Kirisits and Schwanda 2015). However, circumstantial evidence from lab tests on mycelial growth of the pathogen on media containing leaf extracts of different host species, suggest that *F. ornus* might be less susceptible to the disease that the other European ash species (Carrari et al 2015). The discovery of additive genetic variation in susceptibility (Kjaer et al. 2012, McKinney et al. 2012, Enderle et al. 2015) encourages wider screening of native ash species and gives hope for selection and breeding of resistant clones of European origin (McKinney et al. 2014).

As in the case with elm susceptibility to DED, ash susceptibility to *H. fraxineus* seems to depend on host phenology and seasonal variation in growth rhythm. Ash genotypes less affected by *H. fraxineus* are more frequently found among trees that shed leaves earlier (McKinney et al. 2011). To explain this observation, McKinney and colleagues have hypothesized that rapid leaf senescence shortens the infection period and reduce the available time for hyphal growth in woody tissues. Whichever the mechanism behind, this source of resistance might be exploited for selection and breeding of resistant clones, possibly in combination with other types of resistance.

In order to obtain ash clones resistant to *H. fraxineus* to be used as ornamental trees or for plantation in artificial forests, and in general for aims other than reintroduction in natural forests, susceptible individuals of native ash species bearing valuable phenotypes at interesting traits might be crossed with genotypes from resistant ash species of foreign origin. Risks linked to introgression of non-locally adapted genetic variation at other traits that might decrease the mean fitness of native populations (Keller et al. 2000) should be considered. The pathogenicity of *H. fraxineus* to different ash species has been little investigated so far, but available studies indicate variable resistance between and within non-European ash species. Manchurian ash (*Fraxinus mandshurica* Rupr.) is generally reported to be asymptomatic in the native range in Asia (McKinney et al. 2014). In Europe, necrotic stem lesions have been observed at a very low incidence on naturally infected ornamental *F. mandshurica* var. *mandshurica* Rupr. trees (Drenkhan et al. 2014). The Central Asian ash species, *F. sogdiana* Bunge is susceptible to natural infections in Europe (Drenkhan et al. 2015). *F. mandshurica* var. *japonica* resulted susceptible when the pathogen was inoculated in the stem, suggesting that defense mechanisms against *H. fraxineus* act in leaves and/or before penetration (Gross and Holdenrieder 2015). Among American ash species, the black ash (*Fraxinus nigra* Marshall) seems to be highly susceptible, while the white ash (*Fraxinus americana* L.) shows only minor symptoms after infection (Drenkhan and Hanso 2010). The green ash *Fraxinus pennsylvanica* Marshall seems moderately susceptible and definitely less susceptible than *F. excelsior* (Drenkhan and Hanso 2010; Gross and Sieber 2016, Kowalski et al. 2015).

F. excelsior and *F. angustifolia* are interfertile, naturally hybridise in contact zones (Fernandez-Manjarres et al. 2006), and can be crossed with *F. mandshurica* that belongs to the same section of the genus, i.e. section Fraxinus. *F. pennsylvanica* and *F. ornus* belong instead to two different sections, i.e Melioides and Ornus, respectively. Both a systematic assessment of resistance to *H. fraxineus* in the genus *Fraxinus* and a detailed survey of crossability between *Fraxinus* species remain to be done.

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Natura 2000 Habitats Dominated by Ash and Elm, Invaded by Alien Invasive Fungi on the Gotland Island of Sweden: an Overview

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Abstract

Natura 2000 sites in Gotland are unique and possess very high nature conservation values. Both ash and elm are the typical tree species in several key habitats of Natura 2000 sites and accommodate a high number of associated (red-listed) species. Recent invasion of Dutch elm disease and ash dieback pathogens threatens elm and ash existence, and threatens the integrity of Natura 2000 sites. Management measures undertaken are aimed primarily at sustaining ash and elm, and associated biodiversity, and thus aimed at sustaining high nature conservation values of Natura 2000 sites.

Keywords: nature conservation, *Fraxinus*, *Ulmus*, invasive pathogens, *Hymenoscyphus*, *Ophiostoma*

Introduction

The Swedish island of Gotland is 2.994 km^2 in size and is fully encompassed by the Baltic Sea. The coast line is 770 km long. Gotland is located about 90 km east of the Swedish mainland and about 130 km west of Latvian coast. Gotland is made up of a sequence of sedimentary rocks of limestones and shales (Laufeld 1974). The highest elevation on the island is 82 m above sea level. Geographical position of the island, specific climate, which is maritime and therefore mild, and nutrient rich calcareous soils have given many valuable habitats and the establishment of many Natura 2000 areas unique in a European context. Natura 2000 is a network of valuable natural areas that have been built up within the European Union (EU). The aim with Natura 2000 areas is to protect birds, habitats and species. Natura 2000 areas were established across EU to address two directives: the Birds Directive (Directive 79/409/EEC), introduced in 1979, in order to protect the birds, and the Habitats Directive (Directive 92/43/EEC), introduced in 1992, to protect valuable habitats and species. Natura 2000 areas on Gotland include 129 sites based on Habitat Directive, and 30 sites based on Birds Directive (Figure 1A). The area of all these sites is of ca. 200.000 ha.

Importance of ash and elm to Natura 2000 sites

Many Natura 2000 sites in Gotland are exceptionally rich in deciduous trees with elm (mainly *Ulmus minor*), ash (*Fraxinus excelsior*) and oak (*Quercus robur*) being most characteristic. Notably, there are at least one million elms in

Gotland, which is the largest wild population in northern Europe (Östbrant et al. 2009). Natura 2000 sites in Gotland include several key habitats of the Habitats Directive Annex I: 6530* - Fennoscandian wooded meadows, 9020* - Fennoscandian hemiboreal natural old broad-leaved deciduous forests, 9070 - Fennoscandian wooded pastures and 9010 - Western taiga.

Among these habitats 6530*, 9020*, 9010* are priority habitat types. Both ash and elm are the core species and pivotal constituents of the habitat types 6530*, 9020* and 9070. They constitute dominant elements comprising the whole landscape structure. Moreover, specifically under Gotland conditions ash and elm are the typical species featuring habitat type 9010*, which makes coniferous taiga stands of the island unique on the worldwide scale. Each of those four habitat types harbour wide range red-listed and vulnerable species directly associated with ash and elm including two species of birds, ten of bats, 37 of beetles, six of butterflies, 12 of fungi and 23 of lichens and mosses. Besides, elm and ash make up almost 70% of the old growth tree layer on Gotland and is significant even in a national context given that 17% of the Swedish population of old elms and 24% of the old ash are on Gotland (Skogsstyrelsen 2016).

From the cultural and historical perspective, Gotland represents the richest region of Sweden in terms of both pollard meadow landscape and noble hardwood dominated land where ash, elm and oak constitute a crucial component (Mebus 2006). During the last 1500 years they have been intensely used for pollarding, hay harvesting and pasture (Hultengren 2006). Consequently these tree species are of exceptional cultural and historical value. Prior to establishment of protected areas including Natura 2000 sites, the biodiversity associated with broadleaved woodlands, wooded pastures and wooded meadows has long been under serious threat from the intensification of agriculture, abandonment of grazing or conversion to conifer plantations (Jönsson et al. 2011). At present, all Natura 2000 sites in Gotland are protected with elaborated individual nature conservation plans (Lansstyrelsen Gotlands Län 2016). Nature conservation plans describe the natural values that should be preserved, the objectives of the conservation of the site and the measures needed to achieve them. The plan also describes the measures that may threaten the species or habitats that are protected under Natura 2000 conservation plan.

In the last few years, however, elm and ash populations in Gotland are threatened by the alien invasive fungi causing Dutch Elm Disease (DED) and Ash Dieback (ADB). DED and ADB result in loss of elm and ash trees and associated biodiversity especially in the habitats of the Habitat Directive Annex I 6530*, 9020*, 9070 and to a lesser extent in 9080, 6210, 6280, 9180 and 8210. The virulent form of DED was observed for the first time in Gotland in 2005 (Menkis et al. 2016b), while ADB invaded Sweden including Gotland in 2001 (Barklund 2007).

Dutch elm disease in Gotland

DED is a lethal vascular wilt disease caused by the pathogenic *Ophiostoma* (Ascomycota) fungi, which during the last 100 years have destroyed billions of elm trees worldwide (Phillips and Burdekin 1982). In Gotland, DED is comprised of two distinct fungal pathogens, the less virulent *O. ulmi*, and the virulent *O. novo-ulmi.* Conidia of the DED pathogens are vectored by the elm bark beetles (*Scolytus* spp.) (Ploetz et al. 2013), and in Gotland, *Scolytus multistriatus* is the only known vector (Menkis et al. 2016a). Despite the fact that DED was present for decades in Europe including mainland Sweden, the elm population in Gotland remained unaffected. The geographical position of the island in the Baltic Sea has likely prevented natural dispersal of DED.

After introduction in 2005, the DED was observed for the first time in the north-eastern part of Gotland, and in the following years, it rapidly spread in all directions, generally following the major distribution of elms including a number of Natura 2000 sites (Menkis et al. 2016b). A rapid spread of DED was particularly notable during the first three years. Consequently, the area with DED-diseased trees in Gotland increased from 15.8 km^2 (0.5%) in 2005 to 1446.2 km^2 (48.3%) in 2008, and its diameter expanded from 5.5 to 85.6 km, respectively (Menkis et al. 2016b). During 2005–2008, 4278 DED-diseased elms were felled and destroyed. During the period between 2009 and 2013, the implementation of a new combat strategy resulted in the felling and destruction of 17903 DED-diseased and visually healthy elms growing in their vicinity. The use of the herbicide killed stumps and root systems of harvested elms. Implementation of this control strategy resulted in the number of DED-diseased trees and the area of Gotland infected by DED remaining similar each year (Menkis et al. 2016b).

The ongoing control measures resulted that incidents of DED have stabilised and spread to new areas was largely arrested. In 2009, both DED pathogens *O. ulmi* and *O. novo-ulmi* were present in Gotland. However, in 2012, occurrence of *O. ulmi* was found to be very occasional (Menkis et al. 2016a), suggesting that, as elsewhere, it is being replaced by *O. novo-ulmi* (Brasier et al. 2004). Moreover, *O. novo-ulmi* was found to be the second most common fungus vectored by *S. multistriatus* (Menkis et al. 2016a). Due to ongoing incidents of DED, all species of elms including *U. minor*, which is the major elm species in Gotland, are now red-listed in Sweden as critically endangered (CR) (ArtDatabanken 2016a). Besides, *U. minor* is generally more susceptible to DED than other *Ulmus* species.

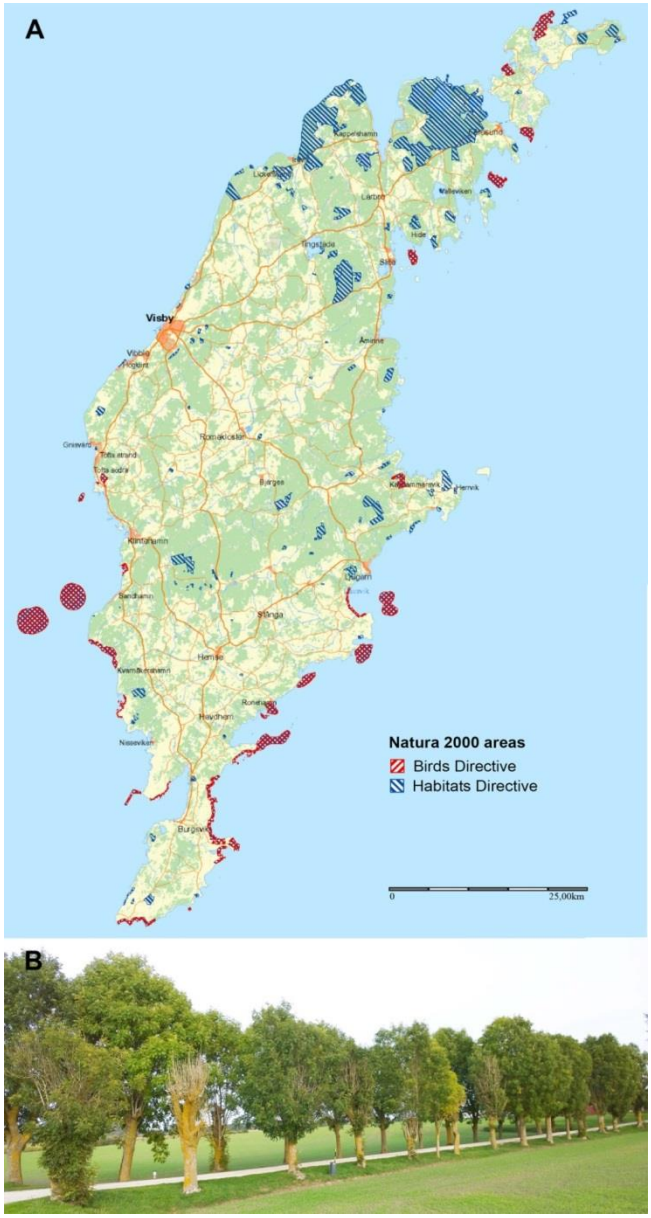

Figure 1. A. A map of Gotland showing Natura 2000 areas based on Birds Directive and Habitats Directive. **B.** Alley of *Fraxinus excelsior* in Gotland year 2015 showing variable degree of tolerance of individual trees to ash dieback pathogen *Hymenoscyphus fraxineus*

Ash dieback in Gotland

ADB is caused by *Hymenoscyphus fraxineus* (Queloz et al. 2011), which is invasive in Europe (Husson et al. 2011), and which causes severe damage to *F. excelsior*, often resulting in mortality (Pautasso et al. 2013). Its origin is the Asian Far East (Zhao et al. 2012, Zheng and Zhuang 2014). It appears that the pathogen invaded Europe in early 1990s together with saplings of *Fraxinus mandshurica* imported to Poland and via airborne spores spread from there in all geographic directions (Bakys et al. 2009). ADB invaded Gotland approximately at the same time as mainland Sweden (2001-2002) leading to dieback and decline of many ash trees. Evaluation of ADB damage in Gotland showed that it largely resembles situation observed elsewhere in Europe with ca. 70% of susceptible or dead individuals in the population (Jonsson and Thor 2012). Only, a small fraction of the *F. excelsior* population can be expected to survive due to inheritable resistance (Pliūra et al. 2011) or specific phenological traits such as early leaf senescence (McKinney et al. 2011). Despite the damage, observations from Gotland demonstrate a variable degree of tolerance of individual trees to ADB, i.e. from dead to healthy-looking (Figure 1B), suggesting that breeding for resistance may be possible. As a result of ADB, since 2010 *F. excelsior* is red-listed in Sweden as strongly endangered (EN) (ArtDatabanken 2016b).

Besides the loss of elm and ash, the species diversity associated with these tree species in woodlands, wooded pastures and wooded meadows of Natura 2000 sites are threatened strongly by the DED and ADB. Many species linked to these trees and high conservation value is being lost when the old elm and ash trees die. Assessment of associated lichens in Gotland showed that dieback of ash trees results in loss of lichen community viability, with significant local reductions in species richness and shifts in lichen species composition (Jonsson and Thor 2012).

Sustaining ash and elm in Natura 2000 sites

To address recent situation arising from invasion of DED and ADB in Gotland, various inventories, update of the conservation plans and concrete management measures are undertaken that favour elm or ash in Natura 2000 sites. To exemplify Natura 2000 sites on Gotland and measures implemented, the descriptions are provided below for Allekvie and Hörsne sites.

Allekvie wooded meadow

Allekvie wooded meadow is 11.25 ha in size and includes four habitats of the Habitat Directive Annex I: 4.2 ha of 6530*, 3.2 ha of 6210, 3.1 ha of 9020* and 0.6 ha of 9070 (Figure 2A). Allekvie wooded meadow is rich in deciduous trees and lies in the area, which has been managed as a meadow for a long period, possible for more than two thousand years. Approximately one third of the area is managed traditionally where the leaves and twigs are collected in the spring and the hay is cut and removed in the summer. The other parts are grazed or have become deciduous woodland. Deciduous trees in the meadow consist primarily of *F. excelsior* (37 veteran ash trees), *U. minor* (32 veteran elms), *Q. robur* (13 veterans), *Malus sylvestris*,

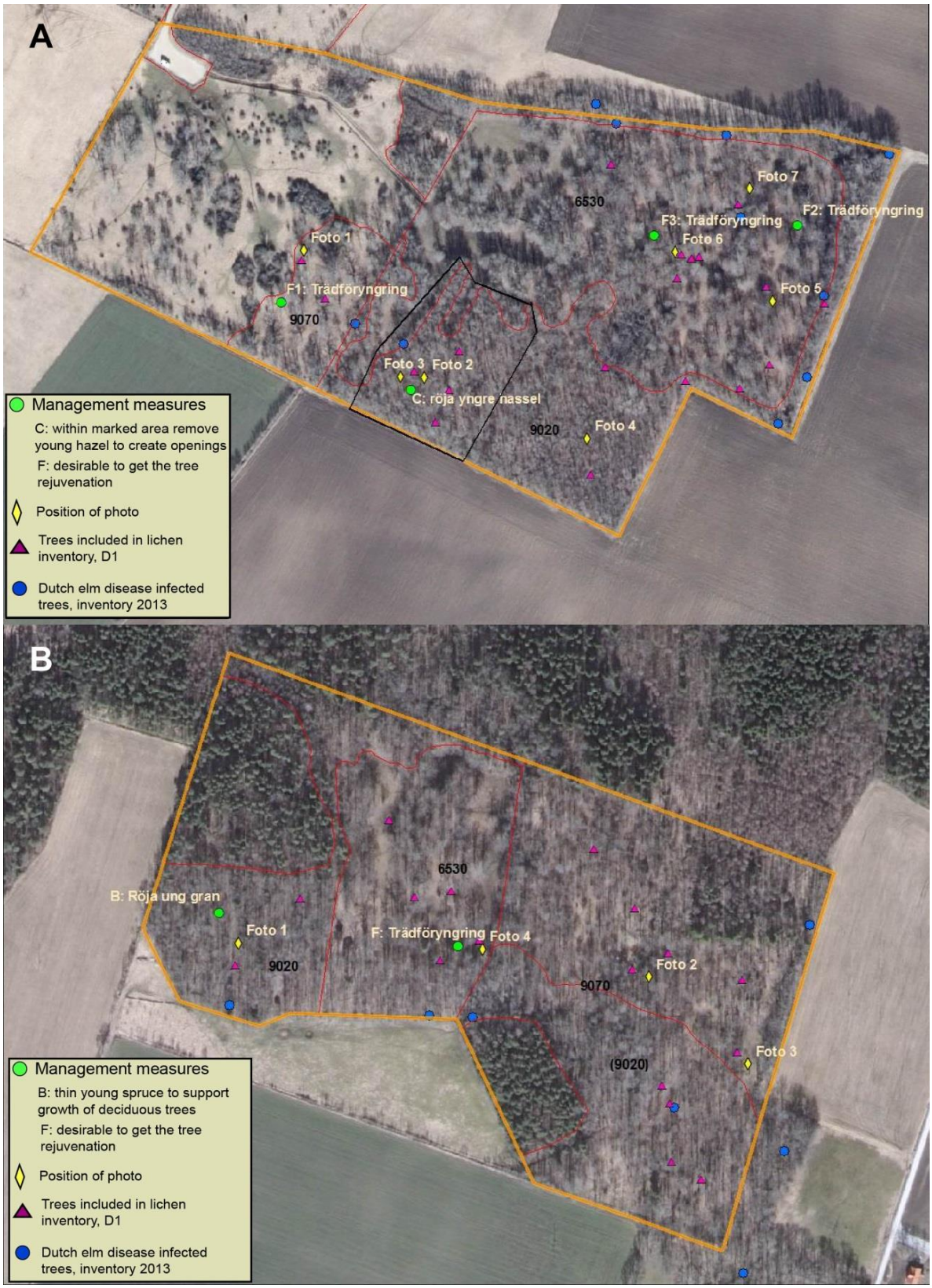

Figure 2. A. Allekvie wooded meadow and **B.** Hörnse wooded meadow in Gotland with planned management measures to sustain trees of ash and elm and associated biodiversity (maps are adapted from the restoration plans of Länsstyrelsen Gotlands Län)

Sorbus aucuparia and *Betula pendula*. Many of the ash and elm trees in the area look like old pollards. Ash and elm are two of the most important tree species in the meadow, which provide habitat for many species and give the site its character. Between the trees there are islands with *Corylus avellana* and other shrubs such as *Cornus sanguinea*, *Rhamnus cathartica*, *Rosa* spp., *Prunus spinosa* and *Crataegus* spp. The meadow has a rich plant and fungal flora. At least 27 species from the Swedish Red Data Book have been recorded.

Specifically, the wooded meadow of 6530* habitat is characterised by very high nature conservation values. In particular, many rare soil fungi, but also many species of lichens are present on the trees. The nature conservation values are linked to the old meadow trees of ash, elm and oak, and a continued hay meadow management and tree continuity. Lichens linked to ash and elm trees are rich in species. The broad-leaved forest of 9020* habitat was regrown with younger trees and hazel. The value lies mainly in the old-growth trees of ash, elm and oak. Lichens, mosses, wood-decay fungi and soil fungi are examples of species-groups that thrive here. Lichen flora associated with ash and elm is less species rich, probably due to inappropriate light conditions. Epiphytic lichen *Lobaria pulmonaria* is quite common and thrive on most trees, so for the sake of lichen alone the continuity of ash and elm are not necessary. However, there are other nature conservation values that would be lost if ash and elm disappear, and the goal is to maintain continuity of these tree species. The wooded pasture of 9070 habitat is characterized mainly by oak and ash trees. The value lies primarily in the rough oaks with high conservation values. A number of ash trees are declining.

Implemented concrete management measures to promote elm or ash are shown in Figure 2A. Management measures include: within the selected area in the south there is much of younger hazel growing next to the older hardwoods. To create more light in the area, and to get less sharp transition between the open meadow and hardwood forests and to open valuable hardwood trees, younger hazel should be cleared; in the meadow part on the north-east, it is desirable to have the tree rejuvenation of ash and elm. If healthy seedlings grow up, they should be fenced in order to become replacement trees in the future.

Hörsne wooded meadow

Hörsne wooded meadow is 9.68 ha in size and includes three habitats of the Habitat Directive Annex I i.e. 1.5 ha of 6530*, 5.8 ha of 9020* and 2.38 ha of 9070 (Figure 2B). The primary aim of the conservation plan for the Hörsne wooded meadow is to maintain favourable conservation status at a biogeographic level for the habitat types and species (according to the Habitat Directive and Bird Directive) included in the Natura 2000 site. Hörnse wooded meadow is a traditionally managed meadow. It is the remains of a once much larger wooded meadow area. Continuous management has taken place here for at least 2000 years. The meadow is criss-crossed by old boundary markers which can be seen today as lines of stones. The remains of a fossilised field can be seen here. The site has probably contained deciduous trees for at least 5000 years. The tree layer is dominated by *F. excelsior*, *U. minor* and *Q. robur*. The shrub layer is dominated by *Corylus avellana*. Many of the trees have been pollarded. The meadow is surrounded by woodland, which means that it has an even and humid micro-climate. The open parts of the meadow contain high biodiversity of plant communities. 1.5 hectares are managed as a meadow and 5.8 hectares have developed into deciduous forest. The meadow hosts species rich lichen and fungi flora and is also a good site for bats. At least 18 species from the Swedish Red Data Book have been recorded from the site.

On the site, both ash and elm are given as typical species for the habitats 6530* and 9020*. The ash and elm trees have been pollarded and have grown on poor soils, which means they are relatively small and have hollows and bark structure suitable for many epiphytic lichens and mosses. These hollows are important breeding sites for *Ficedula albicollis*, which is found here. It is also an important site for bats and *Eptesicus nilsonii* has been confirmed from this site. It is likely, however, that more protected species can be found if further study is undertaken. The species *Cossonus parallelepipedus* has been found in this site, which is a small weevil, which lives in hollow trees and especially in *Ulmus*. *U. minor* and *F. excelsior* are thus very important tree species on this site.

Specifically, the wooded meadow of 6530* habitat is characterised by many ash and elm trees several of which are veteran trees. Many species of lichens are attached to ash and elm, indicating desirable continuity of these tree species as well as continued and extended meadow management. 9020* habitat represent overgrown wooded meadow with elements of older trees. Dense lower layer of younger trees is composed of deciduous trees and of young spruce. Many species of lichens are attached to the ash and elm, making continuity of these species desirable. 9070 habitat is a wooded pasture with high nature conservation values, mainly linked to ash and elm, but also to oak and aspen. Many species of lichens are found on ash and elm, and their persistence dependent on the continuity of these tree species and continued grazing.

Implemented concrete management measures to promote elm and/or ash are shown in Figure 2B. Management measures include: in the western area of hardwood forest there are a lot of younger spruce trees, which should be cleared before it grows other and shadows the deciduous trees; in the meadow, it is desirable to get the tree rejuvenation that in the future can provide the natural conservation

values linked to the old-growth trees. If healthy seedlings appear, they should be saved and where appropriate fenced in order to become replacement trees in the future.

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Tests with Wood-Decay Fungi to Control Sprouting from Cut Stumps Infected by Dutch Elm Disease

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Abstract

The Dutch elm disease pathogen *Ophiostoma novo-ulmi* in year 2005 invaded the Swedish island of Gotland, which possesses large and valuable population of elms. The control of the disease is accomplished when infected elms are harvested and destroyed, and stumps are treated with the glyphosate herbicide to kill the stumps and the root systems, and prevent further spread of the disease to the neighbouring trees via root contacts. The aim of the present study was to test an alternative method to control the stump sprouting by deploying two species of saprotrophic wood-decay fungi as biological control agents. The study was carried out during three consecutive years 2014, 2015 and 2016. Fungal inoculum of *Chondrostereum purpureum* and *Stereum hirsutum* was prepared by cultivating vegetative mycelia in liquid nutrient medium and then formulating into a gel. Each year, the inoculum was applied at the beginning of the growing season on the surface of fresh stumps. In total, 250 stumps were treated with saprotrophic wood-decay fungi and 250 stumps were left as non-treated control. Assessment of stump mortality was carried out ones after each growing season. Results showed that the mortality of stumps (without living sprouts) was low in each treatment and year (between 4.1% and 25.6%) and did not differ significantly from those in the control. In conclusion, the method tested had very limited or no effect on the mortality of elm stumps, and thus, appears to be unsuitable to control the spread of Dutch elm disease via root contacts and sprouts.

Keywords: biological control, Dutch elm disease, *Ophiostoma*, saprotrophic wood-decay fungi, sprouting control, *Chondrostereum*, *Stereum*

Introduction

The Swedish island of Gotland has large and highly valuable populations of elms (mainly *Ulmus minor*), which currently is threatened by the invasive Dutch elm disease (DED) (Menkis et al. 2016b). DED is an aggressive vascular wilt disease, which during the last hundred years have destroyed billions of elm trees worldwide (Phillips and Burdekin 1982). Despite the DED was present in Europe for decades (Brasier et al. 2004), it invaded Gotland only in 2005 (Menkis et al. 2016b). Geographical isolation of the island in the Baltic Sea was suggested to be the limiting factor preventing natural arrival of the disease, which was probably brought to the island with DED-infected elm wood (Menkis et al. 2016b). DED is caused by the pathogenic *Ophiostoma* (Ascomycota) fungi, which are vectored by *Scolytus* bark beetles (Ploetz et al. 2013, Menkis et al. 2016a). However, the disease also possesses the ability for secondary spread from tree to tree via root grafts (Santini and Faccoli 2015). The DED fungi reproduce by a yeast-like budding process, and the bud spores are distributed in the sap stream and spread rapidly throughout the current xylem. The fungi cause wilting and death, both by the plugging of the conducting system and by the production of toxins. A typical internal symptom of the disease is the formation of a brown ring in the infected sapwood resulting

from the formation of tyloses and gels in the xylem vessels (Santini and Faccoli 2015). In Gotland, the control of the disease is accomplished when infected elms are identified, harvested and destroyed, and stumps are treated with the glyphosate herbicide to kill the stumps and the root systems, and prevent further spread of the disease to the neighbouring trees via root contacts (Menkis et al. 2016b).

However, the use of synthetic herbicides involves the potential hazards due to toxicological and environmental risks. Besides, the use of synthetic herbicides may be restricted in certain areas. Therefore, alternative methods to control sprouting are desirable and would allow limit the input of synthetic herbicides. The deployment of indigenous saprotrophic fungi to control stump sprouting can be seen as environmentally friendly approach (Dumas et al. 1997, de Jong 2000). For example, a saprotrophic fungus *Chondrostereum purpureum* was shown to possess a considerable potential as a biocontrol agent to control stump sprouting in several deciduous tree species by applying vegetative mycelium on freshly cut stumps (Lygis et al. 2012 and references therein). *C. purpureum* aggressively colonises the cambium in an injured area causing mortality of stump sprouts (de Jong et al. 1990). Another saprotrophic fungus, *Stereum hirsutum*, was also shown to be an aggressive and common colonised of wounds in deciduous trees (Vasiliauskas 1998). It was also found in association with unusual decline of tanoak sprouts (McDonald et al. 1988), and therefore, may possess the capacity to control stump sprouting. Despite the numerous field tests and the development of some saprotrophic fungi into the commercial products to control stump sprouting (de Jong 2000), the information on the efficacy of such fungi to control sprouting from elm stumps is scarce.

The aim of the present study was to test an alternative method to control sprouting from elm stumps by deploying saprotrophic wood-decay fungi *C. purpureum* and *S. hirsutum* as potential biological control agents.

Materials and Methods

Study sites

The study was carried out at Ganthem (year 2014), Vallstena (2015) and Endre (2016) sites situated in the mid-eastern part of Gotland island, Sweden (Table 1).

All sites were in close proximity (between ca. 9 and 11 km from each other) and characterised by similar climatic conditions. However, the site at Vallstena included a number of open areas, while sites at Ganthem and Endre were less exposed. Forest stands were dominated by *Ulmus* spp. with *Pinus sylvestris* L., *Picea abies* (L.) Karst., *Betula pendula* Roth, and *Alnus* spp. in admixture. The sites were characteristic to Gotland in terms of landscape and trees species composition, and were in the areas characterised by a high incidence of DED.

Table 1. Sites in Gotland and the number of fresh elm stumps treated during 2014-2016 with saprotrophic wood-decay fungi. The geographical positions of the study sites at Ganthem, Vallstena and Endre are shown in parenthesis.

Treatment	Site			All
	Ganthem (N57°30' E18°37') 2014	Vallstena (N57°36' E18°39') 2015	Endre (N57°35' E18°30') 2016	
Chondrostereum purpureum	48	43	34	125
Stereum hirsutum	49	42	34	125
Control	97	85	68	250
Total	194	170	136	500

DED-diseased elms were identified based on external and internal disease symptoms (Menkis et al. 2016a, Menkis et al. 2016b) during the surveys that were carried out by the Swedish Forest Agency in each preceding growing season. After identification, DED-diseased elms were felled during the dormancy period (November to April), transported to the local power plant, chipped and burned. Stumps of felled trees were treated with the herbicide glyphosate in order to kill root systems and to prevent further spread of the DED via roots and sprouts (Menkis et al. 2016b). However, the stumps in protected areas such as Natura 2000 sites and on land of organic farming were not treated with the herbicide due to legislation requirements. Instead, these stumps were used for the treatment with selected saprotrophic wood-decay fungi native to Gotland. In total, there were 194 such stumps in 2014, 170 in 2015 and 136 in 2016, resulting in 500 stumps altogether. Each year, half of stumps were treated with mycelia of saprotrophic wood-decay fungi and half were left as non-treated control (Table 1). The stumps used for the treatment and non-treated control stumps were selected randomly resulting in intermixed distribution at each site.

Preparation of fungal inoculum and inoculation

Fungal cultures of *Chondrostereum purpureum* strains P2.2 and *Stereum hirsutum* strain S8 were obtained from the culture collection of the Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala. Inoculum of each fungal species was produced separately. Firstly, the fungi were sub-cultured on Hagem agar medium (Stenlid 1985) in order to check viability and sterility of the cultures. Sub-cultured fungi were maintained in 9 cm diameter Petri dishes at room temperature (ca. 21°C) in the dark. For the production of fungal inoculum, which comprised vegetative mycelia, 5 l Erlenmeyer flasks were used each containing 2 l of liquid Hagem media. Ten agar plugs 0.5 × 0.5 cm in size with established fungal mycelia from an actively grow-

ing colony were aseptically inoculated in each flask and incubated at room temperature in the dark for four weeks.

Fungal inoculum was prepared one day before application in the field. Mycelium of each fungal species was harvested from liquid cultures through filtering, placed in a Moulinex Q50 blender (SEB, Ecully, France) and homogenised for two minutes. In order to prevent rapid desiccation of fungal mycelia following application on the stumps, homogenised mycelia was formulated into a gel by mixing at a ration 1:10 with a water suspension of Agrisan gel (Flügel GmbH, Osterode am Harz, Germany), which was prepared following recommendations of the producer. Agrisan gel is based on the poly-acrylic acid potassium salt crosslinked superabsorbent polymer and is commonly applied to plant roots at their outplanting. Prepared fungal inoculum was stored at 4°C until used in the field.

Each year, inoculation was done ones in the middle of May by applying prepared fungal inoculum on the entire surface of fresh stumps using a trowel. Each fungal species was inoculated separately. The control stumps were treated in the same way only by applying a water suspension of Agrisan gel alone. The treatments were applied each year on new stumps. The number of stumps treated with saprotrophic wood-decay fungi and non-treated controls in each year are shown in Table 1. To check vitality of the inocula, after the application in the field was completed remaining inoculum was brought back to the laboratory and from each treatment the respective fungus was re-isolated onto Hagem medium.

Assessment of stumps

In order to evaluate the effect of different treatments on mortality of stumps, the presence or absence of sprouting from the stumps was used as a measurement. In the absence of living sprouts the stumps were scored as dead. Each year, the assessment was done in the middle of September by visual inspection of all treated and non-treated control stumps. The assessment for production of fungal sporocarps and the detection of fungi in wood samples was not carried out.

Statistical analyses

The impact of each treatment on stump mortality was evaluated within each year by comparing the actual observations (dead/living stump data) in different treatments and a non-inoculated control using chi-square tests (Mead and Curnow 1983). Similarly, the impact of site/year was evaluated by comparing the actual observations of the same treatment among different sites/years. As each of the datasets was subjected to multiple comparisons, confidence limits for *p*-values of chi-square tests were reduced corresponding number of times as required by the Bonferroni correction (Sokal and Rohlf 1995).

Results

The mortality of stumps at Ganthem site after the growing seasons 2014 was 6.3% in *C. purpureum* treatment, 4.1% in *S. hirsutum* treatment and 6.2% in non-inoculated control (Figure 1). Comparison by chi-square test showed that different treatments and a control did not differ significantly from each other. The mortality of stumps at Vallstena site after the growing seasons 2015 was 25.6% in *C. purpureum* treatment, 21.4% in *S. hirsutum* treatment and 23.5% in non-inoculated control (Figure 1), thereby all of these did differ significantly from each other. The mortality of stumps at Endre site after the growing seasons 2016 was 11.8% in *C. purpureum* treatment, 14.7% in *S. hirsutum* treatment and 13.2% in non-inoculated control (Figure 1), and chi-square test showed that different treatments and a control did differ significantly from each other.

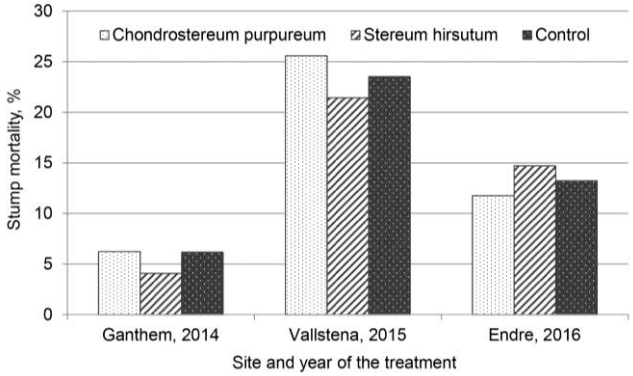

Figure 1. Mortality of elm stumps after the first growing season following inoculation with saprotrophic wood-decay fungi *Chondrostereum purpureum* and *Stereum hirsutum*.

Assessment of stump mortality after the second growing season following application of the treatments showed that the number of dead stumps remained largely unchanged (data not shown). Within each treatment (*C. purpureum*, *S. hirsutum* or control), comparison among different years showed that stump mortality was significantly lower in 2014 vs. 2015 ($p<0.04$), but did not differ significantly when compared 2014 vs. 2016 or 2015 vs. 2016.

Discussion

The results showed that the mortality of elm stumps was relatively low in each treatment and year (Figure 1). Moreover, within each year, the stump mortality in inoculation treatments was similar to those in the control (Figure 1), demonstrating that the method tested was largely unsuitable to control the stump sprouting, and thus, unsuitable to control the spread of Dutch elm disease via root and sprouts. By contrast, the glyphosate herbicide treatment is known to be highly efficient, thereby resulting in high rates

of stump mortality. The observed variation in stump mortality among different sites/years (Figure 1) was likely due to particular environmental conditions of each site. It was noted that the majority of dead stumps were in direct exposer to the sun while the stumps with sprouting were often in the shadow of the living trees. Consequently, the higher stump mortality at Vallstena (year 2015) (Figure 1) was likely due to their higher exposer to the sun as compared to those stumps at Endre (year 2016) and in particular at Ganthem (year 2014) sites. The latter suggests that environmental conditions and in particular sun radiation, but not the stump inoculation with saprotrophic wood-decay fungi, were largely responsible for the observed levels of stump mortality in different treatments and years.

Previously it was shown that the stump treatment with *C. purpureum* as compared to controls resulted in significantly higher levels of stump mortality in *Populus tremuloides* and *P. grandidentata* (Dumas et al. 1997), *Betula pendula* and *Acer negundo* (Lygis et al. 2012), *Alnus rubra* (Becker et al. 2005), *Sorbus aucuparia* (Hamberg et al. 2011) and *Prunus serotina* (de Jong 2000). In many cases the effect of this biological control treatment on mortality of stumps was as high as of synthetic herbicides. However, such positive effect of the stump treatment with *C. purpureum* appears to be host specific. For example, no effect of the treatment was observed on *Robinia pseudoacacia* and *Hippophae rhamnoides*, which was suggested to be due to either resistance of these trees to *C. purpureum* infection or significantly delayed response (Lygis et al. 2012). Taken together, the above demonstrates that the stump treatment with *C. purpureum* is not equally efficient on different tree species. In agreement, the result of the present study confirmed the latter observation at the same time showing that elm as a tree species exhibits little or no response to the stump treatment with tested wood-decay fungi.

In conclusion, the method tested had very limited or no effect on the mortality of elm stumps, and thus, appears to be unsuitable to control the spread of Dutch elm disease via root contacts and sprouts.

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Boxwood Blight in Turkey: Impact on Natural Boxwood Populations and Management Challenges

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Abstract

There are approximately 1000 ha of natural populations of *Buxus sempervirens*, a small evergreen tree widely used in ornamental landscaping, in Turkey. These populations usually occur as an understorey in forests. Since the outbreak of boxwood blight epidemics in the Eastern Black Sea region in 2011 approximately 90% of the trees in the affected areas have died. In this paper we discuss the possibilities to save boxwood in Turkey.

Keywords: boxwood blight, Turkey, disease management

Introduction

Importance of Buxus spp. in Turkey

Boxwood (*Buxus sempervirens* L.) is a small evergreen tree, widely used in ornamental landscaping as individual specimens or in hedges, parterres and groups. It is among one of the most important ornamental plants in the municipalities in Turkey. Seedlings are produced by state and municipality nurseries and to less extent by private ones (Anon. 2015). In addition to ornamental plantations there are approximately 1000 ha of native boxwood populations. These populations, comprising mostly of *Buxus sempervirens* L., usually occur as an understorey in forests. The distribution covers the Black Sea and the Marmara Sea regions but there are also small populations in the Mediterranean region. These populations have suffered significant damage over centuries due to harvesting of the valuable wood and unplanned cutting of shoots for floricultural usage. As part of efforts of sustainable usage of boxwood harvesting is regulated and some exceptional populations have been selected as genetic reserves.

Boxwood blight and its spread

Traditionally, boxwood was regarded to be free of serious pests (Henricot et al. 2008). In mid-1990s a new disease, boxwood blight, appeared on boxwood plants in the United Kingdom and spread throughout Europe (Henricot et al. 2000, Henricot 2006). The disease is caused by two closely related fungi of unknown origin, *Calonectria pseudonaviculata* (Crous, J.Z. Groenew. and C.F. Hill) L. Lombard, M.J. Wingf. and Crous 2010 (syn. *Cylindrocladium buxicola* Henricot, G1 clade) and *Calonectria henricotiae* Gehesquière, Heungens and J.A. Crouch (G2 clade) (Gehesquière et al. 2016). Near-clonal genetic background of both species indicates that they are invasive alien species introduced as single asexually spreading isolates (Gehesquière et al. 2016). Since boxwood is among one of the most popular ornamental plants in the world, spread especially via trade of plants probably contributed to the rapid spread of the disease (Henricot and Culham 2002). Distribution of the disease is not limited to Europe; boxwood blight is also known in New Zealand since the late 1990s (Crous et al. 2002) and it was first observed in North America in 2011 (Douglas 2011, Ivors et al. 2012). Box-

wood blight affects many commercial boxwood species and their varieties, among which *B. sempervirens* and its varieties are the most susceptible ones (Henricot 2006, Henricot et al. 2008, LaMondia 2015).

Both fungal species can grow in temperatures ranging between 5 and 30° C, with high growth rates at temperatures ranging from 15 to 25 ^{o}C and observed optimum at 25 ^{o}C (Henricot and Culham 2002, Gehesquière et al. 2016). Mycelial growth of *C. henricotiae* is faster than that of *C. pseudonaviculata* at 20 ^{o}C and higher temperatures (Gehesquière et al. 2016). The life cycle of the fungi in optimal conditions can be as short as one week. The spread of the fungi occur asexually by conidia, which are sticky indicating that they are disseminated by rain splash or a vector. At warm temperatures (18-25 ^{o}C) and high humidity spore germination starts three hours and penetration of the leaves five hours after inoculation. Penetration occurs directly or via stomata by hyphal growth, without formation of specific infection structures (Henricot 2006). Mycelium of the pathogens can survive prolonged periods up to several years in leaf litter on the soil surface or buried in the soil. Survival can be facilitated by formation of microsclerotia (Henricot 2006, Dart et al. 2015).

In this paper we give an overview of the health situation of natural *Buxus sempervirens* populations in Turkey since the outbreak of boxwood blight and discuss some disease management options to save the remaining boxwood trees as well as the serious threat caused by another invasive pest, the box tree moth.

Boxwood blight epidemic in Turkey

Disease outbreak and spread in Turkey

In Turkey, boxwood blight was first detected and identified in the end of 2011, in the Eastern Black Sea region in Trabzon and Artvin provinces (Akıllı et al. 2012). It is not known how and when the pathogen was introduced to Turkey. Nevertheless, the disease seemed to have made a jump from Western and Central Europe to the Eastern Black Sea region and Iran (Gorgiladze et al. 2011, Akıllı et al. 2012, Mirabolfathy et al. 2013). According to official reports boxwood blight epidemics in the region broke out more or less simultaneously: in 2010 in Georgia (Gorgiladze et al. 2011), in 2011 in north-eastern Turkey (Akıllı et al. 2012), and in 2012-2013 in Southern Russia (EPPO 2016). It is possible that the disease has been present in the Eastern Black Sea region for several years before the reported disease outbreaks, as the earliest, unpublished observations of symptoms similar to boxwood blight are from the Caucasus in 2007 (Marc Kenis, Iryna Matsiakh, personal communication). Although details about techniques used in identification of the pathogen in connection of these observations are not known, the observations are most likely correct. However, several factors, including the complexity of natural ecosystems, a time lag between the arrival of an invasive pathogen and outbreak of a disease as well as trade of boxwood, may make tracing of the boxwood blight outbreak to its source difficult or impossible (cf. Brasier 2008).

The break out of the epidemics in north-eastern Turkey was observed in November 2011, one year after detection of the disease in Georgia in Mtirala National park located 15-30 km from the Turkish border (Gorgiladze et al. 2011). At that time, the disease was spreading epidemically in the natural *B. sempervirens* populations in Trabzon and Artvin provinces. Devastating effects were visible in the end of 2012 in some of the populations near the Black Sea coast; in affected areas up to approximately 90% of the boxwood plants were completely defoliated (Lehtijärvi et al. 2014). Distribution of the disease was approximately 200 km along the coast from Georgian borders towards west and 3 to 25 km along the steep, moist river valleys to the south. However, possibly due to scattered distribution of the *B. sempervirens* populations in the river valleys the populations located further south (inland) were still free of infection or showed only the very first signs of arrival of the epidemic.

Impact of boxwood blight on the natural populations

In November 2012, the degree of damage in the affected areas (Lehtijärvi et al. 2014) indicated that the boxwood blight epidemics threatened the existence of the natural boxwood populations in the Eastern Black Sea region of Turkey. Boxwood trees growing as understory and dense groups were often completely defoliated. At that time some of the severely defoliated box wood plants were producing epicormic shoots, mostly at the lower part of the trunk. However, it is unclear which proportion of the plants was able to recover. A revisit to one of the sites in 2015 indicated that such a recovery was not common. Owing to the optimal conditions for the life cycle of the fungus during several months in the affected area repeated infection of the foliage and shoot axis in both the epicormic and normal shoots is likely a reason for failure in recovery. The progress of the damage followed a pattern: defoliation progressed from base to the top of the trees, and in case of complete defoliation, drying of the tree from top to down. A minor proportion (≤10%) of the boxwood plants had survived the disease. They were scattered and had at least a limited green top in the canopy. The situation seems to be similar throughout the affected areas also in 2016 (Metin Karadağ, personal communication). Natural boxwood vegetation in the near future is likely to consist of single boxwood individuals scattered in the landscape.

Signs of potential genetic resistance in populations and individuals

In the end of 2012, one to two years after the outbreak of the epidemics in north-eastern Turkey, even in the most severely affected sites single boxwood plants with variable proportion of green canopy were present (Lehtijärvi et al. 2014). Similar observations made in March 2015, in one of the locations, indicated that some of the plants were either more resistant or had escaped the disease. The trees with no or little signs of defoliation were often growing in special locations in the affected stands: under rock cliffs, scattered in on a hill slope with other tall plants and on slopes facing southwest to west. These observations indicated that in spots where air currents carrying conidia within rain splash could not reach, or where the leaf wetness period was shorter due to sunshine infection pressure on the plants was lower.

Ongoing and planned projects / research in Turkey

National Pest Risk Analysis report concerning boxwood blight was prepared in 2015. In addition, survey work on distribution of the disease in Turkey has been conducted and a distribution map is under preparation (Anon. 2015).

Management options to mitigate the impact on natural boxwood populations

Silvicultural management options

During boxwood blight epidemics effects of silvicultural management strategies may be insignificant. However, in post epidemics situation silvicultural methods may help to reduce infection pressure on surviving boxwood individuals. For instance, increased aeration and solar radiation resulting from removal of upper canopy layer could diminish leaf wetness period and thereby lower the new infections. Creation of gaps between boxwood individuals by thinning operations may somewhat reduce infections. The spread of the disease in boxwood hedge can be very rapid in one autumn (Rosander 2012). Within severely defoliated boxwood vegetation recovery via regrowth from epicormic shoots or root suckers could be promoted in a scattered pattern using e.g. 100 m spacing between the plants. It is important that the boxwood plants between these recovery spots are left to die naturally, or if harvested, the remaining stumps killed with a herbicide to create gaps free of boxwood shoots and foliage. Inoculum of boxwood blight in upper soil layer in the recovery spots could be reduced by flaming the surface (Dart et al. 2012).

Alternative *Buxus* species

While usage of less susceptible boxwood species or varieties, such as *Buxus sinica* (Rehder and E.H. Wilson) M. Cheng var. *insularis* (LaMondia 2015) would not solve the issue of saving the natural boxwood populations, replacement of susceptible boxwood with them in ornamental usage would help to reduce the spread of the disease. In long term it would also eliminate reinfection of the natural stands from ornamentals. This would be beneficial if the surviving, possibly more resistant boxwood individuals in the affected region will be used in efforts to recover the original boxwood vegetation.

Chemical control

Due to obvious lack of resistance against the blight among the *B. sempervirens* plants (Henricot et al. 2008) control of the disease with fungicides would be the only short term alternative to save the boxwood populations. However, there are only limited possibilities to use fungicides in the infected areas, due to the risk of the chemicals ending up in the rivers and therefrom to households. Moreover, the climatic conditions in the river valleys during growing season, temperature ranging from 15 to 25 °C and precipitation occurring on average every second day, are optimal for the fungus.

Potential threat caused by other pests on *Buxus sempervirens*

Box tree moth

In addition to boxwood blight, the most threatening one of the few currently-known pests that can have significant influence on boxwood populations is the box tree moth *Cydalima perspectalis* (Walker). This invasive alien species of East Asian origin was first found in 2007 in Germany. To date the distribution of this moth covers most of Europe (EPPO, 2016). Box tree moth had reached western part of Turkey by 2011 when it was found in the European part of Istanbul, and has since then spread at least approximately 300 km eastwards. It is highly likely that distribution of this pest will cover large parts of that of *B. sempervirens* in Turkey in the near future. The moth is spreading from the Caucasus via Georgia towards the Turkish border (Marc Kenis, Iryna Matsiakh, personal communication). The moth can cause complete defoliation and death of a boxwood plant. It is difficult to evaluate what impact the boxwood moth can have in the boxwood populations surviving from the disease epidemic.

Fungal species

Other disease agents observed on boxwood in the forests infected with the boxwood blight were *Pseudonectria buxi* (DC.) Seifert, Gräfenhan and Schroers (syn: *Volutella buxi*), *Puccinia buxi* Sowerby and *Dothiorella candollei* (Berk. and Broome) Petr.(syn: *Macrophoma candollei* (Berk. and Broome) Berl. and Voglino). These fungi were also reported in these forests or in other regions of Turkey or on ornamentals previously (Göbelez and Ka-

raca 1954, Göbelez 1962, Gürcan 1976, Hüseyin et al. 2005, Lehtijärvi et al. 2014, Hüseyin and Selçuk 2014).

Volutella blight, caused by *P. buxi*, was one of the major boxwood diseases and was considered the primary cause of boxwood decline in many countries (Shi and Hsiang 2014). The pathogen usually reported to co-occur on *C. buxicola* infected plants, thus regarded to increase the impact of the disease (Henricot et al. 2000, Šafránková et al. 2012, 2013). However in an inoculation test, a non-significant effect of their co-occurrance was found (Oskay et al. 2015).

Phytophthora citricola Sawada is another important pathogen isolated from rhizosphere soil of diseased boxwood seedling from a nursery in Turkey (Aday Kaya 2014). Some other fungi, worth to mention, associated with boxwood reported from Turkey are: *Diplodia buxicola* Sacc, *Rosellinia buxi* Fabre (Hüseyin et al. 2005, Selçuk et al. 2010, Lehtijärvi et al. 2014, Hüseyin and Selçuk 2014).

Possibilities for *ex situ* conservation of Turkish boxwood

Boxwood can be easily propagated with cuttings, which opens up a possibility for *ex situ* conservation of the genetic diversity that still could be recovered from the natural populations. Careful selection of disease free shoots from the surviving individuals could be collected, propagated and cultivated in an area free from the disease. The high variation in climate in different parts of Turkey would allow *ex situ* conservation of a collection of Turkish boxwood genotypes in areas with suitable climate but lacking natural boxwood populations. More expensive alternative would be to maintain a genotype collection in glasshouses located in the dry inner Anatolia where boxwood does not grow naturally. These boxwood reserves could be used in resistance breeding programs and replanting of the areas affected by the epidemic if and when the areas have become free of the disease.

Conclusions

Due to obvious lack of resistance against the blight among the *B. sempervirens* plants (Henricot et al. 2008) control of the disease with fungicides would be the only short term alternative to save the boxwood populations. Although there are several preparates that are effective, none of them is certified for usage in forest in Turkey. Moreover, there are only limited possibilities to use fungicides in the infected areas, due to the risk of the chemicals ending up in the rivers and therefrom to households. Owing to the optimal climatic conditions for the fungus in the river valleys during several months, i.e. high humidity and temperature ranging from 15 to 25 °C, repeated fungicide treatments would be necessary. Combination of silvicultural management of the remaining boxwood and *ex situ* conservation may be the best long time strategy to save the remaining genetic diversity.

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Epidemiology and Management of Pine Pitch Canker Disease in Europe - a Review

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Abstract

Fusarium circinatum is an ascomycete fungus that causes Pine Pitch Canker (PPC) of pines. The disease is causing damages in forests and nurseries all around the world. In Europe, is present in northern Spain, and also has been detected in Portugal, France and Italy. *Fusarium circinatum* seems to require fresh wounds on trees as infection court. Notwithstanding, the susceptibility of these wounds to infection could decrease significantly with wound age. *Fusarium circinatum* has been reported to be phoretically associated with *P. pubescens* in California. In northern Spain, *T. piniperda* is a major candidate for being an effective vector of *F. circinatum* due to the maturation feeding it practices in the crowns of healthy pines and subsequent overwintering. At present there are no means of controlling PPC disease in adult trees in forest or plantations. However, given the seedborne character of *F. circinatum,* some encouraging results have been obtained by the use of different strategies to reduce the presence of the pathogen in pine seeds. For example hot water treatments (51–52ºC for 30 min) were found effective in reducing *F. circinatum* contamination in seeds. Endophytic species which do not cause any damage that could be used for biological control of *Fusarium* spp., have been reported related to pitch canker diseased in *P. radiata* trees. The use of mycoviruses to control fungal diseases of plants could be a promising method when the genetic diversity of the populations of the pathogen is low, for example, when the introduction of the fungus in a region is recent or when sexual reproduction is absent as occurs in PPC. Recently, three different strains of mycoviruses co-infecting a Spanish isolate of *F. circinatum* were found and characterized. More studies are essential to prevent the rapid spread of the disease from southern to northern Europe.

The pitch canker disease pathogen: *Fusarium circinatum*

Fusarium circinatum is an ascomycete fungus belonging to the *Gibberella fujikuroi* clade that causes PPC on pines (Nirenberg and O'Donnell 1998). *Fusarium circinatum* is a seedborne pathogen that can survive both superficially and internally in the seeds, causing seed high mortality rates (Gordon 2011). Seedlings can also show die-back and die due to the girdling, but the main symptoms observed in seedlings are necrosis, chlorosis, wilting of needles, dieback and desiccation of the seedling tip (Viljoen et al. 1994; Martín-Rodrigues et al. 2013). The main symptom of PPC in adult trees is the presence of pitch soaked cankers in trunks and big branches which girdle both

trees and branches (Figure 1) (Wikler et al. 2003). Trickles of resin can also be found on the trunks of diseased trees. The disease can affect the crown when suitable wounds are available for infection (Gordon et al. 2001), causing dieback that can lead to tree dead. However, on small diameter branches a single infection may be sufficient to cause the death of the branch (Gordon 2011). Dieback symptoms are also common on the crown due to the obstruction of water flow caused by the cankers. The wilting and discoloration of needles, which eventually turn red and finally fall off, is a common symptom of the disease as well (Wingfield et al. 2008). The tree finally dies when cankers girdle the trunk or as a result of the loss of structural integrity at the site of the canker formation. In forest nurseries, *F. circinatum.* can reduce germination of seeds, cause pre- and post-emergence damping-off, the wilting of seedlings, shoot and tip dieback, and finally lead to the death of the established seedlings (Viljoen et al. 1994) The increase in the resin production is due to the increment of number of the traumatic resin ducts (TRDs); this fact could benefit *F. circinatum* since epithelial cells surrounding the TRDs have starch that the fungus uses for feeding. The increment in the resin production restricts the water supply and leads to the desiccation of the infected tissue causing the tree dead (Martín-Rodrigues et al. 2013). *Fusarium circinatum* also causes growth reduction in adult trees in forest and plantations leading to great economic and ecological losses.

Fusarium circinatum has a wide geographical distribution. This pathogen was first reported in North Carolina (Hepting and Roth 1946) on *Pinus virginiana* Mill. in southeastern United States, epidemics occasionally occur and are generally associated with abiotic stress (López-Zamora et al. 2007). Hosts may have co-evolved with the pathogen due to the proximity of this region to the area where the pathogen is said to be endemic. Forty years after its initial detection, the disease was recorded in California affecting landscape pines mainly of the species *P. radiata* D.Don but also *P. muricata* D.Don, *P. pinea* L. and *P. halepensis* Mill. (McCain et al. 1987). Some years later, the hosts and the geographic range of the PPC pathogen increased, affecting native stands of *P. radiata* in the Monterey peninsula and making a transgeneric jump to *Pseudotsuga menziesii* (Mirb.) Franco (Storer et al. 1994). Later, it was also detected in Haiti (Hepting and Roth 1953), California (McCain et al. 1987), Japan (Muramoto and Dwinell 1990), South Africa (Viljoen et al. 1994), Mexico (Guerra-Santos 1998), Chile (Wingfield et al. 2002), Korea (Cho and Shin 2004), France (EPPO 2004), Spain (Landeras et al. 2005), Italy (Carlucci et al. 2007), Uruguay (Alonso and Bettucci 2009), Portugal (Bragança et al. 2009), Colombia (Steenkamp et al. 2012) and Brasil (Pfenning et al. 2014). Pitch canker disease poses a threat to pine plantations and natural stands throughout the world (Wingfield et al. 2008), especially *Pinus radiata* D. Don plantations due to the high susceptibility of this pine species (Viljoen et al. 1995). However other *Pinus* species like *Pinus pinaster* Ait. and *Pinus sylvestris* L. (Landeras et al. 2005, Pérez-Sierra et al. 2007) as well as *Pseudotsuga menziesii* (Gordon et al. 1996) are susceptible to the pathogen. In Spain, the presence of *F. circinatum* in Monterey pine plantations and in nurseries has resulted in severe loss and in a reduction of revenues due to the ban on planting susceptible species in infected areas (Real Decreto 637/2006 and 65/2010), the high costs invested in monitoring and control, and the restrictions on the export of timber. At present, the disease is causing damages in forests and nurseries in five regions within Spain; Galicia, Asturias, Cantabria, País Vasco and Castilla y León. The origin of the pathogen introduction in Spain has been deeply studied, showing two significantly differentiated populations regarding all the affected areas (Berbegal et al. 2013) and a clonal population within País Vasco (Iturritxa et al. 2011). According to the results of this study, the *F. circinatum* isolates detected in the first infected nursery in País Vasco in 1997 (Dwinell et al. 1998) and deposited in the Fusarium Research Center collection (Pennsylvania State University) belong to the most common multilocus genotypes of the two groups, MLG32 (Berbegal et al. 2013). Therefore it is probable that one of those introductions occurred in the País Vasco region, and it spread from there to the rest of the regions in northern Spain. The other group of multilocus genotypes is represented by MLG59, which is restricted to the northwest of the country (Berbegal et al. 2013). Galicia may be the second place of origin of *F. circinatum*, taking into account that the disease was reported to be in that region in 1996 (MAPA 1996). Since it was first reported in Spain, several studies have been carried out to identify the factors influencing its distribution on the northern area (Romón et al. 2007a) and in order to prevent the pathogen dispersal (Serrano et al. 2014). But little is known regarding some of the factors influencing the epidemiology of the disease, as for instance, forest management or the specific role of bark beetles vectors. Since the detection of *F. circinatum* (Landeras et al. 2005), some regions in Spain have stopped planting *P. radiata* due to the ban on using *Pinus* spp. and *P. menziesii* for the reforestation of the affected areas (MAPA 2006). The production of *P. radiata* seedlings in

Figure 1. Pitch canker disease symptoms in *Pinus radiata* adult trees: a) pitch soaked canker on a trunk, b) crown dieback, c) trunk transversal section at the canker level, d) broken branch at the canker level

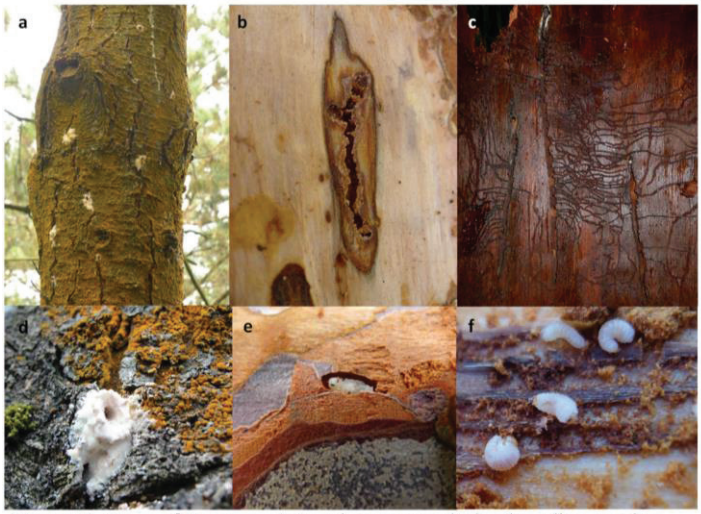

Figure 3. Trace of *Tomicus piniperda* presence during breeding on pine trunks: a) *Pinus radiata* trunk with *T. piniperda* entrance holes, b), c) *T. piniperda* breeding galleries: b) surrounded by necrotic tissue in *Pinus nigra,* c) maternal and larval galleries in *P. radiata* d) detail of a *T. piniperda* entrance hole in *P. radiata* e) pupal chamber belonging to a breeding gallery in *P. nigra, f) T. piniperda* larvae in *P. nigra*

Figure 4. *Tomicus piniperda* within shoot feeding galleries: a) *Pinus nigra* and b) *Pinus radiata*

Figure 6. *Pinus radiata* attacked by *Pityopthorus pubescens.* a) Reddish twigs, b) detail of the gallery burrowed by *P. pubescens*

forest nurseries was also reduced due to the risk of contamination from the pathogen and the consequent loss in crop and yield.

Fusarium circinatum is microscopically characterised by the presence of sterile coiled hyphae, polyphialides in branched conidiophores, non-septate microconidia and multiseptate macroconidia (Figure 2). Sporodochia with macroconidia appear sparsely on carnation leaf agar (CLA) (Leslie and Summerell 2006). In culture on potato dextrosa agar (PDA) *F. circinatum* produces aerial mycelium that is usually white to violet and can produce grey to dark pigmentation (Ganley 2008). The sexual stage, *Gibberella circinata*, has been produced only in culture, but has not been observed in nature. This pathogen has a necrotrophic behavior, since fungi belonging to the genus *Fusarium* do not suffer differentiation of the hyphae for invading the host tissues, i.e. haustoria or appresoria (Mendgen et al. 1996). However, they are characterized by the production of cell-degrading enzymes and mycotoxins. *Fusarium circinatum* produces poligalacturonasa for the degradation of the cell wall and subsequent penetration of the host (Leslie and Summerell 2006). Mycotoxins are secondary metabolites that are released by the fungi after host penetration, e.g. beauvericin, which is the toxin most widely produced by *F. circinatum* and by other species from the genus, as well as fumonisin (Mirete et al. 2003). Beauvericin induces cell death similar to apoptosis and causes cytolisis, having entomopathogenic and phytopathogenic properties and appears to be one of the most widely produced toxins by species of Fusarium (Logrieco et al. 1998). Due to this mechanism, vegetal cells of the host are destroyed forming gaps where conidiophores grow, however the transformation of vegetative mycelia to conidiophores requires a change on the genetic expression pattern. Martín-Rodrigues et al. (2013) suggest that this transformation could occur when the pathogen feeds on the starch of the parenchyma cells in the pith of seedlings and reported, for the first time, the production of conidiophora orientated towards the hollow cavities of the pith at the moment when the first symptoms of disease appeared.

Until now *F. circinatum* is exclusively a pine species pathogen, although some trees of the species *P. menziesii* were found susceptible to the disease (Storer et al. 1994, Gordon et al. 2006). At least 57 pine species have been reported as being susceptible to the PPC pathogen after observing symptoms of the disease on seedlings or adult trees or performing inoculation experiments (reviewed by Wingfield et al. 2008). However, susceptibility varies among pine species. For example, while species like *P. pinea* or *P. canariensis* seem to be resistant to the pathogen (Gordon et al. 1998, Iturritxa et al., 2013), there is evidence that *P. radiata* is the most susceptible species to the disease (Wingfield et al. 2008). It is also the most widely-planted pine in the world (Critchfield and Elbert 1966), Chile being the country with the most surface planted with this species (more than one and a half million hectares). Other countries with large areas of *P. radiata* plantations are Argentina, Uruguay, South Africa, Australia and New Zealand (Fernández and Sarmiento 2004). *Fusarium circinatum* is still absent from the last two countries, and due to the importance of this pine species there, the occurrence of the disease would have serious economic, ecological, and social impacts. In Spain, around 275 000 hectares are planted with this pine (Fernández and Sarmiento 2004), a relatively small area in comparison with native pines (3.6% of the total area covered by coniferous species). However, due to its fast growth and short rotation time, it provides 25% of the conifer timber in Spain (Hermoso et al. 2007). The use of monospecific *P. radiata* plantations is leading to the emergence of pests and diseases that threaten the crops (Dajoz 2001, García-Serna 2014).

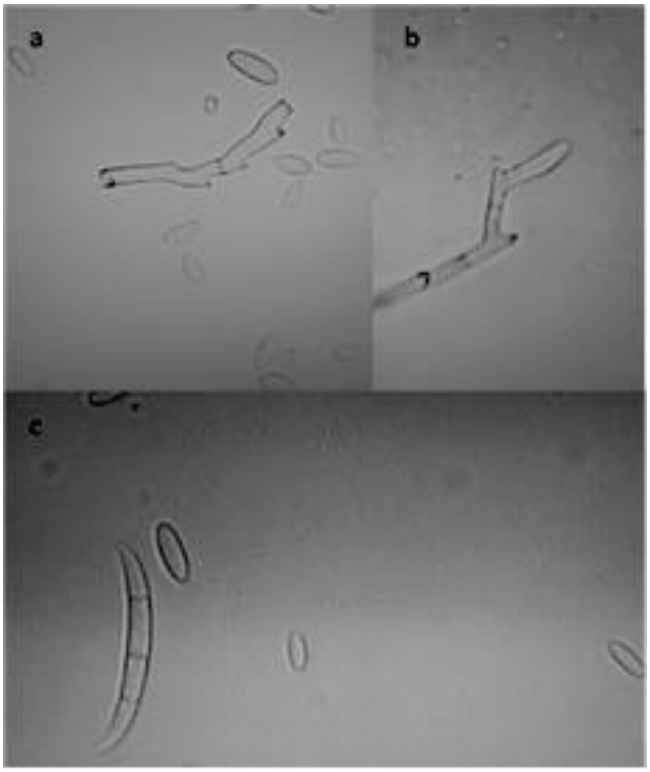

Figure 2. *Fusarium circinatum* microscopical structures on SNA. a) monophialides and microconidia, b) polyphialid, c) macroconidia and microconidia

Apart from pines and *P. menziesii*, the susceptibility of a variety of other plant species including trees and herbaceous plants has been tested, with the pathogen failing to infect them (McCain et al. 1987, Wingfield et al., 2008). On the other hand, *F. circinatum* has been found to infect grasses as a symptomless endophyte (Swett and Gordon 2012). This fact makes the eradication of the disease even more difficult in most parts of the world where the pathogen is well-established; grasses may serve as a reservoir of inoculum which, in turn, influences the occurrence of the disease in pine nurseries and plantations (Swett et al. 2014). An intensive sampling must be done to detect the presence of the pathogen in many other plant species in order to improve the management of the disease. Furthermore, susceptibility to PPC disease in other conifer species must be evaluated to find alternatives to *P. radiata* in the areas where the pathogen is already present.

Factors influencing the epidemiology of pitch canker disease

For a forest disease to occur, a combination of three factors must be present: susceptible plant, infective pathogen and favorable environment (Agrios 1997). However, there are several other events that influence the incidence (number of infected plants), severity and dissemination of a disease. In regard to PPC epidemiology, there are two major aspects that have to be considered: biotic factors and abiotic factors.

Abiotic factors

a. Forest management:

Movement of plant material

Infested seeds as well as latently-infected seedlings can serve as a vehicle for dissemination of *F. circinatum* over long distances (Gordon 2011). However, the development of the disease coming from infected seedlings will depend on the environmental conditions. For instance, Chile, which is known to have *F. circinatum* in the nurseries but not in the plantation forests, was also predicted to have marginal to suitable climatic conditions for pitch canker establishment (Ganley et al. 2009).

Fusarium circinatum has been dispersed around the world, probably, with pine infected seeds (Berbegal et al. 2013). Genetic evidences have shown that Mexico is a plausible source of *F. circinatum* infection found in South Africa (Wikler and Gordon 2000); however in Spain two independent introductions seemed to occur (Berbegal et al. 2013).

Tree host selection

Up to 60 Pinus species have been reported to be susceptible to the PPC (Gordon 2006), among them: *P. radiata*, *P. sylvestris* (Landeras et al. 2005), *P. pinaster* (Vivas 2012), *Pinus nigra* Arn. and *Pinus uncinata* Ram. (Martínez-Álvarez et al. 2014a) in Spain. *Pinus muricata* D. Don in California (Schmale and Gordon 2003), *Pinus halepensis* Mill. and *P. pinea* in Italy (Carlucci et al. 2007), *Pinus rigida* Mill. in Japan (Kim et al. 2009) and *Pinus taeda* L. in Uruguay (Alonso and Bettucci 2009) are also susceptible. Other coniferous trees such as *Pseudotsuga menziesii* (Mirb.) Franco can also suffer from this disease (Gordon et al. 1996).

Although the pitch canker pathogen can cause disease in many coniferous species, not all the hosts are equally susceptible, being *P. radiata* a particularly susceptible species (Correll et al. 1991, Martínez-Álvarez et al. 2014a). Thus, the risk of damage caused by this pathogen could be minimized using, less susceptible species or selecting resistant genotypes (Gordon 2011). Intraspecific variation has been observed regarding PPC susceptibility in a number of pine species. For instance, different *P. pinaster* families showed variation in resistance to *F. circinatum* in the study carried out by Vivas et al. (2012) and Bonello et al. (2001a) demonstrated that induced resistance could appear as a consequence of the previous presence of *F. circinatum*. Thus, the frequency of resistant individuals will influence the extensiveness of damage caused by the PPC (Gordon 2006).

Pruning

Fusarium circinatum seems to require fresh wounds on trees as infection court (Dwinell et al. 1985). These authors suggested that *F. circinatum* inoculum could infect wounds produced by pruning, mowing and harvesting, although little is known on this issue. Notwithstanding, the susceptibility of these wounds to infection could decrease significantly with wound age (Sakamoto and Gordon 2006). Nonetheless, other studies carried out by Correll et al. (1991) suggest that branches with mechanical wounds are not susceptible to infection even if airborne inoculum is present, postulating that airborne spores are unable to infect wounds.

On the other hand, pruning could be considered for removing diseased branches, though this approach is not effective in eradicating the disease (Gordon et al. 2001). Attempts to remove disease causing fungi have been made via tree pruning, though it was shown that this treatment does not completely eliminate the disease from the tree (Moorman and Lease 1999). As such, forest management

should be considered as an important factor for decreasing disease establishment and spread (Waring and O'Hara 2005). The effect of pruning has not been deeply studied in Monterey pine plantations where PPC is destroying the trees, although in Spain Bezos et al. (2012) observed that pruning wounds have an increased chance of becoming infected by the pathogen which could enhance cankers and deformation. Notwithstanding, pruning in Monterey pine diseased plantations is not desirable as a result of stem deformation caused by cankers, making them useless for the wood industry.

b. Environmental conditions

Environmental conditions taking place in both air and soil are determinant for the development of a disease after the contact of a pathogen with its host (Agrios 1997). Regarding PPC development, temperature (20-25ºC for spore germination and fungal growth) and high humidity levels are chief factors for the pathogen to success (Wingfield et al. 2008). Thus, it develops more rapidly in *P. radiata* plots closer to the coast than in plots located in land (Wikler et al. 2003), being the fog also a major factor influencing the disease distribution near the coast (Gordon 2006).

Some other meteorological events can affect the incidence of the disease acting as wounding agents, for instance, hail or wind storms that increase the number of infection courts for the pathogen. Wounds caused by hurricanes or those resulting from wind-thrown needles are also thought to provide an infection court for the pathogen to infect the trees (Kelley and Williams 1982). Environmental conditions also influence dissemination of *F. circinatum* spores, especially wind and rain (Gordon 2011). Dispersal of airborne spores in *F. circinatum* and *Fusarium* spp. not only depends on the wind, but also on the rain, since macroconidia are adapted to the dispersion by wind, but before flight they require to be in touch with raindrops that carry the spores into the air (Deacon 2006).

Biotic factors

a. Fungal communities

Fungal communities inhabiting *P. radiata* trees may be a determinant factor influencing PPC distribution in Spain (Bezos et al. 2013). Fungal communities in forests are formed by endophytes together with saprotrophic and pathogenic species. Knowing the species composition and the factors influencing the presence of different fungal communities is important in terms of understanding the role that fungi play on the regulation of other organisms (Arnold 2007). In general terms, interactions between two fungal species may occur in three different ways: i) by the exclusion of one species through the competition in exploiting resources, ii) the exclusion by antagonism i.e. antibiotic production or parasitism and, finally, iii) by the ability of two species to coexist (commensalism) or to cause a profit to both (mutualism) (Deacon 2006).

The study of fungal species present on PPC affected plantations could be crucial for the biological control of the disease. Endophytic species which do not cause any damage (Arnold 2007) such as *Trichoderma viride* Bissett that could be used for biological control of *Fusarium* spp. (Martínez-Álvarez et al. 2012), have been reported related to pitch canker diseased in *P. radiata* trees. *Trichoderma* spp. have antagonistic properties by means of antibiotic production, chitinase secretion or parasitism, the latter occurs when the hyphae of *Trichoderma* coil round the hyphae of another fungi and eventually penetrates it from these coils (Deacon 2006). *Penicillium* spp. usually appear as saprotrophes in pines, and rarely occur as endophytes in healthy tissue (Zamora et al. 2008), but the role of *Penicillium chrysogenum* Link. in association with *F. circinatum* in *P. radiata* resulted in antagonism and induce resistance against the pitch canker pathogen in the work carried out by Romón et al. (2008).

Other fungal species e.g. *Diplodia pinea* (Desm.) Kickx, which may remain as a latent pathogen in pine trees, have been reported to be associated with *F. circinatum* in *P. radiata* plantations (Bezos et al. 2013, García-Serna 2014). *Diplodia pinea* is a saprotrophic fungi that can act as a parasite in stressed trees causing shoot dieback. In *P. radiata*, the symptoms of this disease are the presence of resin drops and necrotic stem lesions (Chou 1976, García-Serna 2014).

Fusarium species in *P. radiata* stands affected by PPC may play a chief role as endophytes or as plant pathogens, depending on the species. The genus *Fusarium* includes important plant pathogens affecting both forest and agricultural species (Alves-Santos and Diez 2012) because of the production of different types of wall-degrading enzymes (e.g. cellulases, glucanases or glucosidases) and mycotoxins like beauvericin or fumonisins (Mendgen *et al.* 1996, Logrieco et al. 1998). Regarding the association of *Fusarium* species with *F. circinatum*, it was found that *Fusarium lateritium* Nees, which is not pathogenic, inhibited the pathogen growth in vitro when it was introduced as a pioneer (Romón et al. 2008).

b. Bark beetles

Bark beetles (Curculionidae; Scolytinae) have a worldwide distribution affecting forest dynamics,

contributing to nutrient cycling, canopy thinning, gap dynamics, disturbance regimens and successional pathways (Raffa et al. 2015). Several bark beetle species has been reported to be present in Spanish forests (Gil and Pajares 1986), having determinant implications for forest management (López 2007; Fernández et al. 1999a, 1999b).

Bark beetles are associated with several fungal species, in native forests and plantations world-wide, in particular, with endophytic or pathogenic fungi including *F. circinatum* (Lieutier et al. 1989, Jacobs et al. 2004, Kirisits 2004, Romón et al. 2007a). The interaction between fungal pathogens and insects is a complex relationship that has been widely studied, being, in many cases, a mutual relationship between the vector and the fungus that has ecological advantages for both organisms (Paine et al. 1997). These pathogens have been traditionally considered allies of the insects, as they may serve to overcome tree resistance, facilitating the beetle's attack, since the successful colonization of the host by the insect depends on its ability to overcome tree resistance mechanisms (Christiansen et al. 1987, Långström and Hellqvist 1993, Franceschi et al. 2005). As it has been proved that bark beetles can kill trees without any pathogenic fungi, other authors propose that this association could only benefit the fungus, allowing it to get to trees that it would not reach without an insect vector (Six and Wingfield 2011). Lieutier et al. (2009) explained the role of pathogenic fungi in beetle establishment in terms of tree defence stimulation instead of in terms of defence overcoming.

To report the role of an insect species as vector of a pathogen, rules of proof for insect transmission described by Leach (1940) must be properly checked. Thus, i) a close, although not a constant, association of the insect with diseased plants must be demonstrated, ii) it must be demonstrated that the insect also regularly visits healthy plants under conditions suitable for the transmission of the disease, iii) the presence of the pathogen or virus in/on the insect in nature or following visitation to a diseased plant must be demonstrated and iv) the disease must be produced experimentally by insect visitation under controlled conditions with adequate checks. Moreover, bark beetles not only act as vector or phoretic agents, but also they can act as wounding agents when bore their breeding or feeding galleries. Thus, the presence of these insect species in PPC affected stands could increase the incidence of the disease even if bark beetles are not carrying the pathogen.

Several species of *Fusarium* are associated with insects in a mutualistic way, colonizing dead insects like saprophytes or acting as entomopathogens (Teetor-Barsch and Roberts 1983). The importance of *Fusarium* spp. regarding its presence in PPC affected trees is highlighted by its entomopathogenic activity, due to the question less role of bark beetles in *F. circinatum* spreading. These entomopathogenic fungi infecting bark beetles are usually ascomycetes, i.e. *F. oxysporum*, whose infective unite are conidia that germinate on the insects' cuticle and penetrate the hemocele causing the insects death (Vega and Hofstetter 2015). *Fusarium circinatum* has also been reported to be phoretically associated to several bark beetles species in *P. radiata* plantations in northern Spain, e.g. *Pityophthorus pubescens* (Marsham), *Hylurgops palliatus* (Gyllenhal), *Ips sexdentatus* (Boerner), *Hypothenemus eruditus* (Westwood), *Hylastes attenuatus* Erichson and *Orthotomicus erosus* (Wollaston) (Romón et al. 2007a, Bezos et al. 2013). We hypothesize that different bark beetle species living in these plantations could play a different role in the spreading of *F. circinatum.* The differences in their bioecology, e.g. *Hylastes* species feed on roots or trunks of declining trees whereas *Tomicus piniperda* L. feeds on shoots of healthy crowns (López 2007) during maturation feeding. The population levels may also increase until epidemic levels (Raffa and Berryman 1983) determining the spreading of the infections. The most relevant bark beetle species present in *P. radiata* stands in Cantabria, regarding *F. circinatum* distribution, are described below.

1- Tomicus piniperda L.

Tomicus piniperda is a serious pest affecting pines in Europe, Northern Africa and Asia (Långström 1980, Bouhot et al. 1988; Kirkendall et al. 2008) and in the United States ever since it was introduced in 1992 (McCullough and Smitley 1995). Its main host is *Pinus sylvestris* L. but other pine species are also suitable hosts, as, for example, *P. radiata. Tomicus piniperda* is a univoltine species that may present several sister broods. This species can colonize trunks and thick branches of weakened trees where it breeds (Figure 3), colonizing stressed or dying trees that have previously been attacked by other primary pest or pathogenic fungi (Paine et al. 1997).

But, in terms of maturation feeding on the pith of the shoots *T. piniperda* acts as a primary species (Figure 4) (Långström 1982, Lieutier et al. 2015). This bark beetle becomes a primary pest capable of causing sharp reductions in growth and in carbon content, nitrogen loss, malformations and, in cases of high population densities, the death of the host (López 2007). The fact that *T. piniperda* causes weakness in the hosts after feeding on shoots also increases the number of reproductive niches susceptible to colonization, although shoot damage rarely exceed 50% (Långström 1980). In regard to its life cycle in northern

Spain, *T. piniperda* dispersion flight occurs in February, colonizing weakened trees for breeding. Subsequently, emerging young F1 beetles target the tops of nearby healthy trees to practice gonadal-maturation feeding and fat accumulation (Långström 1982). This maturation feeding continues with the hibernation period inside the shoots. In addition, each insect penetrates more than one shoot during the feeding phase, especially in the thicker and fresh current-year shoots (Tiberi et al. 2009). The association between *T. piniperda* and *F. circinatum* has been demonstrated in northern Spain following Leach´s postulates (Leach 1940) where the pathogen appeared on breeding and feeding galleries, as well as on *T. piniperda* exoskeleton (Bezos et al. 2015). That indicates that this species could transport the pathogen and later introduce it both under the bark and into the pith of the shoots. Maturation feeding and overwintering within the shoots are the most susceptible moments for pathogen inoculation.

In northern Spain, *T. piniperda* is a major candidate for being an effective vector of *F. circinatum* due to the maturation feeding it practices in the crowns of healthy pines and subsequent overwintering. Several authors have previously mentioned the association of *T. piniperda* with virulent ophiostomatoid fungi like *Leptographium wingfieldii* Morelet (Lieutier et al. 1989) or like *Ophiostoma minus* (Hedgc.) Syd. & P. Syd. (Långstrom et al 1993, Solheim et al. 2001) and *L. guttulatum* M.J. Wingf. & K. Jacobs (Romón et al. 2014). This association occurs in the absence of mycangia, although some body structures present in the base of setae could be acting as fungi transport frames (Figure 5).

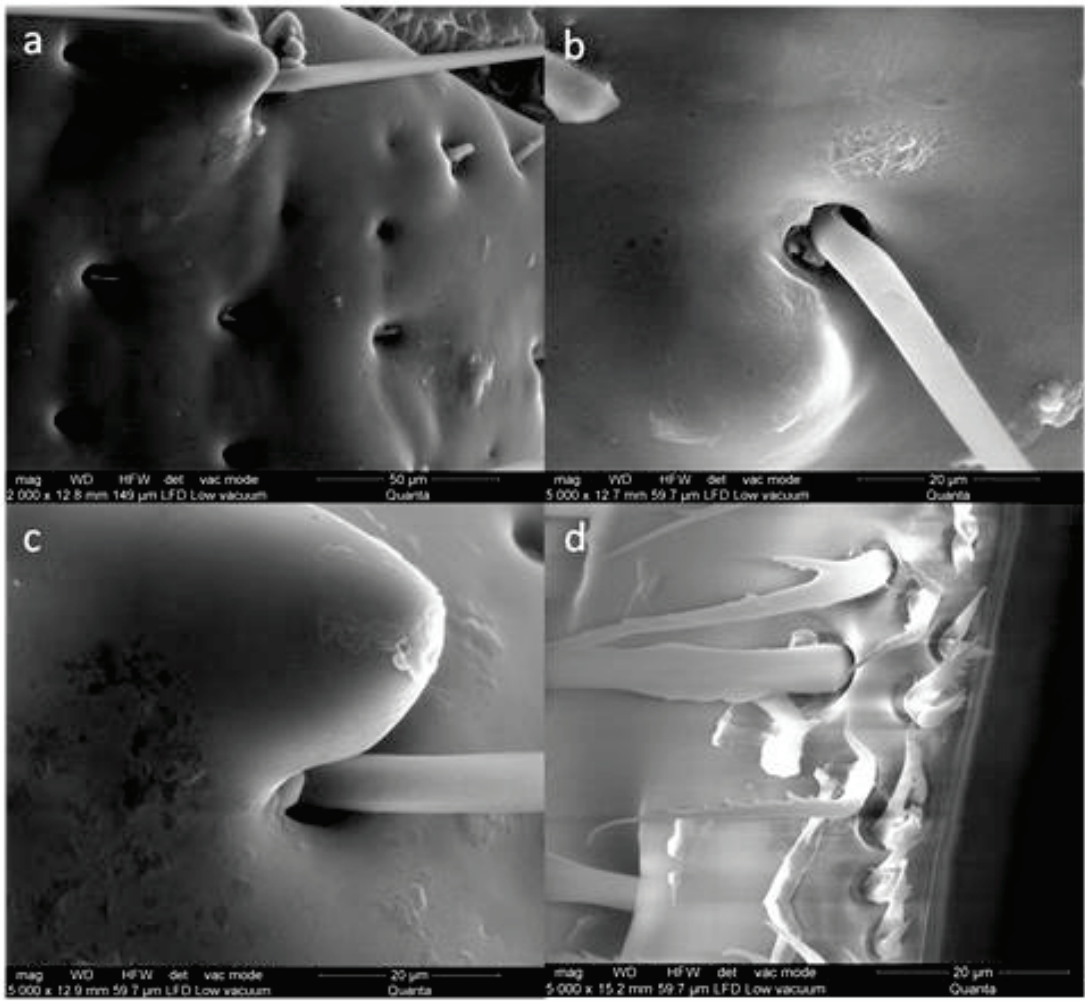

Figure 5. Scanning Electron Microscope pictures of *Tomicus piniperda*'s body structures: a), b) and c) on the elytra, d) at the base of the pronotum

2- Pityophthorus pubescens (Marsham)

Twig beetles, *Pityophthorus* spp., are phloeophagus and myelophagus species (Vega and Hofstetter 2015). This bark beetle species are widely distributed in Europe living in several *Pinus* species as *Pinus pinea* L, *P. pinaster* and *P. radiata* (Gil and Pajares 1986). Most species of this genus colonize mainly week trees with a low economic impact (Vega and Hofstetter 2015). *Pityophthorus pubescens* is present in the Iberian Peninsula that attacks weakly trees. The presence of this insect species on the attacked crowns can be observed by the presence of reddish twigs (Figure 6a). Twig beetles breed in shade-suppressed and broken branches as well as in branches of recently dead trees, but rarely cause tree mortality or even the death of individual branches (Storer et al. 2004). They construct their galleries in the phloem or in the pith of small branches in the host tree (Figure 6b) (Sakamoto et al. 2007). Overwintering in *P. pubescens* has been observed to occur on shoots within *T. piniperda* feeding galleries (Balachowsky 1962).

Fusarium circinatum has been reported to be phoretically associated with *P. pubescens* in Spain (Bezos et al. 2013, 2016; Romón et al. 2007a). The association of *Pityophthorus* spp. with PPC disease has also been observed in other affected areas, as for example in California where the importance of *Pityophthorus setosus* Blackman and *Pityophthorus carmeli* Swaine as *F. circinatum* vectors has been already demonstrated (Sakamoto *et al.* 2007). Bonello et al. (2001b) reported the ability of *Pityophthorus* spp. in discriminating between healthy and pitch canker diseased branches, preferring symptomatic branches due to the increasing of ethylene emission. The relevance of the role of *P. pubescens* in regard to *F. circinatum* spreading has to be assessed taking into account its feeding and breeding habits as well as its population level.

3- Other bark beetles in northern Spain

Ips sexdentatus is a polygamous species with one to five generations per year, depending on the weather conditions (Vega and Hofstetter 2015) completing three generations in the Mediterranean area (López 2007). Most of this insect`s live cycle occurs under the tree bark (Vega and Hofstetter 2015). *Ips sexdentatus* colonize weak and dead *P. radiata* trees in northern Spain, however when population levels increase they can kill healthy trees (Etxebeste and Pajares 2011), although outbreaks usually occur after forest fires or adverse climatic conditions (Gil and Pajares 1986, Fernández and Salgado 1999, Etxebeste et al. 2012).

The association of *Ips* species with fungi has been widely study. Whitehill et al. (2007) found the role of *Ips pini* as a vector of *Diplodia pinea* and several ophiostomatoid species were isolated from *I. sexdentatus'* exoskeleton in the work carried out by Fernández et al. (2004) Romón et al. (2007b) and by Bueno et al. (2010). *Ips sexdentatus* was also found to be phoretically associated with *F. circinatum* in Spain (Romón et al., 2007a; Bezos et al., 2014), with different percentages of specimens carrying the pathogen (0.9%-8.5%). Likewise, the importance of other species like *Ips mexicanus* (Hopkins) and *Ips paraconfusus* Lanier in association with *F. circinatum* has also been observed in PPC affected areas in California, where this species were reported as vectors of the pitch canker fungus (Fox et al. 1991). The importance of the association of *I. sexdentatus* with fungi is highlighted by the presence of mycangia on the insects' exoskeleton.

Among other bark beetle species present on pitch canker disease affected stands there are four species from the genus *Hylastes* present on the Iberian Peninsula. They are monogamous, phloeophagus and colonize mainly weak or felled trees (López 2007). *Hylastes ater* (Payk.), *Hylastes angustatus* Herbest and *Hylastes linearis* Erichson appear at the base of the trunk or roots, whereas *H. attenuatus* also attacks branches. *Hylastes ater* is the most dangerous species since adult beetles carry out their maturation feeding on the stems of seedlings prior to ovoposition. Sopow et al. (2014) demonstrated the *H. ater* ability of attacking unstressed seedlings more frequently than stressed ones; however the attack to stress seedlings caused substantial girdling-induced mortality. Moreover, *H. ater* has been reported to be associated to several pathogenic fungi i.e. *Ophiostoma* spp. *Leptographium* spp. (Eckhardt et al. 2004) as well as *Fusarium* spp (Romón et al. 2007a, Romón et al. 2014). Bezos et al. (2014) found that the lesser *H. attenuatus* was a carrier, although in low proportion (1.60% of specimens carried spores).

Orthotomicus erosus is a polygamous species that generally feed on weak trees, infesting fallen or felled trees, but also attack living trees that suffer from stress due to fire, droughts or diseases (López 2007). Population densities may increase until epidemic levels, what would lead them to overcome food store of weaken trees and attack the healthy ones (Gil and Pajares 1986). Maturation feeding of young adults occurs under the bark, in the phloem of the same tree where they were born or in another one from the same or different species (López 2007). This insect species has been reported to be associated to Ophiostomatoid fungi, i.e. *Ophiostoma* spp and *Leptographium* spp. (Kirisits 2004, Romón et al. 2014) as well as to several *Fusarium* spp., including *F. circinatum* (Romón et al. 2007a).

Measures for disease control: Biological control

At present there are no means of controlling PPC disease in adult trees in forest or plantations. However, given the seedborne character of *F. circinatum,* some encouraging results have been obtained by the use of different strategies to reduce the presence of the pathogen in pine seeds. For example hot water treatments (51–52ºC for 30 min) were found effective in reducing *F. circinatum* contamination in seeds (Agustí-Brisach et al. 2012, Berbegal et al. 2015). Furthermore, several studies found that hydrogen peroxide is a good disinfectant of the contaminated seeds (Dwinell and Fraedrich 1999, Berbegal et al. 2015). Unfortunately, these methods do not prevent the arrival of infected seeds to the forest nurseries and seedlings to the forest. To reduce the impact of the disease, an integrated management approach is necessary, and, at the same time, the role of biological control is crucial due to the advantages it has over the use of chemicals.

Cook and Baker (1983) defined biological control as the reduction of the amount of inoculum or disease-producing activity of a pathogen produced by or through one or more organisms other than man. The organisms mentioned in the definition are (1) avirulent or hypovirulent strains of the pathogen, (2) the host plant manipulated either genetically, by cultural practices, or with microorganisms toward greater or more effective resistance to the pathogen, and (3) antagonists of the pathogen, i.e. microorganisms that interfere with the survival or disease-producing activities of the pathogen.

Biological control of plant diseases has many advantages when compared to the use of chemicals. For instance, fungicides and bactericides have promoted the development of pathogen strains resistant to chemicals, which has been a problem for at least the last thirty years (Dekker and Georgopoulus 1982). Besides, most chemicals are nonspecific and broad-spectrum in their effect, and they can produce undesirable effects on non-targeted organisms, such as the fungal or bacterial endophytes, which can play an important role as antagonists of the pathogens (Spokes et al. 1981). Humans may be harmed as well, specifically when pesticides are employed in agriculture and potentially enter into the food chain (Kniewald 2003). Another advantage is that longer-term control is achieved because biological control agents (BCAs) act as a host-specific control method continually present and constantly impacting the target pathogen. Thus, although it may be expensive to introduce due to research costs, it can be quite economical in the long term (Cook and Baker 1983). The importance of biological control methods is even higher in forests where, day after day, chemical use is more and more restricted (EU 2009a).

As explained before, there are several strategies classified as biocontrols in the fight against pathogens. However, here we will focus our attention only on the control of plant pathogens through the use of fungal endophytes and mycoviruses.

a. Biological control using fungal endophytes

Many definitions for the term fungal endophytes can be found in scientific literature. One of the most accepted definitions says that fungal endophytes are fungi that are able to infect their hosts without causing visible symptoms of disease (Petrini 1991). Typically, they may be divided into three types: (1) pathogens of another host that are non-pathogenic in their endophytic relationship; (2) non-pathogenic fungi; and (3) pathogens that have been rendered non-pathogenic yet are still capable of colonization by selection methods or genetic alteration (Backman and Sikora 2008). Endophytes can produce many benefits for the host plants. For instance, various studies have demonstrated that plants infected with endophytes obtain growth promotion (Barka et al. 2002), resistance to drought stress (Swarthout et al. 2009), tolerance to unsuitable soil conditions (Malinowski et al. 2004), greater access to nutrients (White et al. 1997), and improved defense against herbivorous animals (Carroll 1988) and pathogens (Arnold et al. 2003). Regarding the latter benefit of endophytes, the mechanisms implemented to protect the plant against the infection of plant pathogens can be grouped into: direct effects, indirect effects and ecological effects (Gao et al. 2010). In the case of direct effects, endophytes directly suppress pathogens by producing antibiotics (Richardson et al. 2014) or secreting lytic enzymes (Tripathi et al. 2008). Induction of plant resistance, stimulation of the plant's secondary metabolites and promotion of plant growth and physiology all are indirect effects that the endophytes have on the plant to help it reduce damage caused by pathogens. Finally, examples of ecological effects are the occupation of an ecological niche, as well as hyperparasitism and predation (Gao et al. 2010).

One group of fungi stands out among the endophytes because of their potential as a biological control agent of plant diseases: genus *Trichoderma* (Howell 2003). This group is well known and worldwide in occurrence. One of the most salient characteristics of the group is their ability to parasitize other fungi (Weindling 1932), but they also produce antibiotic substances that are inhibitory to many plant pathogens (Howell and Stipanovic 1983). However,

the principal mechanism in the biocontrol process of *Trichoderma* spp. is the competition for space and nutrients in the rhizosphere (Howell 2003). The growth of these fungi is not restricted to the soil and plant roots, but rather they are also able to colonize the phloem and even the sapwood of trees (Jankowiak 2006). To understand the potential of this group of fungi as BCAs of phytopathogenic fungi, it is important to point out that 90% of the applications performed to control plant diseases have been carried out with different strains of the genus *Trichoderma* (Benítez et al. 2004). There are many examples of *Trichoderma* spp. employed in the successful control of different plant diseases (Latunde-Dada, 1993, Abdullah et al. 2008, Ruano-Rosa et al. 2010) which, in some cases, are caused by pathogens of the genus *Fusarium* (Sivan et al. 1987, Bernal-Vicente et al., 2009, Basak and Basak 2011). *Trichoderma* spp. have potential not only to control fungal pathogens but also bacteria (Phupiewkham et al. 2015).

Regarding tree diseases, the most well-known example of biocontrol is the one carried out by *Phlebiopsis gigantea* (Fr.) Jülich against *Heterobasidion annosum* (Fr.) Bref, considered the most harmful forest pathogen in economic terms in the Northern Hemisphere (Woodward et al. 1998). However, the importance of endophytes as BCAs is not restricted to forests but also extends to nurseries (Capieau et al. 2004). The production of seedlings carrying antagonistic endophytes to the most aggressive pathogens may be the future of biocontrol in forest pathology.

Regarding PPC disease, some experiments have been performed to reduce the impact of the pathogen using fungal endophytes. Antagonistic interactions between *F. circinatum* and the fungal species *Penicillium chrysogenum* Link. and *Fusarium lateritium* Ness. were observed in an experiment performed in the lab by Romón et al. (2008). In this experiment, seedlings emerged from *Pinus radiata* surface-sterilized seeds were coated with 0.5 mL of a 900 000 conidia/mL suspension of *Penicillium chrysogenum*, *F. lateritium* and control treatment in soil simultaneous infected with 0, 450 000, 4500, or 45 pitch canker fungus conidia inoculum levels. Similarly, Soria et al. (2012) showed that two endophytic bacteria (*Bacillus subtilis* Cohn and *Burkholderia* sp.) were antagonists to the pitch canker pathogen. In another experiment, the potential use of *Trichoderma* spp. and *Clonostachys* spp. strains to control *F. circinatum* on *P. radiata* seedlings was evaluated by (Moraga-Suazo et al. 2011). One of the strains of *Clonostachys* sp. tested significantly increased the survival of *P. radiata* seedlings, but no effect was observed with the *Trichoderma* strains. The same conclusions were reached in previous studies in which *Trichoderma* spp. were tested as BCAs of the disease (Dumroese et al. 1988; Mitchell et al. 2004, Martínez-Álvarez et al. 2012). Recently, two fungal isolates belonging to the species *Chaetomium aureum* and *Alternaria* sp. reduced the micelial growth in dual cultures, and the symptoms caused by *F. circinatum* on *P. radiata* seedlings planted in the field, indicating that they may therefore be suitable for use as BCAs of the disease (Martínez-Álvarez et al. 2016).

***b.* Biological control using mycoviruses**

Mycoviruses or fungal viruses are widespread in all major taxa of fungi. Most of them have dsRNA (double-stranded RNA) genomes, although an increasing number of positive or negative ssRNA (single-stranded RNA) and ssDNA (single-stranded DNA) viruses have been isolated and characterized in recent years (Ghabrial et al. 2015). Mycoviruses are, in general, associated with latent infections in their hosts (Ghabrial and Suzuki 2009). However, in some cases they induce different symptoms such as changes in growth, colour, sporulation, and sometimes enhancement (hypervirulence) or attenuation of fungal virulence (hypovirulence) (Ghabrial and Suzuki 2009, Pearson et al. 2009). Hypovirulence in the host's physiology is the most important reason why plant pathologists are interested in mycoviruses and why mycoviruses can be used as BCAs.

The mycovirus that dominates in the context of plant pathology is the Cryphonectria hypovirus 1 (CHV1) (Heiniger and Rigling 1994), which has been successfully used as a BCA for the chestnut blight pathogen throughout Europe (Robin and Heiniger 2001, Turchetti et al. 2008, Zamora et al. 2014). This hypovirus reduces mycelial growth and sporulation of *Cryphonectria parasitica*. It also produces changes in the morphology and colour of the fungus colony (Rigling et al. 1989, Peever et al. 2000). After being infected, the pathogen is only capable of forming superficial healing cankers on stems, allowing trees to survive after the attack (Nuss 1992).

Among mycoviruses, one genus only found in fungi stands out due to its importance in the biological control of plant diseases. This is the genus, *Mitovirus*, which belongs to the family Narnaviridae, a group in which the members have the simplest genomes of any autonomous RNA virus (Ghabrial et al. 2015). Putative members of the genus *Mitovirus* are located and translated in the mitochondria (Polashock and Hillman 1994), where they mostly occur as dsRNA replicative forms (Ghabrial 1998). In some cases they exhibit phenotypic changes and cause hypovirulence in major plant pathogens, such as *Botrytis cinerea* (Wu et al.

2010), *Ophiostoma novo-ulmi* (Rogers et al. 1987) or *Sclerotinia homoeocarpa* (Deng et al. 2003).

Recently, three different strains of mycoviruses co-infecting a Spanish isolate of *F. circinatum* were found and characterized (Martínez-Álvarez et al. 2014b). They belonged to two novel species of the genus Mitovirus, Fusarium circinatum mitovirus 1 (FcMV1) and Fusarium circinatum mitovirus 2 (FcMV2) and are common among the isolates of the PPC pathogen in northern Spain (Vainio et al. 2015).

The use of mycoviruses to control fungal diseases of plants could be a promising method when the genetic diversity of the populations of the pathogen is low, for example, when the introduction of the fungus in a region is recent or when sexual reproduction is absent. This is the case with the pitch canker pathogen in Spain (Berbegal et al. 2013). However there is still a long way to go in the development of a biological control tool using mycoviruses, and it requires first finding a virus producing hypovirulence then finding out the limitations in its transmission.

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Dothistroma Needle Blight on High Altitude Pine Forests in Montenegro

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Abstract

Dothistroma needle blight (DNB) is one of the most serious and widespread needle diseases of pines. Its current distribution was investigated in high altitude native and planted pine forests in Montenegro. The suitability for the disease under different climatic conditions is discussed. Using molecular methods, polymerase chain reaction (PCR) with species specific primers, *Dothistroma septosporum* (Dorog) M. Morelet was detected from needles of *Pinus nigra* Arn., *Pinus nigra* 'Dalmatica' (Visiani) Franco, *Pinus sylvestris* L., *Pinus mugo* Turra, *Pinus heldreichii* H. Christ, *Pinus peuce* Griseb. and *Picea abies* Karst. in different parts of Montenegro, at altitudes between 800 and 2150 m. *Dothistroma pini* Hulbary was detected from *P. sylvestris*, *P. nigra* and *P. mugo* from restricted area in Northwestern part of Montenegro at altitudes between 800 and 1850 m. This is the first report of DNB on *P. mugo* and *P. abies* in Montenegro, and the first time that *D. septosporum* was detected from *P.nigra* 'Dalmatica'. Also, *D. septosporum* was detected through native forests of *P. heldreichii* and *P. peuce*. The climatic conditions and altitudes where DNB was detected were different from what has previously been reported for this region. From this study, we can conclude that DNB is present in high-altitude pine forests throughout Montenegro. However, the intensity of the disease is low due to prevailing environmental conditions, which are probably not favorable for DNB development.

Keywords: *Dothistroma septosporum*, *Dothistroma pini*, *Pinus heldreichii*, *Pinus peuce*, *Pinus mugo*, *Pinus nigra* 'Dalmatica', climate

Introduction

Dothistroma needle blight (DNB), also known as red band needle blight, is one of the most serious and widespread needle diseases of pines (Karadžić 1988, 2004; Barnes et al. 2008, Barnes et al. 2014). DNB is caused by two fungal species, *Dothistroma septosporum* (Dorog) M. Morelet (teleomorph *Mycosphaerella pini* Rostr.), which is considered a cosmopolitan species, and *Dothistroma pini* Hulbary (teleomorph unknown), which has more restricted distribution. Distinguishing the two species from each other based on the morphology of their conidia and microscopic characteristics is almost impossible (Barnes et al. 2004, Ioos et al. 2010).

Dothistroma septosporum is by far the most important invasive pathogen of non-native pine species (Ivory 1994, Karadžić 2004, Barnes et al. 2014). It has been observed on more than 42 pine taxa worldwide and also on other conifers from genera *Abies*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga* (Drenkhan et al. 2016). During the past 50 years, *D. septosporum* has been reported from an increasing number of countries, in Europe as well as in the other continents. The known range of disease has continuously been expanded (Jankovsky et al. 2009, Drenkhan et al. 2014, Drenkhan et al. 2016) and serious epidemics have emerged

around the world (Barnes et al. 2014). Climate change has been proposed as the one of reasons for the increased severity of the disease (Woods et al. 2005, Woods et al. 2016).

Dothistroma pini has a restricted distribution. It has been detected only in the Northern Hemisphere, in North America and Europe. It was found in USA, Russia, Ukraine, Hungary, France, and recently in eight more European countries on different pine species (Barnes et al. 2004, Barnes et al. 2008, Drenkhan et al. 2016). However, distribution of *D. pini* could be much more extensive in Europe (Jankovsky et al. 2009), as the recent research from Balkan Peninsula shows (Lazarević et al. 2014, Drenkhan et al. 2016).

The disease infects the needles and can result in needle defoliation, reduction in stem diameter increment and height growth and, in severe cases the death of tree (Gibson et al. 1964, Karadžić 2004).

Dothistroma needle blight has been known in Montenegro since the late 1980ies (Karadžić 1990, 2004) while in Serbia, the disease has been known since 1955 (Krstić 1958). *M. pini* (teleomorph of *D. septosporum*) was observed there for the first time in 1979 (Karadžić 1986). The disease was, together with other needle pathogens of pine, intensively investigated in the region of Serbia and Montenegro from 1975 to 2005. *D. septosporum*, together with *Sphaeropsis sapinea* (Dyko) Sutton, was recognized then as the main causal agent of decline of *P. nigra* in forest plantations (Karadžić 1986, 1987, 1990, 2004, 2010, Milijašević 1994, 2002). The disease also appeared in forest nurseries and in natural forests. In 1988, there was a severe epidemic of DNB in *P. nigra* plantations near Pljevlja in northern Montenegro (Karadžić 1990, 2004). The disease was also observed on *Pinus sylvestris* L. in the northern part of the country (Karadžić 2010), on *Pinus halepensis* Mill. (Karadžić and Vujanović 1992, Karadžić 2004) and *Pinus pinea* L. (Karadžić 2010) near the Adriatic coast. DNB has only been identified based on morphological characteristics (Karadžić 1990, 2004, 2010, Karadžić and Vujanović 1992) and, hence, it was unknown if DNB in Montenegro was caused by *D. septosporum* or *D. pini*. In recent studies molecular methods, such as polymerase chain reaction (PCR) with species specific primers were used to identify *Dothistroma* spp. directly from the needles, and *D. septosporum* was detected from needles of *P. nigra*, *P. sylvestris*, *P. halepensis*, *Pinus heldreichii* H. Christ. and *Pinus peuce* Griseb., while *D. pini* was detected from *P. sylvestris* (Lazarević et al. 2014).

In the Balkan region, DNB has a one-year life cycle (Karadžić 2004). Conidia are dispersed by rain splash and their dissemination takes place from the beginning of April until the end of October, while the critical period for infection ranges from the beginning of May until the end of June. The length of the incubation period depends on climatic conditions, and in natural conditions it normally ranges from 4 to 6 months (Karadžić 2004). Under controlled conditions, the conidia germinate in temperatures between 5-30°C, with an optimum at 22°C (Karadžić 2004) and germination favors the high humidity (Karadžić 1988). The fungal mycelium develops in temperatures from 3 to 29°C (Karadžić 2004). According to Watt et al. (2009), the upper temperature threshold for growth is 31°C. With sufficient moisture and if the temperature is above 5°C, the fungus can infect needles throughout the year (Sinclair et. al. 1987). Currently, DNB is present in Montenegro, but the severity of the disease does not appear to be high (Lazarević et al. 2014.)

It has been suggested that, in the region of western Balkan, the climatic conditions at altitudes higher than 900 m will be unfavorable for DNB (Karadžić 1986, 1997, 2004), but considering the current world distribution of the disease (Watt et al. 2009. Drenkhan et al. 2016), it is likely though, that DNB could appear at these higher altitudes in the northern parts of Montenegro. Besides, DNB has already been recorded at few localities in high elevation forests of *P. heldreichii* and *P. peuce* in southern part of the country.

The aim of this study was to i) evaluate the current situation of DNB in high mountain forests, primarily in *P. heldreichii*, *P. peuce* and *P. mugo* forests; ii) using PCR with species specific primers, to determine which species of *Dothistroma* is present there iii) to evaluate possible risks posed by the disease on the base of prevailing environmental conditions in different high altitude pine forests.

Materials and methods

Field investigation and sampling

Different types of pine forests in Montenegro, spread in mountain areas, were examined for DNB. There were analyzed the needles of *P. nigra*, *P. sylvestris*, *P. mugo*, *P. heldreichii* and *P. peuce*, from altitudes between 800 - 2150 m above the sea level. *P. nigra* was examined at Mt. Durmitor (1 locality/3 samples), Mt. Prokletije (1 /3), Mt. Orjen (2/6) and Mt. Lovćen (1/5) in native forests or plantations as well as near the city of Pljevlja (2/14), where it was planted as protective or the part of urban greeneries. Forests of *P. sylvestris* L. were examined in Durmitor (3/20) and Prokletije (1/4) mountain massifs, and also near the cities of Pljevlja (2/11) and Rožaje (1/3;). *P. mugo* was sampled on few localities and altitudes throughout mountain massifs of Durmitor (NW MNE; 5/25) and Bjelasica (Central MNE; 1/4), as well as in planted forest at Mt. Lovćen (SW MNE; 1/8). In the case of *P. heldreichii*, it relates to Mt. Orjen (5/17), Mt. Prekornica (2/32), Mt. Žijevo (3/46) and Prokletije massif (2/16), and *P. peuce* at Prokletije massif at Mt. Bogićevica (2/11), Mt. Visitor (1/4) and Mt. Zeletin (1/4). There were also examinet the needles of *P. nigra* ssp. *dalmatica* (Visiani) Franco growing in

forest plantation, near Adriatic Sea, on ca 500 m of altitude (1/8). Additionally, needles of *Picea abies* Karst., from Mt. Durmitor (1/4) were analyzed. In total, there were 44 localities, 248 needle samples, six different species and one subspecies of conifers investigated (Table 1).

The purpose of the field investigation was to examine whether the disease was present in high mountain pine forests, on the different pine species and in the different environmental conditions. The goal also was to confirm using molecular methods that suspected symptoms were caused by *D. septoposrum/D. pini*. Based on previous experience of the disease (our personal observations), it was not always evenly distributed in the forest stand. Furthermore, all the needles on a specific tree are not infected. Therefore, we did not apply a specific sampling schedule and the needles with suspected symptoms of DNB were collected. Such suspected symptoms were red bands, dying needle tips and/or subepidermal acervuli (Karadžić 2004). By chosen localities, the native range of distribution for *P. heldreichii*, *P. peuce* and *P. mugo* in Montenegro is well covered, and it enables an evidence of presence of disease, as well as evaluation of disease intensity on stand level. On the other hand, *P. nigra* and *P. sylvestris* were not being observed in bigger populations or in forest plantations, with pathogens detected from the single trees only.

Needle samples were stored in -20 °C until further molecular analysis.

Climates on researched localities

Montenegro is predominantly mountainous country in South Eastern Europe. It is located in the central part of moderately warm zone in Northern Hemisphere (41º52' and 43º32' latitude North and 18º26' and 19º22 longitude East). Owing to its geographical position, relief dissection, atmospheric circulation and vicinity of the Adriatic Sea, there are big differences within a small area between the climates in costal and high mountains regions. Warm temperature climate (C) is found in lower parts of the country, while the cold climate type (D) is found in higher inland mountain regions, above 1000-1200 m (according to Köppen climate classification, Burić et al. 2014). The warm climate (C) is represented by two climate types, Cs and Cf. Two subtypes: Csa and Csb can be distinguished within the Cs (Mediterranean) climate (Burić et al. 2014). For the purpose of this study, we were especially interested in climates in the mountains near the Adriatic Sea, previously defined as perhumid Mediterranean-submediterranean mountain climate (Stevanović and Stevanović 1995, Walter and Breckle 1985). According to Burić et al. (2014), it was positioned inside Cs/s''/b subtype. Further, humid warm temperate climate type (Cf) is found with subtype Cfb. The cold climate (D) is found in higher regions with Df climate types and two subtypes- Dfb mainly on altitudes up to 1500-1600 m a.s.l. and Dfc, on altitudes above 1600 m a.s.l.
Climates for each locality are given in Table 1. In the text that follows, in combination with prevailing forests for the area, the main characteristics of dominant mountain climates in Motenegro are given in short.

The *P. heldreichii* forests grow in high mountain regions (1200 – 2000 m alt.) exposed to the Mediterranean climate (perhumid Mediterranean-submediterranean mountain climate) (Stevanović et al. 1994). The area is characterised by subalpine climate, cold winters and chilly summers, with average annual temperature of ca 3°C. In the area, winter minimum is below -30°C and summer maximum is above 35°C. The mean annual precipitation is about 2500 mm, but rainfalls during the vegetation season make only ca. 8% of total. During the vegetation season, the rainfall is often followed by 40-70 days-long periods of drought. The rainfall is maximal in late autumn and early winter, while minimal during the summer months, when the drought occurs (Hydrological and Meteorological Service of Montenegro). The *P. heldreichii* and *P. peuce* forests in the Mt. Prokletije (eastern Montenegro) grow under the similar, but colder environmental conditions. During the summer, the forests in this area also sustain the drought, but the temperature extremes are not as high.

At the mountains in northwestern and central Montenegro (Mt. Durmitor and Mt. Bjelasica) coniferous forests dominated with *P. abies*, *A. alba* and *P. sylvestris* (All. *Picetum excelsa montanum*) are present at altitudes of ca 1400-1500 m. Above the 1700-2300 m., high mountain shrubbery vegetation of *P. mugo* appears (Stevanović et al. 1994). The area is characterized by humid boreal climate (Df), cold, long lasting and snowy winters and short and chilly summers. At 1450 m average annual temperature is ca. 4.6°C, air temperature in the coldest month is below -30°C and the temperature of the warmest month goes to 14-15 °C. Mean annual precipitation is between 1250 and 2000 mm. The altitude has the primary influence on temperature (inversely) and also on the occurrence of wet periods throughout the year. The primary precipitation maximum is in the autumn and the secondary is in the spring. Yet the summer precipitation sum is smaller than the winter one (Hydrological and Meteorological Service of Montenegro, Burić et al. 2014).

DNA extraction and PCR

In order to only detect infections while not detecting spores on the needle surface, needle samples were washed prior to DNA extraction. Needles were first washed in 96% ethanol for 30 seconds, followed by 1.5 minutes in 2% sodium hypochlorite and then rinsed twice in 96% ethanol. Each needles' sample was placed in a screw cap tube with a screw and two nuts, freeze dried and then homogenized

Table 1. Occurrence of *Dothistroma* needle blight in Montenegrin mountains

No	Host	Location	Alt.	Latitude	Longi-tude	V	Primeval vegeta-tion	Cli-mate	SP	Ac	PCR	DNB
1	*P. heldreichii*	Ostrog monastry	900	42,669	19,030	UG	*Seslerio-Ostryetum-carpinetum*	Csa	II	-	+	*D. sept*
2	*P. heldreichii*	Mt. Orjen (1)	1050	42,5736	18,6383	F	*Fagion moesiacae*	Csbx	II	-	+	*D. sept*
3	*P. heldreichii*	Mt. Orjen (2)	1600	42,5222	18,5397	F	*Pinetum heldreichii*	Csbx*	II	-	+	*D. sept*
4	*P. heldreichii*	Mt. Orjen (3)	1800	42,5775	18,5858	F	*Pinetum heldreichii*	Csbx*	II	-	+	*D. sept*
5	*P. heldreichii*	Mt. Orjen (4)	1000	42.5072	18,5572	F	*Fagion moesiacae*	Csbx*	II	-	+	*D. sept*
6	*P. heldreichii*	Mt. Orjen (5)	1150	42,5436	18.5240	F	*Pinetum heldreichii*	Csbx*	II	-	+	DNB
7	*P. heldreichii*	Mt. Prekornica (1)	1250	42, 6241	19.1995	F	*Pinetum heldreichii*	Csbx*	III II I	- - -	+ + +	*D. sept*
8	*P. heldreichii*	Mt. Prekornica (2)	1700	42, 6589	19,1853	F	*Pinetum heldreichii*	Csbx*	III II	--	++	*D. sept*
9	*P. heldreichii*	Mt. Žijevo (1)	1300	42,4908	19,5472	F	*Pinetum heldreichii*	Dfc	II I	- -	+ +	*D. sept*
10	*P. heldreichii*	Mt. Žijevo (2)	1950	42,5753	19,5592	F	*Pinetum heldreichii*	Dfc	II I	--	++	*D. sept*
11	*P. heldreichii*	Mt. Žijevo (3)	1750	42,5986	19,5383	F	*Pinetum heldreichii*	Dfc	III II	-- +	+ +	*D. sept*
12	*P. heldreichii*	Mt. Prokletije (1)	1300	42,4414	19,8081	F	*Pinetum heldreichii*	Dfwbx''	III II	+	+	*D. sept*
13	*P. heldreichii*	Mt. Prokletije (4)	2150	42,6145	19,8802	F	*Pinetum heldreichii*	Dfc	II	-	+	*D. sept*
14	*P. mugo*	Mt. Durmitor (4)	1850	43,1719	19,0797	F	*Pinetum mughii*	Dfc	I II	--	++	*D. sept* *D. pini*
15	*P. mugo*	Mt. Durmitor (5)	1750	43,1681	19,0592	F	*Pinetum mughii*	Dfc	II I	- -	++	*D. sept*
16	*P. mugo*	Mt. Durmitor (6)	1650	43,0953	19,0208	F	*Pinetum mughii*	Dfc	II	-	+	*D. sept*
17	*P. mugo*	Mt. Durmitor (3)	1600	43,1730	19,0839	F	*Picetum excelsa montanum*	Dfc	II	-	+	*D. sept*
18	*P. mugo*	Mt. Durmitor (1)	1450	43,1489	19,0942	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. pini*
19	*P. mugo*	Mt. Bjelasica	1850	42,8753	19,6985	F	*Pinetum mughii*	Dfc	II	-	+	DNB
20	*P. mugo*	Mt. Lovćen	1100	42,3813	18,8344	FP	*Fagion moesiacae*	Csbx	II	-	+	*D. sept*
21	*P. nigra*	Pljevlja (1)	820	43,3616	19,3616	UG	*Querco carinetum betuli*	Cfwbx	II I	- -	+ +	*D. sept*
22	*P. nigra*	Mt. Durmitor (1)	1450	43,1489	19,0900	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. sept*
23	*P. nigra*	Mt. Durmitor (1)	1450	43,1489	19,0900	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. pini*

Alt-Altitude (m above sea level); Latitude & Longitude – geographical coordinates in decimal degree (WGS84); V-type of vegetation (F-natural forest, P-forest plantation; UG-Urban greenery/shelterbelts; Primeval vegetation (according to Stevanović et al. 1995); Climate-Climate type according Kőppen system; SP-symptoms present: age of processed needles; Ac-Acervuli present; PCR- PCR applied; DNB – *Dothistroma* species detected by PCR (*Dothistroma septosporum- D. sept*. and *Dothistroma pini- D. pini)*

Table 1. Continued

No	Host	Location	Alt.	Latitude	Longi-tude	V	Primeval vegeta-tion	Climate	SP	Ac	PCR	DNB
24	*P. nigra*	Mt. Orjen (1)	1050	42,5736	18,6383	P	*Fagion moesiacae*	Csbx	II I	--	++	*D. sept*
25	*P. nigra*	Mt. Orjen (6)	820	42,4942	18,5486	P	*Orno-Quercetum ilicis*	Csbx	II	-	+	DNB
26	*P. nigra*	Mt. Lovćen	1100	42,3814	18,8344	P	*Fagion moesiacae*	Csbx	II	-	+	*D. sept*
27	*P. nigra*	Pljevlja (2)	820	43,3530	19,3558	UG	*Querco carpinetum betuli*	Cfwbx	II	-	+	*D. pini*
28	*P. nigra*	Pljevlja (2)	820	43,3530	19,3558	UG	*Querco carinetum betuli*	Cfwbx	II	-	+	*D. sept*
29	*P. nigra*	Mt. Prokletije (1)	1300	42,4967	19,8169	P	*Fagion moesiacae*	Dfwbx''	II	-	+	*D. sept*
30	*P. nigra 'Dalmatica'*	Mt. Vrmac	520	42,4250	18,7490	P	*Orno-Quercetum ilicis pinetosum*	Csa	II I	-	+	*D. sept*
31	*P. peuce*	Mt. Prokletije (2)	1850	42,5828	20,0311	F	*Pinetum peucis*	Dfc	III II I	--	++	*D. sept*
32	*P. peuce*	Mt. Prokletije (3)	2000	42,5650	20,0332	F	*Pinetum peucis*	Dfc	II I	--	++	*D. sept*
33	*P. peuce*	Mt. Prokletije (4)	2150	42,6146	19,8821	F	*Pinetum peucis*	Dfc	II I	--	++	*D. sept*
34	*P. peuce*	Mt. Prokletije (4)	1800	42,6330	19,8359	F	*Pinetum peucis, Abietum, Fagetum*	Dfc	II	-	+	DNB
35	*P. sylvestris*	Pljevlja (1)	820	43,3617	19,3617	UG	*Querco-carpinetum betuli*	Cfwbx	II	- -	+ +	*D. sept*
36	*P. sylvestris*	Pljevlja (1)	820	43,3617	19,3617	UG	*Querco carinetum betuli*	Cfwbx	II	- -	+ +	*D. pini*
37	*P. sylvestris*	Mt. Durmitor (2)	1300	43,2250	19,1386	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. sept*
38	*P. sylvestris*	Mt. Durmitor (1)	1450	43,1455	19,0981	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. sept*
39	*P. sylvestris*	Mt. Durmitor (1)	1450	43,1455	19,0981	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. pini*
40	*P. sylvestris*	Mt. Durmitor (4)	1850	43,1697	19,0786	F	*Pinetum mughii*	Dfc	II I		+	*D. sept*
41	*P. sylvestris*	Mt. Durmitor (4)	1850	43,1697	19,0786	F	*Pinetum mughii*	Dfc	II I		+	*D. pini*
42	*P. sylvestris*	Rožaje	1110	42,8244	20,1711	UG	*Picetum excelsae submontanum*	Dfwbx''	II	-	+	*D. sept*
43	*P. sylvestris*	Mt. Prokletije (1)	1300	42.4414	19.8080	P	*Fagion moesiacae*	Dfwbx''	II I	-	+	*D. sept*
44	*Picea abies*	Mt. Durmitor (1)	1450	43,1455	19,0981	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D.sept.*

Alt-Altitude (m above sea level); Latitude & Longitude – geographical coordinates in decimal degree (WGS84); V-type of vegetation (F-natural forest, P-forest plantation; UG-Urban greenery/shelterbelts; Primeval vegetation (according to Stevanović et al. 1995); Climate-Climate type according Kőppen system; SP-symptoms present: age of processed needles; Ac-Acervuli present; PCR- PCR applied; DNB – *Dothistroma* species detected by PCR (*Dothistroma septosporum- D. sept*. and *Dothistroma pini- D. pini)*

in a fast prep shaker (Precellys 24 Bertin Technologies). About 100-300 µl of the homogenized needle material per sample were used for DNA extraction where 1 ml of CTAB-buffer (3% cetyltrimethylammonium bromide, 2 mM EDTA, 150 mM Tris-HCl, 2.6 M NaCl, pH 8) was added to each sample, which was then incubated for 1 h at 65° C. After centrifugation, the supernatant was transferred to a new tube and mixed with an equal volume of chloro-

form and centrifuged. The supernatant was then transferred to a new tube, precipitated on ice with 1.5 volumes of isoporopanol, washed with 70% ethanol and redissolved in 50 µl milliQ water. The extracted DNA was then purified using JetQuick DNA purification kit (Genomed GmbH), according to manufacturer's instructions. DNA concentrations were measured using NanoDrop (Thermo Scientific) and samples were diluted to 10-50 ng/ul.

Dothistroma septosporum specific primers Dstub2-F (5'-CGAACATGGACTGAGCAAAAC- 3') and Dstub2-R (5'-GCACGGCTCTTTCAAATGAC-3') and *Dothistroma pini* specific primers DPtef-F (5'-ATTTTTCGCTGCTC GTCACT-3') and Dptef-R (5'- CAATGTGAGATGT TCGTCGTC-3') were used for amplification with conventional PCR (Ioos et al. 2010). The thermal cycling was carried out in an Applied Biosystem Gene Amp PCR System 2700 thermal cycler. An initial denaturation step at 95° C for 3 min was followed by 35 amplification cycles of denaturation at 95° C for 30 s, annealing at 60 ° C for 30 s, and extension at 72° C for 60 s. The thermal cycling was ended by a final extension step at 72° C for 10 min (Ioos et al. 2010). To confirm the presence of *D. septopsporum* and *D. pini* in the samples, PCR products were visualized on a 1% agarose gel and compared to a positive control that was included in each PCR run. For *D. septosporum*, the PCR product was about 230 base pairs and for *D. pini* the PCR product was about 190 base pairs (Ioos et al. 2010).

Results

In all, DNB was confirmed at 44 spots throughout high mountain forests in Montenegro. In total, 6 different plant species and one subspecies were investigated (Table 1). Symptoms of DNB were found in 9 mountain massifs and on 28 localities ranging from 820-2150 m of altitude, as well as on one hill near Adriatic coast at 520 m of altitude. DNB was found on: *P. nigra*, *P. nigra* ssp. *dalmatica*, *P. sylvestris*, *P. mugo*, *P. heldreichii* and *P. peuce*, and also on *Picea abies*. *D. septosporum* was identified using species specific primers in needles from all investigated pine species and also from *P. abies*. *D. pini* was identified with species specific primers in restricted area on northwestern part of Montenegro on *P. sylvestris*, *P. mugo* and *P. nigra*.

The disease intensity on stand level is currently low, but variable between different pine species. The infection level could also vary between different climates and also local environmental conditions, even in different years.

The climatic conditions in mountain regions of Montenegro may be less favorable for DNB development. It seems that *P. heldreichii*, *P. sylvestris* and *P. mugo* sustain it well in their native habitats, while the *P. peuce* is might be a more susceptible and more endangered species regarding DNB.

Acervuli were only found in one site, on *P. heldreichii* needles in Mt. Prokletije, where *D. septosporum* was later confirmed by molecular analysis. On the other localities and pine species, acervuli and conidia were not recorded.

Discussion and Conclusions

The results demonstrate that DNB is widespread across the high altitude pine forest in Montenegro (Table 1). Range of *P. heldreichii*, *P. peuce* and *P. mugo* in Montenegro is well covered in here presented research, enabling evidence of presence of disease and evaluation of disease intensity on stand level. *P. nigra* and *P. sylvestris* were observed in urban greeneries or as single trees inside polidominant coniferous forests with pathogens being detected from just the single trees. Throughout the earlier researches in *P.nigra* plantations (Lazarević et al. 2014), no symptoms of DNB were observed at lower altitudes.

Dothistroma septosporum was reported for the first time on *P. heldreichii* and on *P. peuce* from native forest stands recently (Lazarević et al. 2014). During this study, the presence of DNB has been confirmed using species specific primers on *P. heldreichii* on many new localities, also at higher altitudes than before. It was detected in five different mountain massifs at the altitudes between 1300 - 1850 m (Table 1). Typically, the symptoms of DNB were not present on the current year needles, but they were visible on 2, 3 and 4 years old needles. Infected needles had red bands, dying needle tips and/or necrotic, pale green or orange-yellow tissue around or between the red bands. Needles were retained on the twig, and did not become fully necrotic. Fruit bodies were found on rare occasions (Lazarević et al. 2014). The disease intensity on stand level is currently low, but could vary depending on microclimatic conditions. It is sometimes also increased on skeletal soils or on tree line habitats that are nutrient poor, dry and exposed.

The *P. heldreichii* forests grow in high mountain regions exposed to the Mediterranean climate, which is probably limiting for DNB. It could be considered that average daily temperatures, air humidity and precipitation conditions are likely to be favorable for DNB infection and disease development during late spring and early in summer (May, June and the beginning of July). Later in summer, the climate conditions are probably less favorable for spore dispersal and infection. The dry period is coming, with only sporadic rain, and with the maximum day temperatures reaching 40 °C in open forest stands. Moreover, *P. heldreichii* usually grows in open and exposed positions on shallow limestone soils and, characteristically, the trees are sparsely distributed. The relative humidity inside this forest is thus assumed to be low, which probably further limits the development of fruit bodies, conidia dissemination and new

infections (Gadgil 1977). From previous studies, *P. heldreichii* has been reported as slightly susceptible to DNB (Bendarova et al. 2006), which is consistent with our observations.

The occurrence of DNB was observed on *P. peuce* forests on eastern Montenegrin border. For the first time, symptomatic needles were collected from the plants from natural regeneration on ca 1850 m altitude (Lazarević et al. 2014). Later observations showed that symptoms were present across *P. peuce* forests in the region, at altitudes ca 1900-2100 m on natural regeneration and on older trees. On *P. peuce*, the symptoms of DNB were visible on the current year needles as sporadic red dots and bends. Two years old needles become fully necrotic at the end of vegetation period (September) and falling down from the twigs. Intensity of disease is abundant on natural regeneration and on the young trees, and could be even locally increased in vicinity of glacial lake (loc. Bogićevica). The intensity of disease is weaker at lower altitudes in mixed forests, where symptoms of disease are sporadic.

P. peuce forests appears to be more favoring for DNB than the *P. helderichii* sites, despite they grow under similar climate conditions, since the humidity inside the forest stand is higher. *P. peuce* grows predominantly on silicate soils, in pure or mixed forest stands. It forms dense forests, where the humidity is likely to be higher, especially near the forest floor and where temperature extremes are mitigated by the dense forest cover. There are also other factors that may influence the disease, such as host susceptibility. There no previous reports about *P. peuce* susceptibility to disease (Watt et al. 2009), except that Barnes et al. (2009) reported the untypical symptoms and usually brownish needles.

On *P. mugo*, DNB was detected at mountain massifs in Northern and central Montenegro, where it appears as native, on southern border of its range, and also near Adriatic coast, where a small planted *P. mugo* forest exists at 1100 m alt.

On native habitats, at altitudes between 1450 and 1850 m, the symptoms of DNB on *P. mugo* were sporadic on the current year needles, but more visible on 2, 3 and older needles. Infected needles had red bands and dying needle tips. Needles were retained on the twig and examples of defoliation (needle loss) were not observed. Needles did not become fully necrotic except rarely, and then mainly due to presence of other pathogens or insect attack. In many fragmented populations DNB symptoms were even more sporadic, but evident.

Further, *P. mugo* was sampled in Mt. Lovćen, near Adriatic coast. A small *P. mugo* forest is planted here in Mediterranean mountain climate and in micro depression. On this locality, *P. mugo* was heavily infested by *D. septosporum* and other needle pathogens (mainly by *Cyclaneusma* sp.). Needles from the last vegetation had intensively developed DNB symptoms. Needles from one year before were heavily infested and necrotic, in many cases fallen from twigs. On Mt. Lovćen, there is much more rainfalls and site is much warmer during the whole year, than across native *Pinetum mughii* on the North of the country (humid boreal climate). There are no data on the origin of *P. mugo*, planted in early 1960. Hence, host susceptibility could vary and influence a big difference in health status of *P. mugo* on two localities.

Dothistroma needle blight has been observed for the first time in Montenegro in *P. nigra* plantations in the city of Pljevlja (foothills of Mt. Durmitor) in 1979 (Karadžić 1986, 2004). This area with continental climate has been considered to be a typical habitat for DNB (Karadžić 1986, 2004, Watt et al. 2009, Lazarević et al. 2014). During the last decades, DNB has been present here and in wider region particularly in 5 to 25 year old *P. nigra* plantations, established at altitudes up to 900 m (Karadžić 1986, 1997, 2004). During recent research on 50 years old forest plantations of *P. nigra* in northern Montenegro, no symptoms of DNB were observed (Lazarević et al. 2014). Presence of *D. septosporum* was detected by species specific primers on needles of *P. nigra* and *P. sylvestris* from urban greeneries in Pljevlja (Lazarević et al. 2014). It was also detected on planted *P. nigra* on Mt Lovćen, as well as in 10 years old plantation in the eastern part of Montenegro (Table 1).

During here presented investigations, *D. septosporum* was detected on *P. sylvestris* and *P. nigra* on Mt. Durmitor at 1450 m alt. and also on needles of *P. abies*, at the same locality. *P. abies*, which dominated in this forest area, is new DNB host for Montenegro. Needles of *P. abies* from previous vegetation were symptomatic, with red bends, but intensity of infection was low.

Dothistroma septosporum was further detected on *P. sylvestris* on few more localities at altitudes up to 1850 m on Mt. Durmitor, then in eastern part of Montenegro on planted *P. sylvestris* at 1200 – 1300 m asl.

After the first finding of *D. pini* on *P. sylvestris* in city of Pljevlja (Lazarević et al. 2014), researches have continued with more samples from the same region. Consequently, *D. pini* was identified in needle samples collected at altitudes 1300 – 1850 m on *P. nigra*, *P. sylvestris* and also on *P. mugo* on the Mt. Durmitor. Much more DNA samples from different pines and regions in Montenegro were tested with *D. pini* specific primers, but without positive result. Hence, we can consider that *D. pini* is present in restricted area in northern part of Montenegro, on Mt. Durmitor and its surroundings, between 820 and 1850 m of altitude. Despite the presence of both *D. septosporum* and *D. pini*, the level of infection on *P. mugo* and *P. sylvestris* on Mt. Durmitor is sporadic and of low intensity. Both *D. septosporum* and *D. pini* were sometimes recorded on the same host tree.

Finally *D. septosporum* was detected on *P. nigra* ssp. *dalmatica* on the Adriatic coast (Vrmac peninsula in Boka bay, Mt. Vrmac), on altitude of ca 500 m. *P. nigra* ssp. *dalmatica* grows here in even-aged forest plantation of ca 50 years old trees, in Mediterranean climate (Csa). The plantation is declared as seed stand (Isajev et al. 2011). *D. septosporum* was detected from the shoots from the top of the crowns, and also from lowest branches, from current and previous year needles. Symptoms were more obvious on lower branches, but also mixed with other needle pathogens. According to our knowledge, this is the first record of *D. septosporum* on *P. nigra* ssp. *dalmatica*. In the nearness, clear DNB symptoms were observed on *Pinus pinaster* Aiton, what has not been confirmed by molecular methods until now.

According to here presented results, DNB is widely distributed in different mountain pine forests across Montenegro. The disease intensity is currently low. It has been suggested that, in the region of western Balkan, the climatic conditions at altitudes higher than 900m will be unfavorable for DNB (Karadžić 1986, 1997, 2004), and the disease was not recorded higher during the intense investigations in 1975-2005 (Karadžić 1986, 1997, 2004). The fact that the characteristic symptoms with red bands and fruit bodies are rare in these sites makes it difficult to determine if DNB is a new disease in these forests. It is likely that the disease may be overlooked or mistaken for other diseases. It seems that *P. heldreichii, P. sylvestris* and *P. mugo* sustain well on their native habitats, while the *P. peuce* might be more susceptible and more endangered species regarding DNB. Also, it can be expected that with the change of environmental conditions, sensitivity of pine species according DNB would be different, what was evident on more stressful habitats.

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Three-year Dynamics of Common Ash Defoliation and Crown Condition in the Focus of Black Sawfly *Tomostethus nigritus* F. (Hymenoptera: Tenthredinidae)

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Abstract

Black sawfly (*Tomostethus nigritus*), the foliage browsing pest of common ash (*Fraxinus excelsior*) after several years of depression was registered in forest stands, forest shelter belts and urban ornamental stands of the East Ukraine. The aim of this research was to reveal the peculiarities of ash defoliation by black sawfly during three years of outbreak and to recognize the changes in health condition of defoliated trees. Research was carried out for 2013–2015 in Molodezhny park of Kharkiv (50°00' N; 36°25' E) (Ukraine, Forest-steppe natural zone) in two groups of common ash trees (forest belt and compact stand). For each labeled tree, defoliation of the whole crown and separately of upper, middle and lower layers, outer and inner parts of crown was assessed in June of 2013, 2014 and 2015, and additionally in July 2015. Health condition of each labeled tree was assessed in June 2013 and July 2015. Defoliation of common ash trees did not significantly differ in 2013 and 2014. It almost twice increased in 2015 and in average exceeded 90 %. Dependence of defoliation from crown layer, crown part (outer or inner) or tree diameter was not proved statistically, but correlation analysis shows the better health condition of larger trees. The fast recovery of ash crowns after severe damage by black sawfly in 2015 can be explained by favorable weather conditions. Analysis of ash trees distribution by categories of health condition show the improvement of their health after three years of foliage damage by ash sawfly, but forest stand continues to be weakened. Considerable parts of the trees, which belonged to the 2nd, 3rd and 4th categories of health condition in 2013, changed it by one-three grades. Totally 50 % of trees did not change the category of health condition, 35.3 % of trees improved it and 14.7% worsened it.

Key words: black sawfly (*Tomostethus nigritus*), common ash (*Fraxinus excelsior*); defoliation, crown condition.

Introduction

Black sawfly (*Tomostethus nigritus*) is known as common ash (*Fraxinus excelsior* L.) pest in Russia (Belova 1987), Norway (Austarä 1991), Croatia (Matošević et al. 2002), Czech Republic (Mrkva 1965, Holuša, Drápela 2004), Serbia (Glavendekić and Mirić 2009, 2011), Italy (Mitali 2012).

In the East part of Ukraine black sawfly caused a short-time outbreak in 2002 (Meshkova and Davydenko 2013) and after several years of depression severe defoliation of common ash by this pest was registered in forest stands, forest shelter belts and urban ornamental stands in Donetsk (Popov 2009) and Kharkov regions (Meshkova et al. 2013, Davydenko and Meshkova 2014, Kukina et al. 2014, Zinchenko and Kukina 2015) of Ukraine.

Chemical forest protective measures should be based on ecological and economic principals to minimize pesticide usage by tapping the full potential of all preventive methods. The threat for forest from the same pest exists

only in certain regions, forest stands and under certain weather conditions. Therefore it is important to know the effect of foliage browsing insects on tree condition and growth in every region. In connection with it, numerical evaluation of injuriousness of foliage browsing insects must include assessment the defoliation (which characterizes tree resistance to damage) and further tree response to damage (the change of health condition, increment and mortality, which characterize tree tolerance to damage) (Meshkova 2013).

Biological peculiarities of black sawfly and its impact on tree growth and health condition are poorly known, especially for the East of Ukraine. Therefore such studies were started in green stands of Kharkiv (Ukraine), where larvae of black sawfly damage the crowns of common ash for three years successively (2013–2015). Biological peculiarities of this pest in the region were specified in the East Ukraine (Zinchenko and Kukina 2015). It was proved by these authors, that black sawfly is univoltine monophag, which damages only common ash. The beginning of black sawfly swarming in the East Ukraine coincides with the beginning of common ash foliage development (April, 19–25). Mass swarming started April 24, April 28 and April 26 in 2013, 2014 and 2015, respectively (Zinchenko and Kukina 2015). The eggs of ash black sawfly develop about 10 – 13 days and the first larvae appear at the beginning of May, pass 5 instars and complete feeding at the third decade of May (Zinchenko and Kukina 2015). The larvae form cocoons from the end of May to the beginning of June in forest litter under the crowns. The first pupae of black sawfly appeared in 2013, 2014 and 2015 on April 4–6, 7–9 and 12–15 respectively (Zinchenko and Kukina 2015).

To predict the threat to stands from black sawfly it is necessary to study, which stands and trees are the most defoliated by black sawfly, and how such damage affects the health condition of the trees.

Therefore the aim of this research was to reveal the peculiarities of ash defoliation by black sawfly during three years of outbreak of the pest and to recognize the changes in health condition of defoliated trees.

Materials and methods

Investigations were carried out in 2013 – 2015 in Molodezhny park of Kharkiv (50°00' N; 36°25' E) (Ukraine, Forest-steppe natural zone). Besides common ash (*Fraxinus excelsior* L.), the trees of *F. pennsylvanica* var. *lanceolata* Borkh., *Betula pendula* Roth., *Aesculus hippocastanum* L., *Sorbus aucuparia* L., *Ulmus laevis* Pall., *Acer platanoides* L,. *Tilia cordata* Mill. and *Quercus robur* L. grow in this park. From these tree species only common ash was damaged by black sawfly. Therefore only trees of common ash were labeled in 2013 to monitor their defoliation and health condition.

Two groups of common ash trees were monitored: 25 trees in forest belt (alley) and 43 trees in rather compact stand (curtain).

Diameter at breast height was measured in 2013. Defoliation of each tree was assessed visually up to 5 % at the beginning of June 2013, 2014 and 2015, that is after the end of larvae feeding and their descent from the crown to the litter (Zinchenko and Kukina 2015). Defoliation of upper, middle and lower crown layer, as well as defoliation of outer (peripheral) and inner (central) parts of crown were additionally assessed at the same dates.

In July, 2015 additional assessment of defoliation (for the whole tree) and crown condition was carried out to recognize the crown recover, taking into account the period of ash shoot growth (Gordienko et al. 1996).

Category of tree health condition was assessed in June 2013 (the first year of black sawfly outbreak) and in July 2015. Category of health condition was evaluated on a range of visual characteristics (crown density and color, the presence and proportion of dead branches in the crown etc.) according to "Sanitary rules in the forests of Ukraine" (Sanitary rules in the forests of Ukraine 1995). Each tree was referred to one of six categories of health condition (1^{st} – healthy; 2^{nd} – weakened; 3^{rd} – severely weakened; 4^{th} – drying; 5^{th} – recently died; 6^{th} – died over year ago). Index of health condition for forest stand was calculated as mean weighted from trees number of each category of health condition.

Data on air temperature and precipitation for 2013 – 2015 and mean for 1981 – 2010 were taken from meteorological station Kharkiv (49°90' N; 36°30' E).

The statistical analyses included calculation the mean and standard error of defoliation, one-way analysis of variance (ANOVA) and correlation between tree diameter, defoliation and category of health condition (StatSoft Software, Ver. 7)

Results

In the both plots (forest belt and compact stand) defoliation of common ash trees in 2015 was significantly higher than in 2013 and 2014 (Figure 1, 2). In 2013 averaged defoliation amounted 43 and 53.1 %, in 2014 – 57 and 41.4 %, and in 2015 – 93 and 95.8 % in forest belt and compact stand respectively. However, in the both stands both undamaged and totally defoliated trees could be found every year.

Differences in defoliation in forest belt in 2013 and 2014 were not significant ($F_{calc.}$ = 3.1; $F_{0.05}$ = 4,0), while in 2015 defoliation significantly exceeded meanings of previous years ($F_{calc.}$ = 14.4; $F_{0,05}$ = 3.2). Similarly, any significant differences were revealed for compact stand defoliation in 2013 and 2014, but in 2015 it was significantly greater than in previous years ($F_{calc.}$ = 112.1; $F_{0.05}$ = 4.0).

Trend to increase defoliation from 2013 to 2015 is seen for all crown layers, in the outer and inner parts of crown (see Figure 1, 2).

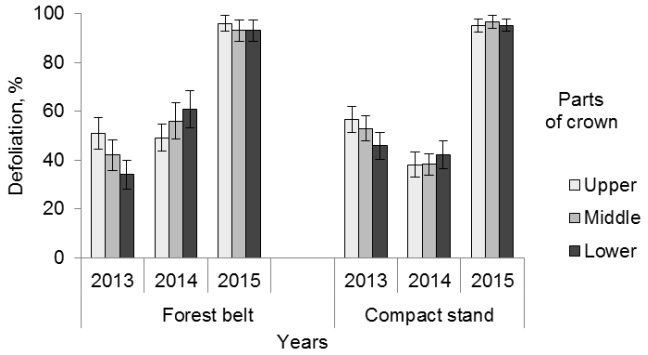

Figure 1. Mean defoliation of different crown layers of common ash in 2013–2015 in forest belt and compact stand (bars mean standard error)

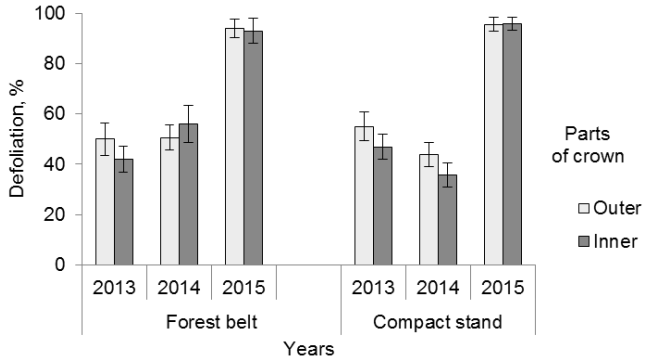

Figure 2. Mean defoliation of common ash in central and peripheral parts of the crown in 2013–2015 in forest belt and compact stand (bars mean standard error)

In 2013 both in forest belt and compact stand the trend was revealed for defoliation decrease from upper to lower crown layer (see Figure 1). In 2014 the trend was reciprocal when defoliation increased from upper to lower crown layer. In 2015 defoliation of all crown layers was about the same, and the difference was insignificant (P > 0.1) for different crown layers in forest belt ($F_{calc.}$ = 1.9; $F_{0.05}$ =3.1) and in compact stand ($F_{calc.}$ = 1.26; $F_{0.05}$ = 3.1).

Defoliation of outer part of ash crown was significantly higher than inner part of crown only in forest belt in 2013 ($F_{calc.}$ = 5.2; $F_{0,05}$ = 4.0). In other years defoliation of inner and outer parts of crown did not differ statistically in forest belt ($F_{calc.}$ = 0.38; $F_{0.05}$ =4.0) and in compact stand ($F_{calc.}$ = 1.1; $F_{0.05}$ = 4.0) (see Figure 2).

Ash stem diameter at breast height (1.3 m) amounted 40 – 80 cm in forest belt and 30 – 70 cm in compact stand. Mean defoliation for groups of trees with different diameter (by 10 cm classes) was evaluated (Figure 3).

Both in the forest belt and in compact stand the trend can be seen in 2013 and 2014 to decrease defoliation with increase of tree diameter. However, statistically this trend is not proved (P > 0.1). In 2015 defoliation of trees from all diameter classes approached 100 %, and even trend to relation between tree diameter and defoliation was not revealed.

Comparison of mean ash defoliation for all years of investigation show significant differences between defoliation in forest belt and in compact stand only in 2014 (P < 0.05, $F_{calc.}$ = 4.8; $F_{0.05}$ = 4.0). In 2013 and in both assessments of 2015, the differences between mean defoliation of ash trees in forest belt and in compact stand were not significant (P > 0.1) (Figure 4).

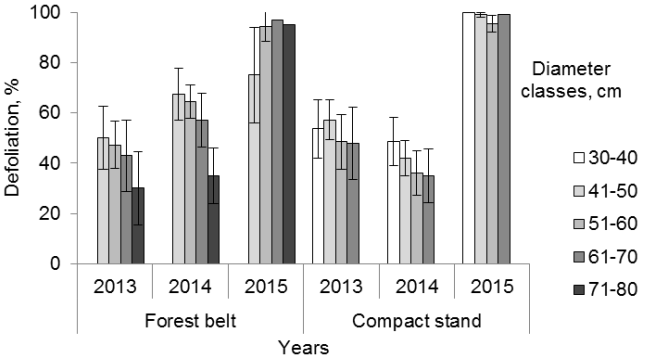

Figure 3. Mean defoliation of common ash of different diameter in 2013–2015 in forest belt and compact stand (bars mean standard error)

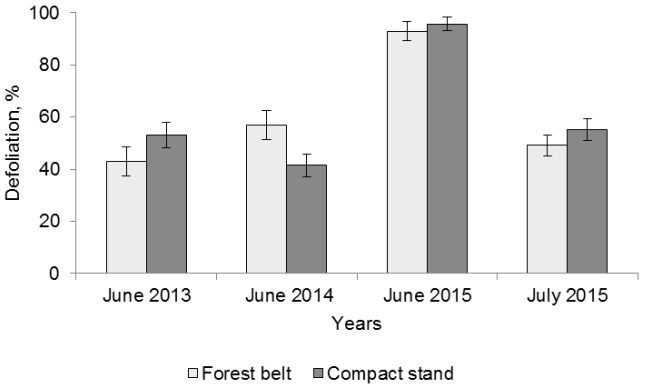

Figure 4. Mean defoliation of common ash in forest belt and compact stand at different asessment datas (bars mean standard error)

Comparison of defoliation assessment data from June and July 2015 shows that ash defoliation has decreased for one month by 1.9 and 1.7 times in forest belt ($F_{calc.}$ = 68.9; $F_{0.05}$ = 4.1) and in compact stand ($F_{calc.}$ = 66.6; $F_{0.05}$ = 4.0) respectively due to crown recover. Difference between defoliation for these two dates is significant (P < 0.0001).

Analysis of weather conditions in 2013 and 2014 shows that air temperature of April – May exceeded the long-term data, and in 2015 notable excess of the long-term

data (per 2.1 °C) was registered only in June, when development of black sawfly has already completed (Figure 5).

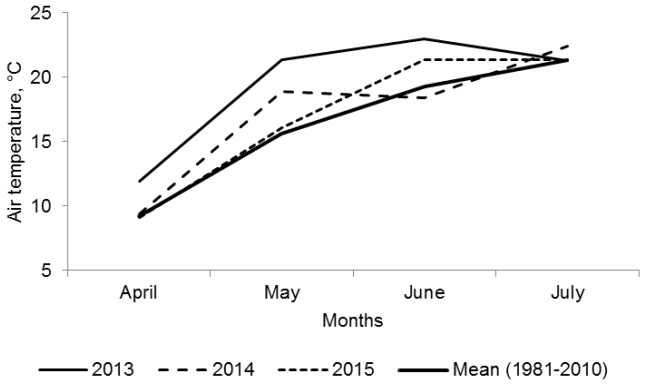

Figure 5. Air temperature of April-July in 2013–2015 and long-term data (meteorological station Kharkiv)

However, precipitation in June 2014 exceeded the long-term data 2.25 times, and in July 2014 was 1.5 times less than by the long-term data (Figure 6).

Precipitations in June 2015 exceeded the long-term data 1.2 times, and in July in 1.75 times, which together with high temperature was favorable for foliage recover (see Figure 6).

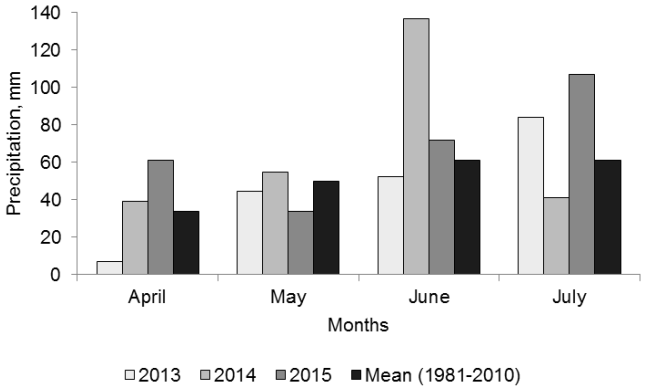

Figure 6. Precipitation in 2013–2015 and long-term data (meteorological station Kharkiv)

Taking into account the lack of significant difference between ash defoliation in forest belt and compact stand, the health condition was analyzed in pooled data.

Assessment of 2013 show (Figure 7) that the trees of the 2nd (weakened) and the 3rd (severely weakened) categories of health condition dominated (39.1 and 32.8 % respectively), and weighted index of health condition for forest stand amounted 2.47.

In 2015, after three years of annual crown damage by black sawfly, the weighted index of health condition defoliation has decreased slightly (to 2.27), i.e. tree condition has become better, and trees' distribution by the categories of health condition has considerably changed (see Figure 7).

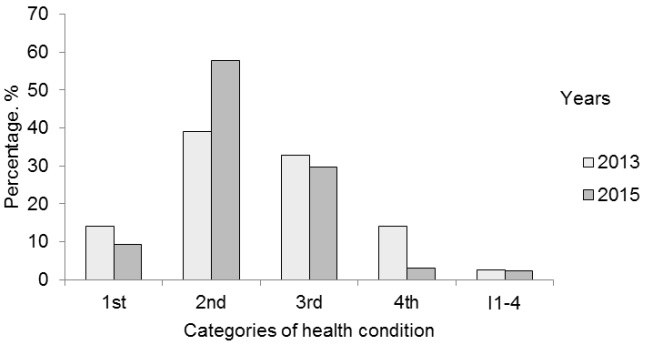

Figure 7. Distribution of ash trees by categories of health condition in 2013 and 2015

As every tree was marked, it was possible to determine how many of them have not change their health condition for three years, improved or worsened it. Totally, 50 % of all trees remained the same category of health condition while the 35.3 % of trees improved their category and 14.7% worsened it (Table 1).

Table 1. Evaluation of trees number, which have not changed, have improved or worsened their health condition for 2013–2015

Health condition in 2013*	Changes in 2015, %**		
	not changed	improved	worsened
1st (9)	11.1±31.4 (1)	–	88.9±11.1 (8)
2nd (27)	81.5±8.3 (22)	11.1± 18.1 (3)	7.4±18.5 (2)
3rd (23)	39.1±16.3 (9)	60.9±13.0 (14)	–
4th (9)	22.2±29.4 (2)	77.8±15.7 (7)	–
Total (68)	50.0±6.1 (34)	35.3±5.8 (24)	14.7±4.3 (10)

* – number of trees in 2013 is given in brackets

** – number of trees in 2015 is given in brackets

The most part of trees (88.9% of trees), which were healthy (the 1st category of health condition) in 2013, have worsened health condition in 2015. The most part of trees (81.5% of trees), which were weakened (the 2nd category) in 2013, have not changed their health condition in 2015. The most part of trees (60.9% of trees), which were severely weakened (the 3nd category) and the most part of trees (77.8% of trees), which were drying (the 4nd category) in 2013, have improved their health condition (see Table 1).

Correlation analysis was carried out with data on tree defoliation, tree diameter and health condition using pooled data from the both plots (forest belt and compact stand) (n = 68 trees).

A significant negative relationship between the tree diameter and its health condition in 2013 (r = -0.28 ± 0.12; P < 0.05) and in July 2015 (r = -0.35 ± 0.12; P < 0.01) was proved. It means that the trees of the bigger diameter had the lesser category of health condition, i.e. the better health condition.

A significant positive relationship is proved between the tree category of health condition in 2013 and in July 2015 ($r = 0.35 \pm 0.12$; $P < 0.01$), the tree category of health condition in 2013 and defoliation in 2013 ($r = 0.52 \pm 0.11$; $P < 0.01$), defoliation in 2013 and in 2014 ($r = 0.51 \pm 0.11$; $P < 0.01$).

Discussion and conclusions

Our investigation show, that both in forest belt and in compact stand every year both undamaged and totally defoliated trees were revealed. It can be explained by the fact that leaf tissue quality varied among and within tree individuals, which is proved for different foliage browsing insects, for example, for *Epirrita autumnata* (Henriksson et al., 2003).

Thickness, water and nutritional content differ both among trees and within an individual tree, between illuminated and shaded leaves.

We took into account that adults of black sawfly hatch from pupae, which are located in the forest litter, about the time of ash bud burst. To lay eggs they choose the shoots with enough developed leaves. It was suggested, that such leaves become earlier in the upper and outer parts of crown.

Only in the forest belt in 2013 defoliation of outer part of ash crown was significantly greater than inner part of crown. In other cases our investigations show the absence of common ash crown layer or crown part (outer or inner) influence on foliage damage by black sawfly larvae (see Figures 1 and 2). The trend was revealed for defoliation decrease from upper to lower crown layer in 2013 and for defoliation increased from upper to lower crown layer in 2014. Such trend may be explained by specific influence of neighboring trees on microclimate inside the crowns which affects the rate of shoot growth, foliage quality and larval survival. Respective data on ash phenology and possible influence of neighboring trees would be considered in a separate paper.

We suggested that microclimate in forest belt and in compact stand would influence on ash defoliation by black sawfly. However, the results show the absence of significant differences between two stands in each of years and similar trend to increase of defoliation in 2015 (see Figures 1, 2).

Common ash defoliation caused by black sawfly in 2013 and in 2014 was significantly correlated, but correlation was insignificant for defoliation of these both years with defoliation of 2015. It may be related with peculiarities of outbreak culmination or by other causes which were not studied here.

Both in the forest belt and in compact stand in 2013 and 2014 the dependence of defoliation on tree diameter was not proved statistically (see Figure 3).

Our research shows the fast recover of ash crown after severe damage by black sawfly in 2015 (see Figure 4).

Such intensive recover of crown condition in 2015 can be explained by peculiarities of weather conditions. As it was shown (Zinchenko and Kukina 2015), in 2015 comparing to 2013 and 2014, the larvae of black sawfly started feeding the most early (on April, 29), and completed the most late (on May, 25). Namely that the foliage was the most severally damaged on the beginning of its development brought to active shoot growth from reserve buds.

Ash shoot growth completes in middle July, therefore weather conditions did not promote foliage recover in 2014. In 2015 both air temperature and precipitation exceeded the long-term data in June, when black sawfly development has already completed, which was favorable for foliage recovery (see Figures 5 and 6).

A significant positive relationship was proved between defoliation and category of health condition of ash trees in 2013, as well as between the tree category of health condition in 2013 and in July 2015, that is after crown recovery. It may be the evidence that health condition of the tree is rather stable, and its recovery occurs at favorable conditions.

Analysis of ash trees distribution by categories of health condition shows the improvement of their health after three years of foliage damage by ash sawfly, but stand continues to be weakened (see Figure 7).

Considerable parts of the trees, which belonged to the 2nd, 3rd and 4th categories of health condition in 2013, changed it on one-three grades. Our results demonstrated that no ash tree has died since the last outbreak of black sawfly in 2002. It means that common ash is rather tolerant to foliage damage by ash sawfly.

We have seen the similar situation during outbreak of European pine sawfly (*Neodiprion sertifer* Geoffr. 1785: Hymenoptera: Diprionidae) in Ukraine (Meshkova and Kolenkina 2016). This pest damages needle of Scots pine of previous year in May, when the shoots of current year continues its development. Only those pine trees died, which were damaged not less than 80 % for three years.

The results of this study provide information that may be used in the development the methods of prediction of black sawfly influence on common ash condition and in decision making for integrated control of this pest. At the same time long-term survey must be carried out to recognize the consequences of this pest on ash stands. Peculiarities of microclimate and phenology of ash trees in different parts of stand must be taken into account.

Acknowledgements

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The Frontline of Invasion: the Current Northern Limit of the Invasive Range of Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), in European Russia

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Abstract

Agrilus planipennis is an aggressive beetle native to Asia, which has recently invaded North America and central Russia. In European Russia, the first specimens of *A. planipennis* were collected in Moscow in 2003 and the Moscow Province is therefore thought to be a likely entry point to Europe. The pest spread along roads and railways and, by 2013, it was recorded in 11 provinces of Russia. The goal of this study was to clarify the current northern range limit of *A. planipennis*. To do this, ash (*Fraxinus excelsior* and *F. pennsylvanica*) trees were surveyed along the federal highway M10 (*Russia*) between Moscow and Saint Petersburg in July 2016. The condition of ash trees and presence of *A. planipennis* was recorded at 15 locations. We found dead ash trees with galleries of *A. planipennis* at six locations (56° 27.799' N; 36° 35.383' E to 56° 47.665' N; 36° 03.584' E). At the more north-western sites ash trees became infrequent and signs of *A. planipennis* were not observed on any ash tree. Beyond the National Park Valdayskiy (58° 00.095' N; 33° 08.550' E) no ash trees were observed for about 100 km. Further north in Leningrad Province, there were fragments of ash forests and many ash trees planted in parks in Saint Petersburg and its suburbs, but no signs of *A. planipennis* were seen. Results of this survey suggested that, for summer 2016, the north-west limit of *A. planipennis* was close to Tver City (about 56° 47' N; 36° 03' E). Further range expansion of *A. planipennis* may have been limited by low host density north-west of Tver City, rather than by climatic factors. However, if *A. planipennis* can overcome low host abundance and reach Saint Petersburg or other settlements with planted ash in Russia or abroad, it will likely cause serious damage, similar to that already observed in Moscow Province or North America.

Key words: *Agrilus planipennis*, ash, Buprestidae, Coleoptera, Emerald ash borer, forest health, forest pest insects, *Fraxinus*, invasive pest

Introduction

Emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is a very aggressive invasive beetle native to Asia. It was accidentally introduced to North America and central Russia in recent years (Haak et al. 2002, Baranchikov et al. 2008, Izhevskiy, Mozolevskaya 2010, Orlova-Bienkowskaja 2013a, 2013b, 2014, 2015, Straw et al. 2013, Herms & McCullough 2014, Volkovitsh, Mozolevskaya 2014, Musolin et al. 2017). The beetle can act as a primary stem pest of species of ash (*Fraxinus*), infesting the lower and middle parts of stems with thick and intermediate bark. Flight of *A. planipennis* occurs mostly in June and a full generation of the buprestid takes one or two years, depending on environmental conditions (Haack et al. 2015, Orlova-Bienkowskaja and Bieńkowski 2016). Susceptibility of species of ash to attack by the beetle is greater in open landscapes (along roads, in parks, etc.) compared to forest (Liu et al. 2003).

In Moscow Province and Moscow City (central Russia), *A. planipennis* has mostly been observed infesting, and quickly killing, 30–60 year old ash trees, including those planted in cities, along boulevards, roads, and in parks and gardens. All dying ash trees had the same sequence of symptoms: wilting began at the top of the tree and spread gradually downwards, until the entire crown was affected.

In European Russia, the first specimens of *A. planipennis* were collected in Moscow City in 2003 (Volkovitsh and Mozolevskaya 2014) and, thus, Moscow Province is thought to be the likely starting point for the invasive European range of this species. The pest easily spread along roads and railway lines, because ash trees are commonly planted along roads and railway lines. Efforts to control *A. planipennis* had been limited and control measures were implemented only recently and only in Moscow parks and boulevards. By 2013, the species was recorded in 11 administrative divisions (provinces) of Russia (Volkovitsh and Mozolevskaya 2014, Orlova-Bienkowskaja and Bieńkowski 2016, Musolin et al. 2017).

In Russia, ash forests are composed of European ash *Fraxinus excelsior* (L.), Caucasian ash *F. angustifolia* (Vahl), Chinese, or Korean, ash *F. chinensis* (Roxburgh), and Manchurian ash *F. mandshurica* (Hance) and they cover less than 0.1% of the total forest area of the country (Musolin et al. 2017). Russian ash forests located in the Russian Far East and in central and southern parts of European Russia cover 402 000 ha and 264 300 ha, respectively (State Forest Registry 2014, Musolin et al. 2017). Ash stands and individual planted trees play important role in urban landscaping and along roads. Green ash *Fraxinus pennsylvanica* Marshall was introduced from North America and now is also widely used for landscaping in cities and in shelterbelts. Emerald ash borer can feed on all ash species, but the pest is much more dangerous to North American and European ash species than to Asian ones (Baranchikov et al. 2014).

In Europe, ash is not a major forest tree species but it does play an important role in urban landscaping. Arrival of Emerald ash borer to Central and Western Europe can cause severe damage and even complete disappearance of ash from forest and urban ecosystems, especially taking into consideration the current problem with ash dieback caused by the pathogenic fungus *Hymenoscyphus fraxineus* (Musolin et al. 2017, Vasaitis and Enderle 2017).

Within European Russia, information is available on the rapid invasive range expansion of *A. planipennis* in western and southern directions (Orlova-Bienkowskaja 2014, 2015, Volkovitsh and Mozolevskaya 2014, Baranchikov et al. 2016a, 2016b), but less is known about the northward spread of the pest, i.e. towards Saint Petersburg and further towards Estonia and Finland.

The main goal of this study was to clarify the current situation with the northern range limit of the Emerald ash borer between Moscow and Saint Petersburg.

Materials and Methods

The most likely route for the Emerald ash borer to spread from central regions of European Russia to Saint Petersburg is the federal highway M10 (*Russia*) that runs between Moscow and Saint Petersburg. This is because the highway has a side planting of *F. excelsior* and *F. pennsylvanica* and numerous ash trees of these two species are planted in urban areas along the highway, whereas the surrounding forests are predominantly coniferous. In order to map the current northern limit of the pest's distribution, planted ash trees were surveyed along the federal highway between Moscow and Saint Petersburg during a two-day trip conducted on July 1–2, 2016 (Fig. 1, Table 1). Fifteen sites were surveyed between Klin town (Moscow Province) and Saint Petersburg. Stands of ash trees (10 trees or more) as well as individual trees were surveyed. Taking into consideration that both *F. excelsior* and *F. pennsylvanica* are susceptible to *A. planipennis* (Baranchikov et al. 2014), we surveyed all ash trees and recorded data on both species together. Dead trees and those displaying such symptoms as dieback or wilting were targeted; however, healthy trees growing nearby without any obvious symptoms of wilting were also examined to record possible early stage infestation. During the survey, factors recorded included: the state of the tree, tree diameter at breast height (1.3 m), the presence of adults and/or larval galleries of jewel beetles (Buprestidae), longhorn beetles (Cerambycidae), bark beetles (Curculionidae: Scolytinae) and other insects under the bark and in wood, the presence of emergence (= exit) holes of beetles and the presence of dead insects under the bark and in wood. Galleries of *A. planipennis* were distinguished from galleries of other *Agrilus* species by D-shaped

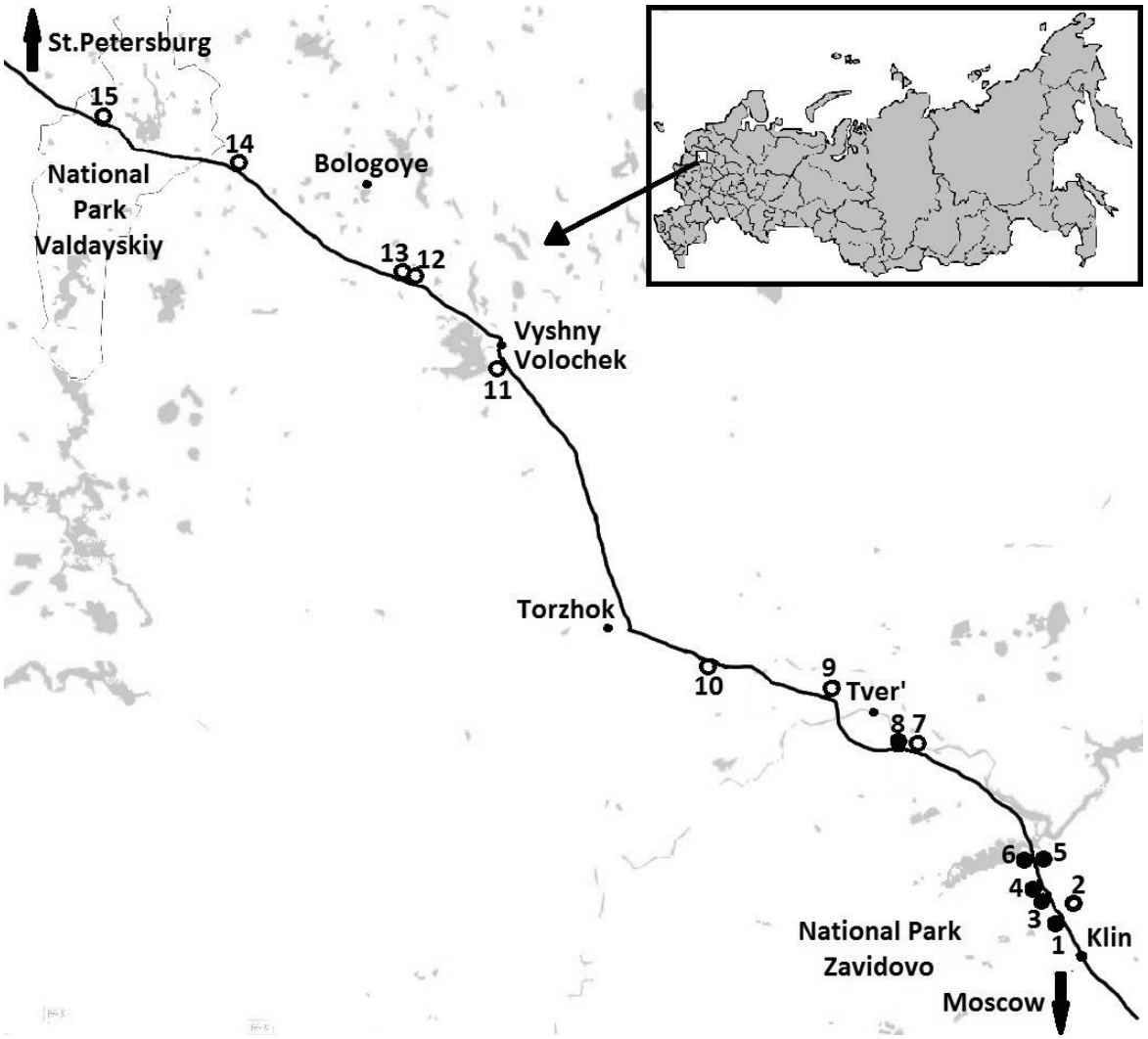

Figure 1. Locations of the inspection sites during the survey of ash trees along federal highway M10 (*Russia*) between Moscow and Saint Petersburg on July 1–2, 2016. Insert is a map of Russia with the region of the survey indicated as a white square. Closed circles indicate sites, at which *Agrilus planipennis* was recorded; open circles indicate sites at which this species was not recorded (see Table 1 for further details)

Figure 2. Typical D-shaped emergence holes of *A. planipennis* on an ash tree. Grid – 1 mm. July 1, 2016 (photo by B. G. Popovichev)

emergence holes and dimensions of the exit holes with the transverse diameter exceeding 3 mm (Fig. 2). This diameter is significantly greater than that of other *Agrilus* species breeding on ash in the region. The GPS coordinates of each site were recorded using a Garmin GPSmap 60Cx navigator (Table 1).

Results and Discussion

The survey began at Klin town (Moscow Province), where *A. planipennis* had already been recorded (Baranchikov 2013, Orlova-Bienkowskaja 2013a, 2013b, 2014, 2015, Volkovitsh and Mozolevskaya 2014). Numerous dead ash trees with galleries of *A. planipennis*

Table 1. Results of the survey of ash trees* along federal highway M10 (*Russia*) between Moscow and Saint Petersburg on July 1–2, 2016

Location №	Location coordinates (and name of a settlement, if appropriate)	Description of ash trees examined	Confirmed presence of *A. planipennis*	Insects recorded
1	56° 27.799′ N; 36° 35.383′ E	2 old dead trees; diameter 24 and 28 cm	Yes	Galleries of buprestids including *A. planipennis* but no adults or larvae of *A. planipennis* collected
2	56° 27.799′ N; 36° 35.383′ E	4 dying trees with dry crowns; diameter 16–28 cm	No	No buprestid galleries
3	56° 27.900′ N; 36° 35.275′ E (Spas-Zaulok)	3 dying trees with dry crowns; diameter 12–26 cm	Yes	Galleries of buprestids including *A. planipennis*, but no adults or larvae collected
4	56° 27.999′ N; 36° 35.160′ E (Spas-Zaulok)	2 dying trees; diameter 32 and 36 cm	Yes	Galleries of buprestids including *A. planipennis*
5	56° 34.765′ N; 36° 29.721′ E	1 dying tree; diameter 26 cm	Yes	1 D-shaped emergence hole of *A. planipennis*
6	56° 35.042′ N; 36° 29.550′ E (Bezborodovo)	13 old dead trees; diameter 36–44 cm	Yes	D-shaped emergence holes; dead adults of *A. planipennis*
7	56° 47.278′ N; 36° 04.909′ E (Pasinkovo)	100+ mostly healthy or weakened trees with only a few dying trees; diameter 16–44 cm	No	No galleries or other signs of infestation
8	56° 47.665′ N; 36° 03.584′ E	2 old dead trees; diameter 28 and 28 cm	Yes	Galleries of buprestids and typical D-shaped emergence holes of *A. planipennis* (Fig. 3)
9	56° 53.203′ N; 35° 46.357′ E (Tver)	hundreds of healthy trees; diameter 16–24 cm	No	No galleries or other signs of infestation
10	56° 58.649′ N; 35° 18.245′ E (Kolesnie Gorki)	100+ mostly weakened trees; diameter 16–24 cm	No	No galleries or other signs of infestation
11	57° 33.205′ N; 34° 34.911′ E (Vyshny Volochek)	100+ mostly healthy and weakened trees; diameter 16–24 cm	No	No galleries or other signs of infestation
12	57° 40.425′N; 34° 19.851′ E	100+ mostly healthy and weakened trees; diameter 16–24 cm	No	No galleries or other signs of infestation
13	57° 40.462′ N; 34° 19.723′ E (Bahmara)	40 alive trees and 4 old dead ash trees; diameter 32–48 cm	No	Entrance holes of *Hylesinus varius* with no galleries under the bark; holes of cerambycids and siricids; 3 dead *H. varius* beetles
14	57° 54.291′ N; 33° 38.188′ E (Endrovo)	100+ trees, mostly healthy and weakened; diameter 16–32 cm	No	No galleries or other signs of infestation
15	58° 00.095′ N; 33° 08.550′ E (Mironegi)	50 weakened and dying trees; diameter 12–28 cm	No	Siricid larvae in wood of two trees

*, both ash species (*F. excelsior* and *F. pennsylvanica*) were surveyed together (see Materials and Methods for details)

were observed around Klin town. Either typical D-shaped emergence holes of *A. planipennis*, its larval galleries or adults were observed at site 1 and sites 3 to 6 (Figs 1 and 2, Table 1). At site 2, four ash trees with heavy wilting symptoms were surveyed, but no galleries or emergence holes of buprestids were observed (Table 1). Dead ash trees with dead or alive basal shoots and with D-shaped emergence holes, characteristic of *A. planipennis*, were recorded up to the southern border of Tver City (close to site 8 at the settlement Pasinkovo, Fig. 3). At the same time, at sites 9–15 hundreds of healthy or weakened ash trees were examined, but no galleries or larvae/adults of *A. planipennis* were ob-

served (Fig. 1, Table 1). Hundreds of ash trees were also surveyed in Tver City (Fig. 1). The great majority of these trees were healthy. Small entrance holes were observed on a few dead ash trees in Tver, which were considered to be the result of unsuccessful infestation attempts by *Hylesinus varius* (F.) (Curculionidae, Scolytinae). Neither emergence holes nor galleries of buprestids were observed in Tver City, despite the recent record of *A. planipennis* in a south-eastern district of the city (Peregudova 2016).

Figure 3. Dead ash trees along the federal highway M10 (*Russia*) between Moscow and Saint Petersburg, near settlement Zavidovo. July 1, 2016 (photo by B. G. Popovichev)

North-west from Tver City, stands of ash trees became infrequent, with distances of about 20–30 km between neighboring groups of trees. There were only individual old ash trees. No ash trees in this area had any symptoms of presence of *A. planipennis*.

A stand of ash trees in the Bakhmara settlement (site 13) consisted of 40 alive and 4 old dead ash trees (Table 1). Entrance holes of *H. varius* were observed on the bark of the dead trees, but no galleries of any beetles were observed under the bark. Three dead individuals of *H. varius* were observed on the bark of these trees. In addition, entrance holes of cerambycids and siricids were also recorded (Table 1).

North of site 12, many planted ash trees were observed, but most of them were healthy without any symptoms of dieback or infestation.

North of sites 14 and 15 (near National Park Valdayskiy), no ash trees were recorded for about 100 km. However, ash might grow inside numerous small settlements along the federal highway. In any case, this gap in the planted ash along the highway might represent a relatively natural barrier slowing the spread of *A. planipennis* further towards the north-west.

The next region along the highway was Leningrad Province where there were fragments of natural ash forests (between Baltic Sea and Ladoga Lake, e.g., in villages Koporje and Orzhitsy) and also many ash trees planted in the historical parks of Saint Petersburg's suburbs (including Alexandrovskiy Park in Pushkin and the park in New Peterhof). In addition, there are many planted ash trees in Saint Petersburg: at Mozhayskaya railway station at Duderhof Heights, Primorskiy Park of Victory, Vyazemsky Boulevard, along large roads at the beginning of Gostilitsy highway, Bolshoy Prospect of Vasilievsky Island and so on. At most of these locations, different symptoms of ash decline have been seen, however, the presence of *A. planipennis* has not been recorded up to date. The major cause of ash death in Leningrad Province is attacks by *Hylesinus crenatus* (F.) and *H. varius*. It is also possible that the initial decline might be caused by the pathogenic fungus *Hymenoscyphus fraxineus*, which was recorded in Saint Petersburg in 2011 (Shabunin et al. 2012, Selikhovkin et al. 2012, Selikhovkin and Musolin, 2013, Musolin et al. 2017).

It should be noted that all ash trees with entrance holes or galleries of *A. planipennis* along the federal highway (Table 1) were most likely infested and critically damaged by this pest in 2015 or earlier. We failed to find fresh infestations of 2016. Rich food potential in the form of numerous ash trees towards the north of Tver City is not used by the buprestid. Our data are in accordance with the observations of Peregudova (2016), who record *A. planipennis* only in the southern parts of Tver City in 2016. Thus, we believe, that the latest advance of the range limit of *A. planipennis* towards north-west took place not later than in 2015, when the species reached south-west districts of Tver City.

Somewhat similar slowing down of the outbreak dynamics was noted in Moscow City and Moscow Province, where after devastating outbreak and expansion of *A. planipennis* in 2003–2014, abundance of the buprestid declined in 2015 due to so far unknown reasons. At the same time, the invasive range expansion actively continues in the southward direction (Baranchikov et al. 2016b).

It might be speculated that the northward range expansion has slowed down because the range had reached its thermal limits. However, it was recently suggested that temperature is not a limiting factor for the Emerald ash borer's range expansion: the highly flexible seasonal cycle of *A. planipennis* might allow this species to move behind the northern limit of ash's continuous range (Afonin et al. 2016). Some slowdown of range expansion might be caused by activity of parasitoids or other so far unidentified factor(s) (Musolin et al. 2017).

Conclusions

Results of the present survey suggest that, for summer 2016, the north-west limit of *A. planipennis* was close to Tver City (about 56° 47' N; 36° 03' E; Fig. 1, Table 1) and this limit has not advanced northward since 2015. Further range expansion of *A. planipennis* may have been limited by low host density north-west of Tver City, rather than by climatic factors. However, if *A. planipennis* can overcome low host density and reach Saint Petersburg or other large cities or small settlements with planted ash in Russia or abroad, it will likely cause serious damage similar to that recorded in Moscow Province or North America in the early 2000s.

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Between Ash Dieback and Emerald Ash Borer: Two Asian Invaders in Russia and the Future of Ash in Europe

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Abstract

Four ash species are native to Russia (*Fraxinus excelsior*, *F. angustifolia*, *F. chinensis*, *F. mandshurica*) while *F. pennsylvanica* was introduced from North America. Ash forests cover 666 300 ha (0.1% of total forest area of Russia) and constitute a volume of 77.91 mln m^3. Ash is widely used in the greening of populated places, around fields and along inter-city roads. We review the current situation with two recent invaders – ash dieback fungus *Hymenoscyphus fraxineus* (Ascomycota) and emerald ash borer *Agrilus planipennis* (Coleoptera). *Hymenoscyphus fraxineus* was likely accidentally introduced from Asia to Western Europe, expanded its range eastward and by 2014 reached Moscow, whereas *A. planipennis* was accidentally introduced from Asia to Moscow Region, expanded its range in all directions but most noticeably southwards. By 2012, *A. planipennis* reached Smolensk Region bordering Belarus, and by 2013, Voronezh Region bordering Ukraine. At least between Belarus and Moscow city, the ranges of invaders overlap. Both species are a threat to the native as well as introduced ash in Europe. We list known records of two invaders in Russia (as of 2016) and for *A. planipennis* also review food plants, seasonal cycle, dispersal, parasitoids and susceptibility of different ash species. We analyze the synergetic effect of two invaders on ash in the area of overlapped ranges and potential losses of biological diversity associated with ash decline and conclude that the future of ash in Europe is precarious. The following directions of actions in Eurasia are proposed: (1) studies of resistance mechanisms to both agents in Asian ash species (first of all, *F. chinensis* and *F. mandshurica*) and hybrids between Asian and European or North-American ash species, (2) studies on selection of resistant ash forms and hybrids (to both agents), (3) controlled introduction of resistant Asian

ash species, (4) slowing down of expansions of *A. planipennis* to Western Europe and *H. fraxineus* within Russia, (5) studies of natural control agents, (6) monitoring of invasions and sanitary condition of ash, and (7) studies on synergetic effect of *H. fraxineus* and *A. planipennis* on ash.

Keywords: *Agrilus planipennis*, ash, ash dieback, Buprestidae, *Chalara fraxinea*, emerald ash borer, forest, forest health, *Fraxinus*, *Hymenoscyphus fraxineus*, pathogen, forest pests, plant resistance

"In terms of invasive forest pests, emerald ash borer may well represent a worst-case scenario."

D. A. Herms and D. G. McCullough (2014)

Introduction – Ash species in Russia

The genus *Fraxinus* L. (ash; Oleaceae) currently consists of 48 accepted tree and shrubby species widely distributed in the tropical and temperate regions of the Northern Hemisphere (Wallander 2012). Three species have wide European distribution: European, or common, ash *Fraxinus excelsior* (L.), Caucasian, or narrow-leaved, ash *F. angustifolia* (Vahl), and South European flowering, or manna, ash *F. ornus* (L.). Among these three species, *F. excelsior* is the most spread ash in Europe (FRAXIGEN 2005, Wallander 2008, 2012).

There are four native ash species in Russia: European ash *F. excelsior*, Caucasian ash *F. angustifolia*, Chinese, or Korean, ash *F. chinensis* (Roxburgh), and Manchurian ash *F. mandshurica* (Hance) (Bulygin 1991, Bulygin and Yarmishko 2000, Usoltsev 2001, Alekseev and Sviazeva 2009). It should be noted that in the earlier Russian literature four other species were sometimes listed that otherwise are considered synonyms, subspecies or variations of the four above mentioned species:

– *F. coriariifolia* (Scheele) as a synonym of *F. exelsior* (Wallander 2012) or subspecies *F. exelsior coriariifolia* (Scheele) Bois (Tree Names 2015),
– *F. oxycarpa* (Willdenow) as a subspecies *F. angustifolia oxycarpa* (Willdenow) Franco et Rocha Afonso (Wallander 2012),
– *F. rhynchophylla* (Hance) as a subspecies *F. chinensis rhynchophylla* (Hance) E. Murray (Wallander 2012),
– *F. densata* (Nakai) as a subspecies *F. chinensis rhynchophylla* (Hance) E. Murray (The Plant List 2013).

As in Western and Central Europe, in Russia, European ash *F. excelsior* is the most widely spread ash species, often dominant in broad-leaved forests in the European Russia and the Caucasus (Bulygin and Yarmishko 2000, Usoltsev 2001). The northern range limit of this species is close to the line Saint Petersburg – Tikhvin – Kostroma – Nizhniy Novgorod (Tkachenko 1952, Timofeev and Dylis 1953). In the East, European ash does not reach the Volga River being limited by Eastern borders of basins of rivers Sura and Khoper (Tkachenko 1952). This species is naturally well regenerated by sprouting. Pure stands, however, are rare. European ash is drought-tolerant and thermophilic and, thus, is often damaged in cold winters. It grows well only on soils rich in lime on overflow lands. The species is widely and actively used for planting of field-protecting belts in central and southern parts of the European Russia.

Caucasian ash *F. angustifolia* is mostly distributed in the Caucasus and in the Crimea. It is smaller in size than *F. excelsior* and has narrower leaves.

Chinese ash *F. chinensis* is distributed in the Russian Far East, in particular in south of Primorye Territory. It often covers dry mountainous slopes (Bulygin and Yarmishko 2000, Usoltsev 2001).

Manchurian ash *F. mandshurica* is up to 35 m in height and up to 1 m in diameter. It is also distributed in the Russian Far East along valleys of rivers, on gentle slopes of hills and rich soils (Tkachenko 1952, Bulygin and Yarmishko 2000).

Green, or red, ash *F. pennsylvanica* Marshall was introduced from North America. It is currently widely used, especially its cold-tolerant forms, for greening in cities and towns in the taiga zone of Russia (Timofeev and Dylis 1953, Bulygin and Yarmishko 2000). One of its numerous forms, namely *F. pennsylvanica* var. *lanceolata* (Borkhausen) Sargent is used for forest cultivation and greening in the steppe and forest-steppe zones in the southern part of the European Russia (Bulygin and Yarmishko 2000) because this form is not only cold- and drought-tolerant but also withstands high soil salinity (Timofeev and Dylis 1953). This ash is often planted in parks, alleys, and along inter-city roads (Bulygin and Yarmishko 2000).

Ash forests cover more than 666 300 ha (about 0.1% of total forest area of Russia) and have volume of about 77.91 mln m^3 (Table 1). These forests usually have a comparatively low stand timber volume (117 m^3 per ha; State Forest Registry 2014).

Distribution of ash forests over the country is far from uniform (Table 1). Such forests are mostly concentrated in two regions of the Russian Federation, namely, central and southern parts of the European Russian (including the Caucasus), and the Russian Far East.

About 264 300 ha of ash forests (i.e., forest lands with ash as a dominant tree species), which is ca. 40% of all Russian ash forests, are concentrated in European Russia. We probably have to add to this number thousands of hectares of *F. pennsylvanica* and to a somewhat lesser degree *F. excelsior* stands in a form of field-protecting belts and along-inter-city-road plantings, the size of which is difficult to estimate. In the north of European Russia and close to

the European ash natural range (in Novgorod, Pskov, and Leningrad Regions), the species covers not more than 300 ha of forest lands, but in cities such as Saint Petersburg (and further south – e.g. in Moscow), ash is often and widely used for greening.

Most of the Russian ash forests and, consequently, ash timber volume is concentrated in the Russian Far East (more than 60% in terms of land and more than 65% of timber volume; Table 1).

In the broad-leaved forests of the steppe-forest zone of the European Russia, *F. excelsior* tends to expand its dominance and the area of ash forests increases. Together with maple *Acer platanoides* L. and linden *Tilia cordata* Miller, ash replaces old oak *Quercus robur* L. stands (Chebotarev and Chebotareva 2015).

Specific plant biomass of ash is 1.5 times higher in the western and southwestern parts of European Russia than in Eastern and Western Europe (Usoltsev 2002). Such forest stands are very good food resources for potential forest pest and pathogen invaders, including those from the Far East of Eurasia.

The disjunction of ash range in Siberia can be easily overcome by existing and sometimes numerous ash trees in form of parks, alleys, field-protecting belts, and along-inter-city-road's plantings in and around populated settlements of different sizes. For example, Green ash *F. pennsylvanica* and Manchurian ash *F. mandshurica* are widely used in Novosibirsk, Barnaul, Tyumen', Yekaterinburg, Krasnoyarsk, Irkutsk, and many other cities and towns. These ash species can well withstand harsh climatic conditions of Siberia.

The vast area occupied by several native and introduced ash species in the European Russia and existing Ural-Siberian "corridor" of planted ash in addition to commercial transfer of resources and materials between different regions of Russia have created good pre-conditions for potential expansion of invasive pests and pathogens associated with ash from the Russian Far East to the European Russia first and then further to Central and Western Europe.

Insects and fungal pathogens associated with ash in Russia

In total, more than 5,000 species of insects associated with forest and urban trees and shrubs are known in Russia (Pests of forest 1955, Maslov et al. 1988, Catalogue 2008, Fauna Europaea 2015). Among them, trophic linkages with ash are known for at least 168 species, what seems to be close to the average number expected for forest tree species. Of these species, 45 can be considered more or less specialized dendrophagous insects and 28 monophagous species of *Fraxinus* (A. V. Selikhovkin, D. L. Muslin, unpublished data). Some of them (mostly, beetles) can strongly affect condition of an ash stand, whereas most of other species colonize only weakened, dying or even dead trees.

Fungal pathogens (and more general – mycobiota) of ash are poorly studied and has never been properly reviewed in Russia. Local, but mostly relatively old literature, lists only 16 largely most common fungal pathogens known to damage ash in Russia and 19 additional species of fungi associated with a wider range of trees including ash (Kuz'michev et al., 2001, 2004, A. V. Selikhovkin, D. L. Musolin, unpublished data). These records need further confirmation.

More species of insects and fungal pathogens associated with ash are known from southern and south-eastern parts of the European Russia as well as from the Far East and Primorye than from other parts of the country. These observations suggest high probability of invasions of pests and pathogens from both west and east.

Ash dieback fungus *Hymenoscyphus fraxineus* in European and Asian parts of Russia

As mentioned above, in Russia species of the genus *Fraxinus* grow both in the European part and Far East. For the European Russia, *F. excelsior* is a native species. It is susceptible to the ash dieback disease caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral et al. (≡ *Chalara fraxinea* T. Kowalski, = *Hymenoscyphus pseudoalbidus* Queloz et al.) (Baral and Bemmann 2014, Baral et al. 2014). The disease quickly kills ash in many countries in Western Europe. It is currently believed that the pathogen was accidentally introduced into Western Europe (likely Poland or one of the Baltic countries) in about 1995 with plant materials (likely, introduction of Manchurian ash *F. mandshurica* (Kowalski 2006, Baral and Bemmann 2014, Drenkhan et al. 2014). As of June 2016, there are several findings of this pathogen in Russia, both in its European and in Asian parts.

In the European part of Russia, *H. fraxineus* was registered for the first time in 2011 in Saint Petersburg, where ash leaf petioles with apothecia of the fungus were found by Dr. T. Kirisits in the Botanical Garden (Dendrarium) of Saint Petersburg State Forestry Technical University and Botanical Garden of Botanical Institute of Russian Academy of Sciences (Gross et al. 2014, McKinney et al. 2014). Despite the pathogen presence, the decline of trees was not obvious at that time and it is not observed now (2016; D. A. Shabunin, unpublished data). The presence of *H. fraxineus* in the Botanical Garden of Saint Petersburg State Forestry Technical University was also confirmed by Drs R. Vasaitis and R. Drenkhan in 2011–2013 (Musolin et al. 2014).

Table 1. Distribution of forest stands (with ash as the main tree species), its area and total growing stock in different subjects (i.e., constituent territories) of the Russian Federation (State Forest Registry 2014)

Administrative divisions of the Russian Federation (by federal district, region [oblast', or province] and territory [krai])	Forest stands with ash as the main tree species	
	Area, thousand ha	Total growing stock, mln m^3
Central Federal District	59.3	9.45
Belgorod Region	9.5	1.04
Bryansk Region	2.2	0.39
Kaluga Region	1.7	0.41
Kursk Region	16.5	2.92
Lipetsk Region	1.3	0.15
Moscow Region	0.3	0.04
Oryol Region	1.6	0.28
Ryazan Region	0.2	0.03
Smolensk Region	1.4	0.26
Tambov Region	0.4	0.05
Tver Region	0.2	0.03
Tula Region	7.9	2.18
Voronezh Region	16.1	1.67
Northwestern Federal District	7.2	1.31
Kaliningrad Region	7.0	1.28
Novgorod Region	0.1	0.01
Pskov Region	0.1	0.02
Southern Federal District	91.2	7.90
Astrakhan Region	8.8	0.68
Krasnodar Krai	31.6	3.80
Republic of Adygeya	6.5	0.95
Republic of Kalmykia	0.4	0.01
Rostov Region	14.8	1.25
Volgograd Region	29.1	1.21
North Caucasian Federal District	46.3	4.64
Chechen Republic	5.3	0.50
Kabardino-Balkarian Republic	3.0	0.36
Karachayevo-Cherkessian Republic	2.8	0.24
Republic of Daghestan	4.6	0.33
Republic of Ingushetia	4.0	0.34
Republic of North Ossetia – Alania	2.5	0.32
Stavropol Krai	24.1	2.55
Privolzhsky (Volga) Federal District	60.3	3.58
Chuvash Republic	2.8	0.15
Nizhni Novgorod Region	0.7	0.08
Orenburg Region	11.2	0.54
Penza Region	4.2	0.57
Republic of Bashkortostan	0.8	0.06
Republic of Marij El	0.3	0.05
Republic of Mordovia	6.2	0.49
Republic of Tatarstan	0.3	0.04
Samara Region	10.2	0.57
Saratov Region	23.5	1.02
Ulyanovsk Region	0.1	0.01

Table 1. (Continued)

Administrative divisions of the Russian Federation (by federal district, region [oblast', or province] and territory [krai])	Forest stands with ash as the main tree species	
	Area, thousand ha	Total growing stock, mln m^3
Far Eastern Federal District	402.0	51.03
Amur Region	0.6	0.06
Jewish Autonomous Region	3.1	0.30
Khabarovsk Territory	85.1	10.27
Primorye Territory	313.2	40.40
Ural Federal District	0.0	0.00
Siberian Federal District	0.0	0.00
RUSSIAN FEDERATION	666.3	77.91

In 2012, ash dieback was recorded in tree stands of the natural monument Dudergof Heights near Saint Petersburg (59°41′52″ N, 30°08′01″ E; Figure 1A; Shabunin et al. 2012). Identification of *H. fraxineus* was confirmed by nuclear DNA sequencing and PCR with a species-specific primer (Figure 2; Shabunin et al. 2012). A mass declining of trees at this site was noted. The fungus caused wilting of branches in adult trees and undergrowth of ash. Different parts of trees were affected to different degree. At some plots, the dead trees without bark were observed in 2012. The entire range of declining trees was seen: from barely affected to declining and dead ones. According to our estimates in Dudergof Heights, tree dying-off process has been going on for at least 5 years. Also, degradation of tissues of a dead tree takes some time. Taking this into consideration, we can assume that the pathogen appeared there no later than in 2005.

In addition to Dudergof Heights, the disease was detected in the State Nature Reserve The Northern Coast of the Neva River Bay near Saint Petersburg in 2013. However, there were no dead ash trees there. In this case, decline of crowns was less than 20%. The ash seed regeneration sized up to 2 m was affected by the disease more than any other age group of ash trees (D. A. Shabunin, unpublished data).

In 2014, Belarusian researchers carried out a survey of ash stands planted along the federal route M1 from the border of Russia with Belarus to Moscow. By that time, in Belarus, more than 54% of ash stands had died most likely because of ash dieback caused by *H. fraxineus* (Zviagintsev et al. 2014), whereas in the European Russia, records of *H. fraxineus* had only been occasional (Shabunin et al. 2012, Baral and Bemmann 2014, Musolin et al. 2014). The survey demonstrated that the road-side alleys were dominated by planted introduced green ash *F. pennsylvanica* and in particular by *F. pennsylvanica* var. *lanceolata*. Native European ash *F. excelsior* was observed only as individual trees on the territory of the park of the Moscow State Forest University (currently named Mytishchi Branch of Bauman Moscow State Technical University; Mytishchi district, Moscow Region) and was only occasionally found in the roadside hedgerows and in forest stands adjacent to the federal route M1 (Zviagintsev et al. 2015).

Visual diagnostics of ash condition identified symptoms of ash dieback everywhere along the federal route M1 from the border of Russia with Belarus to Moscow. The proportion of damaged last-year shoots ranged from 10 to 90%. For precise identification pure cultures of the pathogen were obtained from infected tissues of branches. After two months of cultivation with decreasing temperature, phialides typical for *C. fraxinea* were formed. According to the results of Amplified Fragment Length Polymorphism analysis of pure cultures, species-specific restriction band patterns typical for *H. fraxineus* were identified and it was subsequently confirmed by sequencing (Zviagintsev et al. 2015).

According to these studies, *H. fraxineus* is distributed everywhere along the federal route M1 from the border of Russia with Belarus to Moscow Region and the invasion reached Moscow City at least a few years ago (Zviagintsev et al. 2015). It is interesting to note that the recent studies focused on genus *Hymenoscyphus* in the Moscow Region did not report *Hymenoscyphus albidus* (Gillet) W. Phillips or *H. fraxineus* (Miliokhin and Prokhorov 2007).

A special survey focused on *H. fraxineus* was additionally carried out in the Main Botanical Garden of the Russian Academy of Sciences (Moscow) in 2015. Molecular genetic analysis of nine samples of *F. excelsior* with typical dieback symptoms did not reveal the presence of *H. fraxineus* genetic material. On affected branches and buds five taxa of micromycetes were identified: *Phoma glomerata* (*Corda*) Wollenw. et Hochapfel (dominant, de-

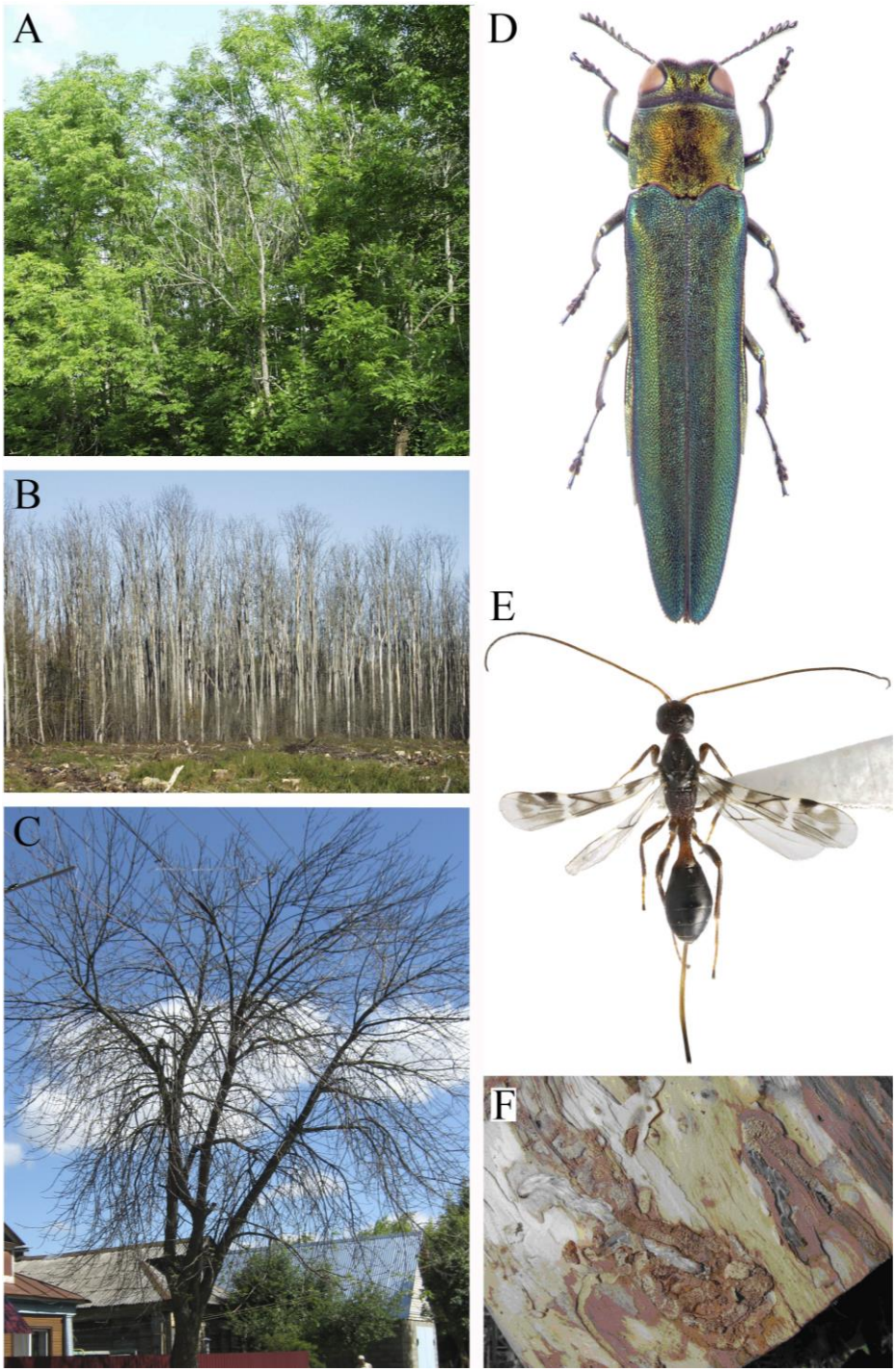

Figure 1. A: The decline of ash in the tree stands of the natural monument Dudergof Heights near Saint Petersburg, 2012 (photo by D. A. Shabunin). **B:** The decline of ash in Kaliningrad Region, 2005. Dead ash tree stand on the background and the sanitary cutting of ash trees on the foreground (photo by D. A. Shabunin). **C:** An ash tree killed by emerald ash borer *Agrilus planipennis*, Voronezh city, Voronezh Region, 2013 (photo by Dr. M. J. Orlova-Bienkowskaja, with permission). **D:** Emerald ash borer *Agrilus planipennis*. The specimen was collected on *F. excelsior* in small town Manihino, Istrinskiy district of Moscow Region on 15 July, 2006 by E. V. Shanhiza (size 10.5 mm; photo by Dr. K. V. Makarov, with permission). **E:** Wasp *Spathius polonicus*, a parasitoid of emerald ash borer *Agrilus planipennis* (photo by Dr. K. V. Makarov, from Orlova-Bienkowskaja and Belokobylskij, 2014, with permission). **F:** Cocoons of emerald ash borer *Agrilus planipennis* parasitized by *Spathius galinae*, under bark of an ash tree, south of Primorye Territory (photo by Dr. G. I. Yurchenko, with permission)

Figure 2. Apothecia of *Hymenoscyphus fraxineus* (from Shabunin et al. 2012; photo by D. A. Shabunin)

tected in 66% of samples), *Cryptococcus* sp., *Eutypa* sp., *Alternaria* sp. and a new undescribed species close to genus *Cryptococcus*, but not *H. fraxineus* (L. G. Seraia, V. B. Zviagintsev, unpublished data).

Recent studies of ash stands in the Voronezh Region revealed their good sanitary condition (Chebotarev and Chebotareva 2015), although outbreak foci of emerald ash borer have been reported in Voronezh city (Orlova-Bienkowskaja 2014a).

Ash forests in the European part of Russia never occupy large areas. Instead, *Fraxinus* spp. are often only secondary mixture species in forest stands. Therefore, decline of ash may often go unnoticed. However, the situation in the Kaliningrad Region was exceptional. In contrast to many other regions, European ash formed pure forest stands there. In 2005, a mass mortality of ash was observed (Figure 1B; Zhigunov et al. 2007). Declining ash stands were surveyed in five forest enterprises. Excavation and examination of root systems of ash trees demonstrated that ash trees with only initial signs of decline in 50–70% were already affected by *Armillaria lutea*. In some cases, the fungus had time to affect 50% of the root system of a particular tree and to form fruit bodies, but the tree crown had to be classified as "having no signs of weakening". The survey showed that the cause of ash trees mortality was the damage to their root system from rot caused by *A. lutea*. An important feature of the disease development is high speed of tree mortality. The forest stands died within one year. There were no trees with different stages of decline, as it is typical for ash dieback disease caused by *H. fraxineus*. Such observation confirms the conclusion that *A. lutea* was the principal cause of ash stands mortality in Kaliningrad Region. The root rot caused by *A. lutea* was detected in all surveyed plots of ash and in the vast majority of plots of oak and even in one plot of aspen. The causes of massive outbreak of *A. lutea* in the region remain unclear.

In 2005, the signs of crown damage by *H. fraxineus* were not found. The current state of ash forests in the Kaliningrad Region is unknown.

In the natural forests of north-western and central regions of the European Russia ash species are not numerous (Table 1). They are represented by small scattered stands or mixed with other tree species. Consequently, natural forest stands cannot be considered sufficiently reliable transmission vector or media of *H. fraxineus* from the western border of Russia to the eastern part of the *F. excelsior*'s range. It is most likely that ash trees widely and actively planted along the inter-city roads and agricultural fields greatly promoted spread of *H. fraxineus* from the western border to Moscow and likely further eastward.

Currently, the expansion of another Far Eastern invader, namely emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is observed in ash stands in Moscow and several nearest administrative divisions (see below). Unfortunately, emerald ash borer disguised the traces of dieback caused by *H. fraxineus* in Moscow.

As mentioned above, two ash species, namely Chinese ash *F. chinensis* and Manchurian ash *F. mandshurica*, are naturally distributed in the Far East region of Russia (Bulygin 1991, Bulygin and Yarmishko 2000, Usoltsev 2001). Our studies demonstrate that none of these two species is susceptible to ash dieback caused by *H. fraxineus* (D. A. Shabunin, unpublished data). We examined several stands of *F. mandshurica* in Terneiskiy district of Primorye Territory and along the route Khabarovsk – Dalnerechensk – Rudnaya Pristan' – Amgu (federal routes A370, A179, A181, and local roads). Even though *H. fraxineus* was detected in the collected samples, the decline of ash was not recorded. On October 14, 2015, we found an anamorph state of the fungus (*Chalara fraxinea*) in the vicinity of Plastun settlement in Terneiskiy district of Primorye Territory (45°06' N, 135°26' E) on fallen leaves of *F. mandshurica* (D. A. Shabunin, unpublished data).

A perfect stage of *H. fraxineus* on *F. mandshurica* was also collected on August 17, 2005 in the Nature Reserve Kedrovaya Pad' (Khasanskiy district of Primorye Territory) by E. S. Popov (Baral and Bemmann 2014).

There is also a record of findings of *H. fraxineus* (as *H. pseudoalbidus*) among seven species of *Hymenoscyphus* in the Far East of Russia by Dr. A. V. Bogacheva (exact location and date are not reported; Bogacheva 2015).

Furthermore, presence of *H. fraxineus* was confirmed by molecular methods in green leaves from crowns of *F. mandshurica* from three locations in Primorye Territory (46°37' N, 134°56' E; 46°37' N, 135°23' E; 44°36' N, 134°52' E). The presence of *H. fraxineus* on non-symptomatic ash suggests a possible role of the species as

an endophyte in its native environment (Marčiulynienė et al. 2013, Cleary et al. 2016).

Thus, the findings of *H. fraxineus* in different locations in the Russian Far East give a basis for a preliminary conclusion that the fungus is widespread in the region and it is likely that the Russian Far East is a part of the species' native range.

Overall, the condition of the ash forests in Russia and the status of *H. fraxineus* are poorly studied and further research is urgently needed.

Morphological features of* Hymenoscyphus fraxineus *collected in Russia

Studies of *H. fraxineus* collected in ash stands in the natural monument Dudergof Heights near Saint Petersburg (Shabunin et al. 2012) revealed that some features of our material differ from those documented in the original description (Baral et al. 2014). In the fungus samples, a heterospory was noted in ascospores and in conidia. This phenomenon has not been recorded before. Shabunin et al. (2012) described the specimens and discussed the revealed differences in ascospores.

In the Russian Far East specimens (from fallen ash leaves), the *C. fraxinea* conidiophores were also observed under conditions of a moist chamber. Conidiophores appeared only on leaf plates. The fungus did not contaminate the rachises and did not form black stroma. On the same leaf rachises, fructifications of different fungi were also seen (*Cladosporium* spp., *Alternaria* sp.). In these specimens, one more type of conidia was observed. The conidia were hyaline elongated, cylindrical, rounded at the upper end and truncated at the lower end, 5.9–6.8 x 1.8–2.2 μm (Figure 3). Thus, heterospory was found in both perfect and imperfect stages of the fungus.

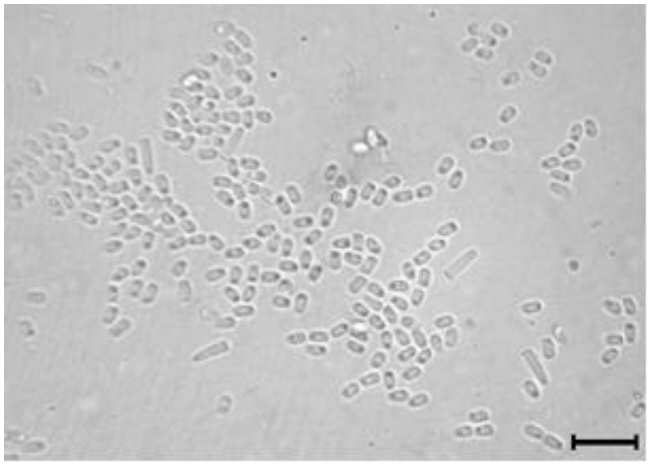

Figure 3. Conidia of *Chalara fraxinea*. The specimen was collected in Terneiskiy district of Primorye Territory (bar = 10 μm; photo by D. A. Shabunin)

Emerald ash borer *Agrilus planipennis*

Whereas currently *H. fraxineus* is spreading from Western Europe to the Central Russia, another extremely important enemy of ash, a beetle emerald ash borer *Agrilus planipennis* (Coleoptera: Buprestidae; Figure 1D), is moving in the opposite direction. This species has demonstrated its devastating potential as a pest of ash: in North America, the beetle was accidentally introduced to the region of the Great Lakes in the late 1980s or early 1990s and already by 2015 the invasive North American range of the pest has covered 24 states of USA and two Canadian provinces. Currently, emerald ash borer is the most damaging forest pest in USA: the annual damage caused by this species exceeds 3 billion US dollars (Haack et al. 2015).

Native and invasive ranges and food plants

Outside Russia the native range of *A. planipennis* covers 9 provinces of North and North-Eastern China, Taiwan, Japan (from Hokkaido to Shikoku), and South Korea (Volkovitsh and Mozolevskaya 2014).

Within its native range, the buprestid is known to feed on different species of ash. In Japan and South Korea, *Juglans mandshurica* Maxim., *Pterocarya rhoifolia* Siebold et Zucc., and *Ulmus davidiana* Planch. were reported as food plants of *A. planipennis*, but these records need to be re-checked because at least in USA in the experimental host range studies emerald ash borer failed to complete development on species of *Ulmus*, *Juglans*, and *Carya* (Anulewicz et al. 2006). Within its invasive (i.e., secondary) ranges in North America and Europe (Russia), the beetle feeds exclusively on different species of ash (Yurchenko et al. 2007, Herms and McCullough 2014, Volkovitsh and Mozolevskaya 2014).

Before the beginning of the current millennium, *A. planipennis* was known to exist only in the territory of the Russian Federation due to limited records. All of them were from the southern part of Primorye Territory where a limited number of specimens were collected in 1935–1999 (Alexeev 1979, Jendek 1994, Yurchenko et al. 2007, Volkovitsh and Mozolevskaya 2014). In 2004, the species was found at the south of Khabarovsk Territory on a waste area ranging from the city of Khabarovsk and its vicinity to the village of Dzonki (100 km from Khabarovsk down along the Amur River; Yurchenko 2010).

Previously, being a very rare species in the Russian Far East, *A. planipennis* was associated exclusively with weakened and dying local Manchurian ash *F. mandshurica* and Chinese ash *F. chinensis*. The harmful activity of the buprestid in that region was first noticed in 2004: *A. planipennis* appeared to be the main factor of dieback of introduced North American Green ash *F. pennsylvanica* on the streets of Vladivostok city (Yurchenko 2010), where rather mature trees with stem diameter of 20–40 cm were

infested. Detailed study of dead trees of introduced North American ash in parks and arboretum in Khabarovsk demonstrated that they had been killed by emerald ash borer within the preceding 5–10 years at the age of 28–35 years (Yurchenko 2010).

In European Russia, first beetles of *A. planipennis* were collected in June of 2003 on streets of Moscow (Volkovitsh and Mozolevskaya 2014). Within the next two years, a few more beetles were found. In 2005, these samples were identified as *A. planipennis* by Dr. A. V. Alexeev (А. В. Алексеев), a leading Russian expert on Buprestidae (Izhevskii and Mozolevskaya 2010). It was finally recognized that emerald ash borer was responsible for the recent intensive ash weakening and dieback all over Moscow city (Baranchikov et al. 2008, Mozolevskaya et al. 2008). Within the following years, the pest was quickly spreading from Moscow in all directions. In 2006, 10 beetles were collected as far as 30 km to the west of the Moscow Ring Highway (Volkovitsh and Mozolevskaya 2014). In 2009, ash trees killed by the buprestid were found in many settlements of Moscow Region; the most westward location of ash dieback was registered in Mozhaisk, 100 km from Moscow (Baranchikov et al. 2010b). In 2010, the beetle was found in Kaluga Region, in 2012 – in Smolensk and Ryazan Regions (Baranchikov and Kurteev 2012, Baranchikov 2013), in 2013 – in Vladimir (Baranchikov 2013), Tver, Tula, Oryol, Voronezh, Yaroslavl, and Tambov Regions (Figures 1C, 4 and 5; Orlova-Bienkowskaja 2014a,b). Thus, currently (as of June 2016), the invasive range of *A. planipennis* covers territory of 11 administrative divisions of the Russian Federation (Figure 6).

Figure 4. Galleries of emerald ash borer *Agrilus planipennis* larvae under bark of a dead ash tree, Pushkino city, Moscow Region, 2016 (photo by D. L. Musolin)

It is important to note that in these regions ash (as an exclusive food plant of emerald ash borer) can be found almost only as artificially planted trees in cities, towns and other populated places, along roads and highways, and in field-protecting tree belts. Native stands of the European ash *F. excelsior* are very rare and limited in size in these regions. Nevertheless, in 2014, an outbreak of *A. planipennis* was for the first time recorded in a natural stand of *F. excelsior* in Moscow Region (Smirnov 2014). The situation will likely become much worse if and when the pest reaches the neighboring Kursk, Belgorod, Rostov, Volgograd, and Saratov Regions, where proportion of ash stands is as high as 3–7% of the total forest area (Figure 6).

Figure 5. Emergence holes of emerald ash borer *Agrilus planipennis* adults on bark of an ash tree, Oryol city, Oryol Region, 2013 (photo by Dr. M. J. Orlova-Bienkowskaja, with permission)

In 2011, *A. planipennis* was mentioned in a review paper as being reported in Sweden (Dobrowolska et al. 2011). The same information was recently repeated in another review (Thomas 2016) and caused a panic in Western Europe (Marshall 2016). However, further investigation revealed that the specimen had been incorrectly identified and, thus, as for 2016, *A. planipennis* is not recorded in Sweden (EPPO 2016, Skovsgaard 2017).

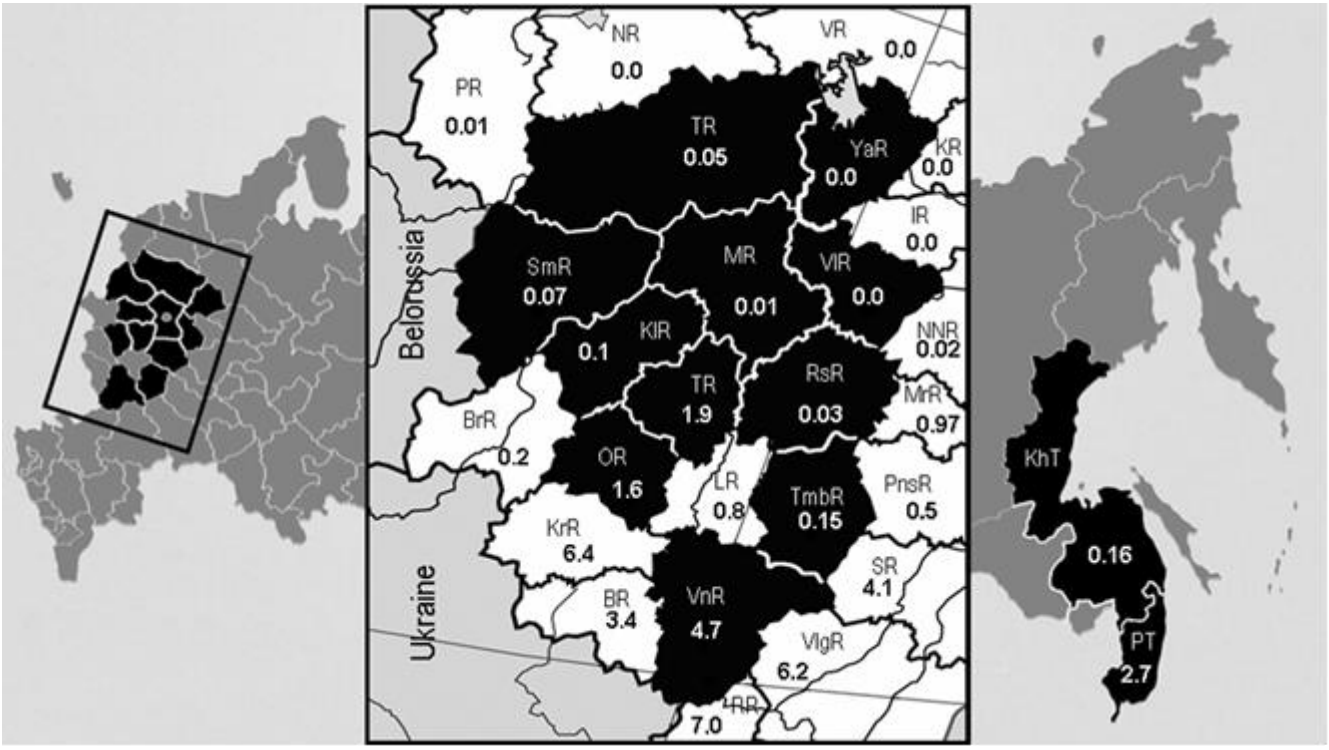

Figure 6. The current distribution of emerald ash borer *Agrilus planipennis* in the Russian Federation. The regions where *A. planipennis* has been recorded are shown in black. Figures refer to the proportion (in percent) of ash forest in the total forest area of each administrative division (State Forest Registry 2014), thus estimating food resources for the monophagous pest. Administrative divisions: In the native range: KhT – Khabarovsk Territory, PT – Primorye Territory; In the invasive range: PR – Pskov Region; NR – Novgorod Region; VR – Vologda Region; TR – Tver Region, YaR – Yaroslavl Region, KR – Kostroma Region, SmR – Smolensk Region, MR – Moscow Region, VlR – Vladimir Region, IR – Ivanovo Region, NNR – Nizhny Novgorod Region, BrR – Bryansk Region, TR – Tula Region, KR – Kaluga Region, RsR – Ryazan Region, MrR – Republic of Mordovia, KrR – Kursk Region, OR – Oryol Region, LR – Lipetsk Region, TmbR – Tambov Region, PnsR – Penza Region, BR – Belgorod Region, VnR – Voronezh Region, SR – Saratov Region, RR – Rostov Region, VlgR – Volgograd Region

Seasonal cycle

In Moscow Region flight of *A. planipennis* starts at the very beginning of June after accumulation of approximately 240 degree-days of the effective temperatures calculated using a tentative lower developmental threshold of 10°C, data from Orlova-Bienkowskaja and Bieńkowski (2016) and local weather data (WeatherArchive.ru 2016). The mean accumulated sum of effective temperatures (240 degree-days) (further confirmed by the timing of the flight beginning in Moscow Region in 2013–2014) is rather close to 250 degree-days of effective temperatures calculated for the population of the species from Michigan, USA (Brown-Rytlewski and Wilson 2004). In Michigan and Canada, the flight period continues for 3–6 weeks (Bauer et al. 2004, Lyons et al. 2004). Females feed on leaves of ash for 10–14 days before copulation (Rodriguez-Saona et al. 2007). Eggs are laid into cavities on the ash bark individually or in small groups and larvae hatch in two weeks at a temperature of 25°C. Larvae build curved and oriented along the stem galleries which widen towards their ends and filled with sawdust (Figure 4). The species has four larval instars. In the cooler climates of the most parts of the invasive range the complete larval development takes two years, whereas in the warmer regions (e.g., Tianjin, China) most individuals complete development within one year (Orlova-Bienkowskaja and Bieńkowski 2016). Larvae spend the 1st winter in their earlier instars and the 2nd winter as pre-pupae in sapwood (if the bark is thin) or in the outer bark (if the bark is thick). In Michigan, USA, pupation starts from mid-April to beginning of May and first new-generation adults emerge in three weeks (Bauer et al. 2004).

Rates of dispersal

To predict expansion of an invasive pest, it is necessary to know its possible spreading rates. There are many approaches and methods of calculation, estimation or modelling of rates of invasive species' range expansion (Tobin et al. 2015). Validation of these methods and confirmation of results require numerous and repeated records over a wide area around the secondary range center during several years. Such data are not yet available for the invasive range

of emerald ash borer in the European Russia. Published data on new records (e.g., Volkovitsh and Mozolevskaya 2014) help us understand the current species' distribution, but characterize mostly activity of researchers rather than that of the invasive pest. The first published regional records of an invader might be well behind the actual year of the first appearance of the pest if the absence of the pest was not carefully checked in the previous years. Thus, Straw et al. (2013) estimated the rate of expansion of *A. planipennis* as 40 km/year based purely on two literature records: the species was reported in 2009 in Mozhaisk (Baranchikov et al. 2010a) and in 2012 in Vyazma (Baranchikov and Kurteev 2012) which is located 130 km from Mozhaisk. However, the cited papers did not prove or even mention that the reported years were the first years when the species appeared in these towns.

In 2014, Y. N. Baranchikov and colleagues carried out a detailed observation of ash stands at the western front of emerald ash borer invasion along the federal route M1 between Mozhaisk and Smolensk (Baranchikov et al. 2016). The westmost mass localization of dead ash trees with emergence holes of the buprestid's adults was found in Vyazma (the same location as two years before that). Samples in the form of stem disk (cross section) were taken from dead ash trees (diameter of 12–14 cm) and healthy ones (from the nearest location where there were no signs of emerald ash borer, 18 km east of Vyazma). Analysis of the samples using dendrochronological cross-dating of dead trees (Cybis Dendrochronology 2014) allowed to conclude that all ash trees died very quickly (there was no decrease of radial increment a year preceding the dieback). It demonstrated that the first ash trees died in 2010 whereas all the rest of the trees in the sampled group (i.e., 90%) died in 2011 (Baranchikov et al. 2016).

Table 2. Estimations of rates of emerald ash borer *Agrilus planipennis* dispersal (within its invasive ranges)

Observation (location)	Speed of distribution	Reference
Reconstruction based on the dendrochronological methods (USA)	a slow phase – 6.5 km/year a fast phase – 20.0 km/year	Siegert et al. 2014
Movement of point of 40% canopy thinning, 2003–2006 (USA)	10.6 km/year	Smitley et al. 2008
Movement of visible canopy thinning (USA)	14.6 km/year	Gandhi et al. 2007
Reconstruction based on the dendrochronological methods (Russia)	10.0–12.0 km/year	Baranchikov et al. 2016

It was recently demonstrated in Michigan, USA, that noticeable damage caused by *A. planipennis* might be recorded only 10–15 years after the first appearance of the invader in a forest stand (Siegert et al. 2014). Thus, we can assume that emerald ash borer was introduced in Moscow circa 1990. It took the invader approximately 20 years to reach Vyazma. Based on these data, it was possible to roughly evaluate a mean rate of the front of invasion spreading westwards as 10–12 km/year. This figure is close to similar estimations previously done in the US (Table 2).

It should be mentioned that the single- or double-row planting of ash trees along roads (highways) is likely to increase the dispersal rates of the invader. Similarly, increase of the dispersion degree of food plants (by protecting of some trees with insecticides or by pre-emptive ash removal) might also increase the probability of long distance dispersal of emerald ash borer (Mercader et al. 2011).

Parasitoids

So far, it is known that in the Russian Far East, *A. planipennis* is naturally controlled by a species of egg parasitoid *Oobius* sp. (Hymenoptera: Encyrtidae) and three species of larval ectoparasitoids: *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), *Atanycolus nigrivensis* Voinovskaja-Krieger (Hymenoptera: Braconidae: Braconinae), and *Spathius galinae* Belokobylskij et Strazanac (Hymenoptera: Braconidae: Doryctinae) (Belokobylskij et al. 2012, Duan et al. 2012). It seems that the last species, namely recently described *S. galinae*, turned out to be the most effective parasitoid of emerald ash borer (Figure 1F). The species is preadapted to the climatic conditions of the northern regions of USA and southern regions of Canada which made it possible to start a release of this braconid in North America in 2015 (Anonymous 2015) and gave some perspective of the use of this agent to control emerald ash borer in both North America and Europe.

It was also noticed that in the invasive ranges, local parasitoids recently started to infest *A. planipennis.* In North America, at least 24 species of local Hymenoptera have been recorded to parasitize emerald ash borer, although so far with a very low efficacy (Taylor et al. 2012). In Europe, braconid *Spathius polonicus* Niezabitowski (Hymenoptera: Braconidae: Doryctinae) turned out to play an important role in the control of emerald ash borer (Figure 1E). This specialized parasitoid feeds only upon buprestids and occurs over a wide range from Kazakhstan to Spain. In some locations in Moscow Region, this parasitoid is reported to be responsible for mortality of 50% of *A. planipennis* larvae (Orlova-Bienkowskaja and Belokobylskij 2014). Two other braconids, namely, *S. exarator* (L.) and S. *rubidus* (Rossi), were also recorded in Europe. These widely polyphagous species feed on insects from different orders and occur over the entire Palaearctic. *Spathius rubidus* occurs in North America as well (Gninenko and Klyukin 2014).

Susceptibility of different ash species to emerald ash borer

Not all ash species are equally susceptible to emerald ash borer. Detailed examination of the ash collection in the Main Botanical Garden of the Russian Academy of Sciences (Moscow) was carried out in 2014 and revealed that only two Asian ash species, namely, Chinese ash *F. chinensis* and Manchurian ash *F. mandshurica*, were resistant to emerald ash borer. Some Asian ash species in the collection died in 2010–2014, but these trees did not have any signs of infestation by *A. planipennis.* At the same time, the pest buprestid killed trees of both North-American (*F. pennsylvanica* and *F. americana*) and European ash species (*F. excelsior*, *F. angustifolia* and *F. ornus*; Figure 7). Death of three European ash species mostly or completely was caused by *A. planipennis*. This finding strongly suggests that the fate of the European ash species will be bleak when the pest spreads further west (Baranchikov et al. 2014).

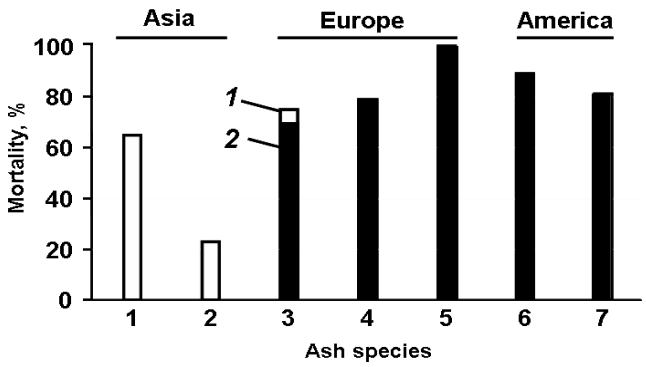

Figure 7. Mortality of Asian, European, and American ash (*Fraxinus*) in the Main Botanical Garden of the Russian Academy of Sciences (Moscow) in 2010–2014. *1* – dead trees without any signs of infestation by *A. planipennis*; *2* – dead trees with galleries and/or emergence holes of *A. planipennis*; 100% – all specimens of each ash species in the collection (see *n* below); continents of origin of ash species are shown above the histogram. Species of *Fraxinus* (number of specimens in the collection in 2010): 1 – *F. chinensis* (*n* = 35), 2 – *F. mandshurica* (*n* = 20), 3 – *F. excelsior* (*n* = 64), 4 – *F. angustifolia* (*n* = 19), 5 – *F. ornus* (*n* = 3), 6 – *F. pennsylvanica* (*n* = 54), 7 – *F. americana* (*n* = 37) (data from Baranchikov et al. 2014)

A similar conclusion was reached by our American colleagues who experimentally studied American, European, and Asian ash species in the plantation in Michigan, USA, in 2010–2014. They revealed that *A. planipennis* killed 95.0–100.0% of three European ash species (*F. excelsior*, *F. angustifolia*, and *F. ornus*) and 35.0–100.0% of five American ash species (*F. pennsylvanica*, *F. nigra*, *F. latifolia*, *F. americana*, and *F. quadrangulata*). At the same time, mortality of Asian *F. mandshurica* and a hybrid *F. nigra* x *mandshurica* was only 20.0% (Herms 2015). The nature of resistance of Asian ash species to emerald ash borer is currently under intensive study, but far from sufficient understanding (Villari et al. 2016).

Ash dieback fungus and emerald ash borer: Overlap of ranges and relationships between two invaders

As demonstrated above, the secondary ranges of ash dieback fungus *H. fraxineus* and emerald ash borer *A. planipennis* have overlapped at least along the federal route M1 from the border of Russia with Belarus to Moscow. We do not know yet how far *H. fraxineus* has penetrated into surrounding forests and residential places, whereas *A. planipennis* have been recorded in numerous cities and towns (Orlova-Bienkowskaja 2014a, b) as well as at least one natural ash stand (Smirnov 2014).

It has been reported that the emerald ash borer readily attacks ash trees that had been weakened by disease, fire or girdling (Herms and McCullough 2014), so trees previously infected by *H. fraxineus* would have a higher risk of being attacked by the pest. Emerald ash borer infests ash trees with diameter more than 5 cm (EPPO 2013) but leaves their epicormic shoots uninfested (Orlova-Bienkowskaja 2014b). In response to infestation, most of the attacked and dying ash trees start to produce massive epicormic shoots that, together with young trees, are not suitable for development of the borer's larvae. It has also been noticed that ash epicormic shoots are more heavily infected by *H. fraxineus* than branches in tree crowns (Zviagintsev et al. 2015). Low resistance of epicormic shoots and young trees to the fungus that can result in fast development of disease and death of a tree has been reported in other regions as well (Pliūra et al. 2011, Schumacher 2011, Yaruk and Zviagintsev 2015). Thus, putting together these observations, we expect that a cumulative effect of two invaders on ash in the zone of overlapped ranges will be devastating for both native and introduced from North America ash species.

It should be mentioned, however, that in basically all populations of all non-Asian ash species, individual trees demonstrate resistance to invaders. Incidence of resistance towards *H. fraxineus* is significantly higher (1.0–5.0%; Klooster et al. 2014) than that to *A. planipennis* (about 0.1%; McKinney et al. 2014). These trees are precious material for selection of resistant ash. It has never been studied whether resistance to the two invaders has anything in common.

Two invaders damaging ash and potential losses of biological diversity

Ash plays an important role in natural communities in different regions of the world. Thus, according to the U.S. National Vegetation Classification, 16 species of *Fraxinus* form or function as important components in

150 plant community types (Wagner and Todd 2015). In the UK, the European ash *F. excelsior* is a part of 61 plant community types and at least 953 species of biota are identified as having some associations with this species (Mitchell et al. 2014).

Reasonably comprehensive estimation of damage that might be caused to local biodiversity by complete loss of ash has never been done. However, in a few countries such damage has been preliminary estimated (Table 3). Thus, extinction of ash in the UK will likely destroy food resources of at least 46 obligatorily or highly associated with ash monophagous insect species, mostly lepidopterans and hemipterans (Table 3; Littlewood et al. 2015). According to two similar studies carried out in USA, mono- and oligophagous insects associated with *Fraxinus* also mostly belong to Coleoptera, Lepidoptera, and Hemiptera (Table 3; Gandhi and Herms 2010, Wagner and Todd 2015). Our list of insects associated with *Fraxinus* in Russia (A. V. Selikhovkin, D. L. Musolin, unpublished data) consists of 168 species and strongly dominated by coleopteran and lepidopteran species (Table 3; polyphagous species are also included). The total number of species is much higher than those reported for the UK and USA (Table 3), but it should be kept in mind that in the different studies, different approaches and procedures were applied.

It should be mentioned that direct effect of emerald ash borer on abundance and diversity of particular species or ecological guilds of biota has never been fully estimated. Some researchers expect that limitation of food resource will directly lead to elimination of phytophagous animals. However, Gandhi and Herms (2010) stressed that elimination of ash in stands would affect diverse guilds of ash-associated arthropods differently. Seed-feeders, folivores, sap-feeders, leaf-miners, and gall-makers will likely experience more or less linear population decline as ash mortality increases. Seed-feeders might suffer first as seed production declines quickly in ash trees infested by *A. planipennis*. On the other hand, when emerald ash borer increases availability of suitable hosts, populations of wood-borers and bark beetles that colonize and utilize declining and dead ash trees will likely initially increase (Gandhi and Herms 2010). This theoretical prediction was recently very well supported by field observations in Russia: European species of ash-associated xylophagous beetles *Agrilus convexicollis* Redtenbacher (Coleoptera: Buprestidae) and *Tetrops starkii* Chevrolat (Coleoptera: Cerambycidae) have noticeably expanded their natural ranges towards east and inhabited stands of *F. pennsylvanica* and *F. excelsior* previously weakened by emerald ash borer (Orlova-Bienkowskaja 2015).

Table 3. Structure of phytophagous insect fauna associated with *Fraxinus* in the UK, USA and Russia

Order	Number of phytophagous insects species associated with ash in different countries (number of species per order and percentage in the total) [1]							
	UK[2]		USA[3]		USA[4]		Russia[5]	
	species	percent	species	percent	species	percent	species	percent
Coleoptera	3	6.5	18	25.7	24	25.8	70	41.6
Diptera	7	15.2	11	15.7	9	9.7	4	2.4
Hemiptera	12	26.1	17	24.3	25	26.9	20	11.9
Hymenoptera	3	6.5	5	7.1	3	3.2	7	4.2
Lepidoptera	18	39.2	19	27.2	32	34.4	62	36.9
Thysanoptera	3	6.5	0	0.0	0	0.0	1	0.6
Orthoptera	0	0.0	0	0.0	0	0.0	4	2.4
Total	46	100.0	70	100.0	93	100.0	168	100.0

[1] – for each country, two insect orders with the highest numbers of species are underlined;
[2] – for UK, data from: Littlewood et al. (2015). Monophagous species (only *Fraxinus* as a food plant) and oligophagous species (*Fraxinus* and 1–3 other genera as food plant) are counted;
[3] – for USA, data from: Gandhi and Herms (2010). Monophagous species (only *Fraxinus* as a food plant) and oligophagous species (*Fraxinus* and 1–3 other genera as food plant) are counted;
[4] – for USA, data from: Wagner and Todd (2015);
[5] – for Russia, data from: A. V. Selikhovkin, D. L. Musolin, unpublished data. Not only monophagous and oligophagous but also polyphagous species are included.

Conclusions: the future of ash in Eurasia - if any

As demonstrated above, two Asian forest invaders quickly spread in Russian ash stands: *H. fraxineus* was likely accidentally introduced first to Western Europe and is now expanding its range eastward, whereas *A. planipennis* was as well accidentally introduced first to Moscow Region and is now expanding its range in all directions but most noticeably southward (where host plant becomes more readily available) and westward. At least between the Republic of Belarus and Moscow (i.e., over Smolensk and Moscow Regions) the ranges of two invaders overlap. Taking into consideration that both invaders are devastating and that they have already almost wiped out European and North American ash species in Western Europe (*H. fraxineus*) and North America (*A. planipennis*), the synergetic effect of the two actors is likely to be fatal to local ash.

As D. A. Herms and D. G. McCullough (2014, p. 23) put it when reviewing the impact of emerald ash borer on trees and people, "the future of the ash resources in North America is precarious". We can say the same about the fate of ash in Europe, twice as much.

Based on the presented review of the current situation between two centers of invasions, we believe that the urgent efforts in Eurasia should be focused on the following issues:

- studies of resistance mechanisms to both agents in Asian ash species (first of all, Chinese ash *F. chinensis* and Manchurian ash *F. mandshurica*) and hybrids between Asian and European or North-American ash species,
- studies on selection of resistant ash forms and hybrids (to both agents),
- controlled introduction of resistant Asian ash species,
- slowing down of expansion rates of emerald ash borer to Western Europe and ash dieback within Russia,
- studies of natural control agents,
- detailed monitoring of invasions and sanitary condition of ash in forest stands, residential places, botanical gardens (arboreta), and road-side plantings, and
- studies on synergetic effect of *H. fraxineus* and *A. planipennis* on ash.

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