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Department of Forest Mycology and Plant Pathology

Studies on the microflora associated with the seeds of European ash (*Fraxinus excelsior*) and the infection biology of the pathogen *Hymenoscyphus pseudoalbidus* causing ash dieback

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Programme: NM012 Forest as Natural Resource – Master's programme
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Abstract

Since the mid-1990's, decline and death of European ash (*Fraxinus excelsior*) has been occurring throughout central, northern and eastern Europe. The causal agent of this destructive disease has been identified as *Chalara fraxinea*, the anamorph of the ascomycete *Hymenoscyphus pseudoalbidus*. Besides the fundamental role of airborne ascospores produced from apothecia on leaf petioles of previous year on the ground in local spread of the disease, the movement of diseased ash material is also a pathway for introducing the pathogen into new areas. The disease on *Fraxinus* spp is concerning for countries outside of the current zone of infestation including North America with the potential pathway for introduction through importation of infected plant material. Currently, the importation of ash plants to the United States for planting is forbidden, although seeds are permitted. Previously *H. pseudoalbidus* was isolated from different parts of wilting or dying European ash trees including stems, branches, shoots, roots, leaves and leaf petioles; however its occurrence in seeds is not known. The first objective of this study was to investigate the presence of *H. pseudoalbidus in* the seed collected from more resistant and susceptible clones of symptomatic *F. excelsior* in southern Sweden and to identify the fungal communities associated with *F. excelsior* seed. This was achieved by using molecular methods including tests with *H. pseudoalbidus* -specific primers and 454- sequencing. The results showed that *H. pseudoalbidus* was detected from the seeds of both more resistant and susceptible clones of *F. excelsior* at equal frequencies (30%) using *H. pseudoalbidus* -specific primer. The most frequently taxa detected from the seeds were *Alternaria infectoria* (93%), *Cladosporium sp.* (74%), *Coniothyrium fuckeli* (72%), *Cryptococcus sp* (93%), *Didymella fabae* (72%), *Phomopsis sp* (80%) and *Phoma sp* (72%). In addition, sequences obtained from larvae found in some damaged seed were identified as the ash shoot moth (*Pseudargyrotoza conwagana).*

Windborne ascospores occurring at highest frequencies in July to mid-August are known to be the main source for initiating new infections on the host. Leaves and petioles are the first infection court for establishment of new host infections. After leaf fall in the autumn, the fungus produces a typical black pseudosclerotial layer on the petiole surface and overwinters inside. Considering the essential role of petioles for spread of the disease, a previous investigation has focused on studying one-year-old leaf petioles for elucidating the life cycle of the disease, though less investigation has been made in fresh petioles of *F. excelsior*.

Hence, to gain better knowledge of the reproduction biology of the pathogen *H. pseudoalbidus*, the second objective of this study was to investigate the genetic structure and population dynamics of *H. pseudoalbidus* in fresh leaf petioles collected from a single symptomatic *F. excelsior* tree in Sweden. DNA extracted from 2 cm length segments of petioles were tested with 13 microsatellite markers. The results confirmed the existence of multiple haplotypes. In addition, the analysis of molecular variance (AMOVA) test and principle coordinate analysis (PCA) confirmed high genetic variability of *H. pseudoalbidus* within individual petioles and low genetic variation among petioles.

1. Introduction

The dieback of European or common ash (*Fraxinus excelsior* L.) *is* a major concern for countries within Europe and beyond where *Fraxinus* spp. are known to occur. The problem was first observed in the mid-1990's in Poland and Lithuania (Przybyl, 2002, Kowalski, 2006). By 2002, large-scale dieback of over 30,000 ha of *F. excelsior* resulted in mortality of approximately 60% of ash stands in Lithuania (Bakys et al., 2009b). Since then, numerous outbreaks have been reported throughout large parts of Europe (Timmermann, 2011). Massive dieback of ash stands has produced critical problems in Denmark, which has led to the concept of not planting more ash trees (Kjær et al., 2012). In Sweden, massive dieback and rapid loss of *F. excelsior* during the last 10 years has resulted in *F. excelsior* now being Red-Listed with the Swedish Species Information centre with vulnerable status at risk for species extinction (http://www.slu.se/en/collaborative-centres-and-projects/artdatabanken/about-us/organization/).

The causal agent of the disease is the ascomycete fungus *H. pseudoalbidus* (anamorph *Chalara fraxinea*) (Kowalski, 2006; Queloz et al., 2010). Ash petioles have an essential role in the epidemiology of the disease. Apothecia of *H. pseudoalbidus* are formed on the pseudosclerotial layers of petioles from the previous year in the leaf litter (Timmermann, 2011), but also sporadically on dead shoots of 1-3 years old ash seedlings (Kowalski and Holdenrieder, 2009). Ascospores are wind-transmitted and initiate new infections on leaves and leaf petioles during the summer (Timmermann, 2011). Due to high ecological, biodiversity and aesthetic values of *F. excelsior*, the Norwegian Food Safety Authority implemented regulations concerning the movement of ash seedlings, propagation material and wood for the purpose of preventing the dissemination of the disease (van Opstal, 2011). Furthermore, since the pathogenicity of *H. pseudoalbidus was* repeatedly confirmed on *Fraxinus* spp. throughout Europe, the fungus was added to the European and Mediterranean Plant Protection Organization (EPPO) Alert List in 2008 (van Opstal, 2011).

Previous studies by Kjaer et al. (2012) and McKinney et al. (2012b) have shown that there is a significant genetic variability among susceptible clones of *F. excelsior* in Denmark, whereby more resistant clones show less symptoms of the disease. This was also supported by Stener (2012) who showed that ash dieback disease among the Swedish populations of *F. excelsior* is strongly genetically controlled and resistance is inheritable. Interestingly, results of all studies suggest that most ash trees are damaged by the disease and rarely some individuals remain relatively healthy.

In the new global trade system, the movement of infected plant material has become a key factor in spreading diseases to new areas. Elmer (2001) described a correlation between the movement of seeds and introducing pathogens to a new environment (Elmer, 2001). For example, movement of *Asparagus officinalis* seeds led to the introduction the plant pathogenic fungus *Fusarium proliferatum* to the United States, Canada and Australia (Elmer, 2001). To date, *H. pseudoalbidus* has been detected in a

variety of different tissues including leaf stalks, stems, leaves and bark of *F. excelsior* (Bakys et al., 2009a*)*, though no investigation has been made with seeds. Thus, the first aim of this study was to investigate the presence of *H. pseudoalbidus* in seeds of *F. excelsior* to determine the potential risk for introducing the pathogen via movement of seeds to new areas.

1.1. The geographical distribution area of the disease

Ash dieback is present throughout the natural distribution range of *F. excelsior* in central, northern and eastern Europe. The disease was first reported in Poland and Lithuania in the mid-1990's (Bakys et al., 2009b). In the years following, the disease spread to affect *Fraxinus* spp. in more than 25 countries throughout Europe (Timmermann, 2011). The disease in U.K and Ireland has most recently been reported. Despite the occurrence of the disease in all parts of Europe, the only country, which is still free from the disease, is Spain. Among the Scandinavian countries, the first observation of the disease was made in Sweden in 2001, and by 2004 the entire distribution of *F. excelsior* was affected by ash dieback disease (Timmermann, 2011).

1.2. Symptoms of the disease

The most obvious symptoms are characterized as: wilting and premature shedding of leaves; necrosis of leaves, buds, leaf stalks, leaflet veins and bark; top and shoot dieback; cankers on shoots, branches, and stems; and sapwood discoloration (Bakys et al., 2009b, Timmermann, 2011). The recent study by Husson et al. (2012) confirmed the presence of *H. pseudoalbidus* on ash logs leads to the discoloration of the root collar and lower stem. Ascospores dispersed in summer occur at higher frequencies from the end of July to mid- August from apothecia fruiting on petioles from the previous year in the leaf litter and initiate new infections on leaves and leaf petioles. Following initial penetration, the fungus spreads into the phloem and xylem of shoots and causes bark cankers and death of the distal part of shoot (Gross et al., 2011, Timmermann, 2011). Since no apothecia are formed on the shoot and stem, or only very rarely as reported by Kowalski and Holdenrieder (2009), it is conceived that shoot and stem infections are dead end for the fungus (Pautasso et al., 2013). Dieback of trees occurs in all age classes, regardless of the site index and the host habitat (forest stands, landscapes, nurseries, urban areas and road sides) (van Opstal, 2011, Bakys et al., 2009a, Schumacher et al., 2010). The occurrence of the disease in young stands may lead to stand mortality in just a few years, whereas in older stands the disease becomes a chronic problem and the vigor of trees will be reduced (Timmermann, 2011).

1.3. The causal agent and life cycle of the disease

Isolations from different symptomatic tissues of *F. excelsior* confirmed that an ascomycete fungus, *Chalara fraxinea* T. Kowalski (Kowalski, 2006) was the causal agent of the disease. Three years later, the study by Kowalski and Holdenrieder (2009) which was based on the culturing of ascospores, morphological comparison and nuclear ribosomal internal transcribed spacer (ITS) sequencing, identified the teleomorph as *Hymenoscyphus albidus*, a saprotroph on ash petioles in the forest litter, known since

1851 in Europe (Kowalski and Holdenrieder, 2009). Subsequently, the study by Queloz et al. (2011) described the causal agent of the disease as a new pathogen based on the DNA sequence of the ITS region, calmodulin gene and translation elongation factor 1-α and also ISSR markers. Apothecia and ascospores of *H. pseudoalbidus* play an important role in the life cycle of the pathogen and dispersal of the disease (Timmermann, 2011). The recent study by Bengtsson et al. (2012) confirmed that the life cycle of *H. pseudoalbidus* is heterothallic, suggesting recombination of the fungus before producing asci and ascospores, and the existence of high gene flow among *H.* pseudoalbidus isolates from Sweden, Austria, Lithuania, Denmark, Hungary, Germany, Poland, Finland, Czech Republic and Norway (Bengtsson et al., 2012) . So far, the life cycle of the disease was only partly known, but the recent study by Gross et al. (2012) described it as follows: Ascospores dispersed from apothecia fruiting on petioles from the previous year in the leaf litter are windborne and initiate new infections on green leaves in summer. Following the leaf penetration of the fungus, an appressoria will be formed and lead to tissue colonization and growth of the fungus into the petiole. After leaf fall, conidia are produced on petioles in the autumn at low temperature. The fungus forms a dark pseudosclerotial layer on the petiole and overwinters inside the petiole. The fertilization of the fungus is accomplished hypothetically by means of conidia acting as spermatia. In the next growing season, new apothecia are formed and initiate new infections on leaves and petioles (Gross et al., 2012) (Fig. 1).

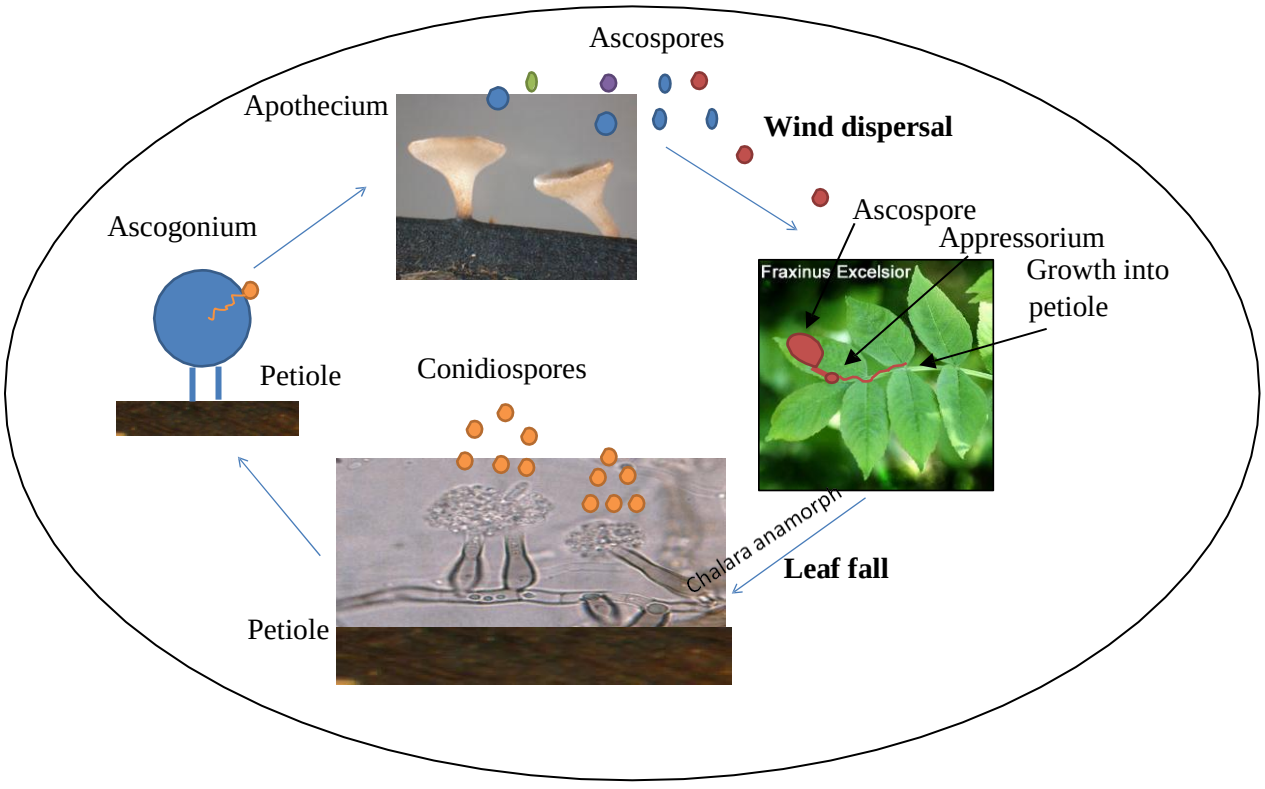


Fig. 1. Hypothetical life cycle of *H. pseudoalbidus*

Due to the vital role of petioles for establishment of new infections on trees another aim of this study was to better understand the mating system, life cycle and genetic structure of *H. pseudoalbidus* within petioles.

1.4. The comparison between *H. albidus* and *H. pseudoalbidus*

Although *H. albidus* and *H. pseudoalbidus* are very similar ecologically and morphologically (Bengtsson et al., 2012), the study by Queloz et al. (2011) confirmed that *H. albidus* is non-pathogenic and *H. pseudoalbidus* is the pathogenic species responsible for causing dieback on ash since apothecia of *H. albidus* were only collected from diseased-free areas, whereas apothecia of *H. pseudoalbidus* were collected only within diseased stands . The recent study by Gross et al. (2012) confirmed the homothallic life cycle of the *H. albidus*. The heterothallic life cycle of *H. pseudoalbidus* will lead to the production of new genotypes and bolster the pathogen's potential to be more virulent (Bengtsson et al., 2012).

1.5. Objectives

To gain a better understanding of some aspects of the infection biology of the fungus, the thesis was comprised of two studies:

I) Microflora associated with *F. excelsior* seeds
II) Population dynamics of the *H. pseudoalbidus* in ash petioles

In study I, the primary objectives were to:

1) Determine the presence of *H. pseudoalbidus in* seeds of *F. excelsior*
2) Detect the fauna and fungal community associated with seed of *F. excelsior* trees affected by ash decline
3) Determine if there is any variation among the fungal communities associated with seeds having morphologically different levels of damage
4) Determine if there are any differences in fungal communities among different genotypes (clones) of *F. excelsior* having different levels of susceptibility to *H. pseudoalbidus.*

The first objective was achieved by testing seed with *H. pseudoalbidus*-specific primers (Johansson et al., 2010), previously developed in the lab at the Department of Forest Mycology and Plant Pathology of Swedish University of Agricultural Sciences in Uppsala which permits a reliable detection of the pathogen directly from infected plant tissue. The latter objectives were achieved using molecular methods based on DNA extraction, PCR and 454 sequencing.

In study II, the main objectives were to:

1) Determine the population dynamics and genetic structure of the fungus in ash petioles
2) Determine if there is any genetic variation among developed lesions on petioles. This was achieved by applying molecular methods, DNA extraction, PCR reactions and microsatellite markers obtained from (Bengtsson et al., 2012, Gross et al., 2012).

2.0. Materials and methods

2.1. Study I: Detection of *H. pseudoalbidus* and fungal community analysis using 454-sequencing

2.1.1. Sample collection and preparation

Seeds from 12 different genotypes (clones) of *F. excelsior* having different levels of susceptibility to ash dieback based on previous field surveys (Stener 2007) were collected from two ash orchards in southern Sweden. Of the selected clones to be used in this study, six were identified as being more resistant to *H. pseudoalbidus* (e.g. Fig 2) and six were susceptible (e.g. Fig 2). While all clones are affected to some degree by *H. pseudoalbidus,* more resistant clones typically show less symptoms of dieback in the crown and fewer bark cankers than susceptible clones.

Fig. 2. Example of a (a) more resistant ash clone and (b) susceptible ash clone at the Trolleholm seed orchard in southern Sweden (Photo by Michelle Cleary)

In the laboratory, seeds were removed from their respective samaras. Based on morphological observations, seed samples were categorized into three groups: damaged, undamaged and suspect. Damaged seeds had an exit hole, which represented evidence of an insect infestation. Undamaged seeds appeared to have a normal brown seed coat. Suspect seeds appeared to have some discoloration on the surface suggesting possible fungal associations (Fig. 3)

Fig. 3. Category of seeds from more resistant and susceptible clones of *F. excelsior* based on their respective morphology (a) Damaged, (b) Undamaged, (c) Suspect

2.1.2. Preparation of samples for DNA extraction

Three replicate samples containing two seeds belonging to each damage category (Damaged, Undamaged and Suspect) were prepared in individual Eppendorf tubes for each of the 12 clones (i.e. 18 seeds per clone, 216 seeds in total).
Each Eppendorf tube contained two seeds, one screw and two nuts (Fig. 4). The tubes containing seeds were kept in a freezer at -20^0 C and then the samples were freeze-dried for 24 hours.

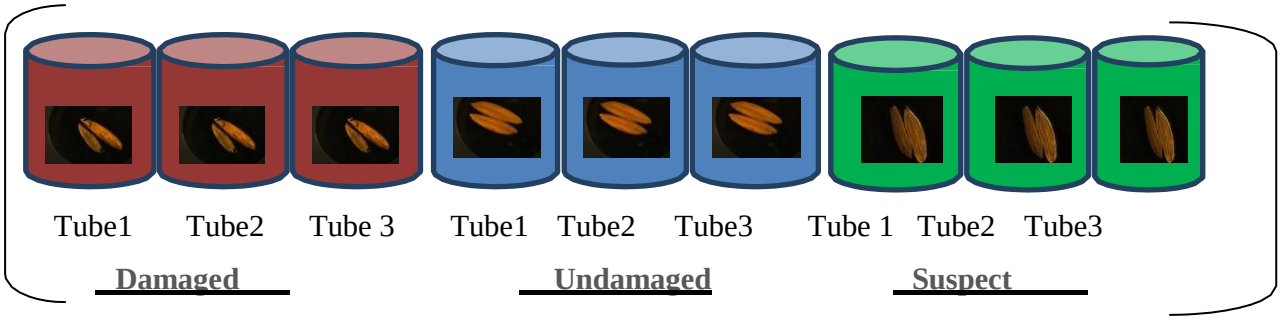


Fig.4. Illustration showing preparation of Eppendorf tubes of an individual seed clone of *F. excelsior* for the three categories (Damaged, Undamaged and Suspect) for the purpose of DNA extraction.

2.1.3. DNA extraction

The freeze-dried samples were homogenized with a fast prep machine. DNA was extracted using the CTAB method (Velegraki et al., 1999). 1mL CTAB buffer (3% Cetyltrimetylammoniumbromide, 2mM EDTA, 2.6 M NACL, 0.15 M TRIS-HCL; pH 8.0) added to each tube, then homogenized and incubated at 65^0 C for 1 h. During the incubation at 65^0 C, the content of tubes was vortexed every 15 min. The tubes were then centrifuged at 10,000 rpm for 5 min. Subsequently the supernatant was removed to a newly marked centrifuge tube. Chloroform (500 µL) was added for cleaning the DNA and the mixture was centrifuged at 10,000 rpm for 7 min. After centrifuging, 500 µL of the supernatant was removed to a newly marked centrifuge tube. For the purpose of precipitating the DNA to a pellet, 2-propanol (1.5 V) was added to the supernatant, centrifuged at 13,000 rpm for 10 min and then the supernatant was poured off in the sink. Subsequently, the pellet was washed with 70% ethanol (500µL), centrifuged at 13,000 rpm for 5 min and the ethanol was then discarded. Next, the pellet was allowed to dry on the bench and then was dissolved in 50-µl milliQ H_2O. Afterwards the DNA-concentration of each sample was measured using a Nanodrop spectrophotometer. Samples were diluted to the concentration 0.5 ng/µl of DNA.

2.1.4. Amplifying DNA using the PCR reaction with ITS primer

PCR was performed using the primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). Total volume of 10 µL PCR reactions consisted of PCR buffer, 0.2 mM dNTPs, 2.75 mM $MgCl_2$, 0.025 U Dream Tag Polymerase, 0.2 µM of each primers and 0.25 ng/µl template DNA.
The reaction was initialized with a denaturation step at 95^0 C for 5 min followed by 35 amplification cycles of denaturation at 94^0 C for 30 s, annealing at 57^0 C for 30 s and extension at 72^0 C for 30 s. The PCR reaction was accomplished by a final extension step at 72^0 C for 7 min (Fig.5).
PCR products were separated by gel electrophoresis on 1% agarose gel (agarose D1) in SB buffer. The gel was loaded with 5µl of each sample and 3µl of a DNA marker and run at 300 V for 20 min. Finally the gel products were visualized under UV light.

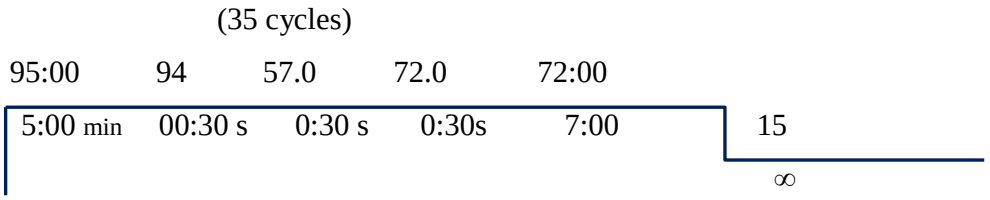


Fig.5. ITS program of PCR reaction used for ITS1 and ITS4 primers

2.1.5. Amplifying DNA using *H. pseudoalbidus* -specific primer

The PCR run was accomplished by applying *H. pseudoalbidus specific* primers: forward (5'-AGCTGGGGAAACCTGACTG-3') and reverse (5'-ACACCGCAAGGACCCTATC-3') (Johansson et al., 2010) and PCR reactions with 10 µL volume containing PCR buffer, 0.2 mM dNTPs, 2.75 mM $MgCl_2$, 0.025 U Dream Tag Polymerase, 0.2 µM of each primers and 0.25 ng/µl template DNA. The reaction started with an initial denaturation step at 95^0 C for 5 min followed by 35 amplification cycles of denaturation at 94^0C for 30 s, annealing at 62^0 C for 1 min and extension at 72^0 C for 30 s. The reaction was finished by an extension step at 72^0 C for 7 min (Fig.6).
To confirm that the PCR reaction based on the *H. pseudoalbidus* -specific primer was valid, one sample containing the mycelium of *H. pseudoalbidus was* used as a positive control in the PCR run.

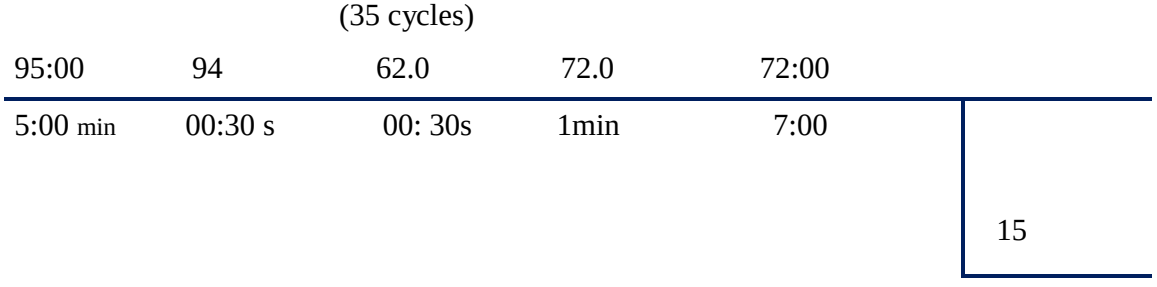


Fig.6. ITS program of PCR reaction used for *H. pseudoalbidus* -specific primers

The mycelium of a known isolate of *H. pseudoalbidus* collected in Sweden was grown on malt extract agar (20g malt extract, 15g agar per one l dH_2O) (Fig. 7). The cultures were kept for 1 month in a dark room at 20^0 C. Mycelia from fresh cultures of H. pseudoalbidus *were* placed in two centrifuge tubes and homogenized with fast prep machine. 1mL of CTAB (3% Cetyltrimethylammoniumbromide, 2mM EDTA, 2.6 M NACL, 0.15 M TRIS-HCL; pH 8.0) was added to remove membrane lipids and incubated at 65^0C for 1 hour. Chloroform (500µl) and 2-propanol were added and the dry pellet was dissolved in 50 µl milliQ H_2O. The DNA concentration was measured using NanoDrop spectrophotometer. DNA Samples were diluted to the concentration 0.5 ng/µl.

The applied method of PCR performance was in accordance with Johansson et al. (2010). The PCR was run with total volume of 10µl containing PCR buffer, 0.2 mM dNTPs, 2.75 mM $MgCl_2$, 0.025 U Dream Tag Polymerase, 0.2 µM of each primers (forward and reverse *H. pseudoalbidus* primers) and 0.25 ng/µl of *H. pseudoalbidus DNA*. The PCR reaction was carried out following the protocol described in Fig. 6.

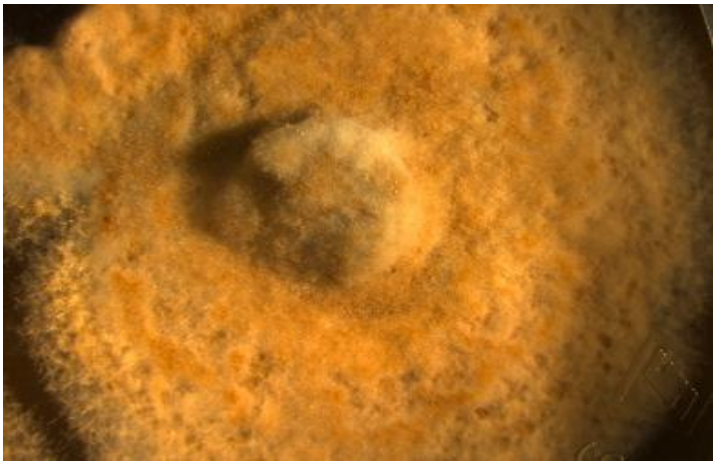


Fig.7. 1-**month-old culture of *Hymenoscyphus pseudoalbidus,* isolate *nf4 tube 55:7:14* Photo: H. Hayatgheibi**

2.1.6. Identification of the insect damage on ash seeds

Morphological examination of damaged seeds by Entomologist Dr. Åke Lindelöw, (Department of Ecology, Swedish University of Agricultural Sciences in Uppsala) suggested the most probable cause of damage was the ash shoot moth, *Pseudargyrotoza conwagana*.

To confirm this, DNA of larvae associated with damaged seeds was extracted according to the previously described methods. DNA amplification was performed using the Lepidoptera specific primer (http://www.ncbi.nlm.nih.gov/nuccore/331255858): Forward (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') and Reverse (5'ATTCAACCATCATAAAGATATTGG-3'). PCR was run with total volume of 50µl containing PCR buffer, 0.2 mM dNTPs, 2.75 mM $MgCl_2$, 0.05 U Dream Tag Polymerase, 0.04 µM of each primers (forward and reverse primers) and 0.25 ng/µl of DNA template. The PCR reaction was performed as follows: initial denaturation step at 94C for 5 min, 35 amplification cycles of denaturation at 94^0 C for 30 s, annealing at 60^0 C for 30 s and extension at 72^0 C for 30 s and a final extension step at 72^0 C for 7 min.
The Gene JET kit was used for purification of PCR products and then was sent to Macrogen (Seoul, Korea) sequencing in both the forward and reverse directions.
Sequences were aligned and edited manually using SeqMan program (DNASTAR) and compared with the sequences available in the GeneBank database (NCBI) using Blast.
H. pseudoalbidus -specific primer was also tested on the DNA of insect larvae to determine existence of the fungus *H. pseudoalbidus* in the larvae. The PCR reaction with a total volume of 10µl was performed following the same method as that on seed (Fig.6).

2.1.7. PCR dilution and cycle validations tests for 454 sequencing

In order to achieve optimal PCR performance, one representative DNA extract from each of three categories (Damaged, Undamaged and Suspect) was chosen as a validation test sample. From each category six replicates were used. A master mix consisting of 10 µM PCR buffer, 2000 µM dNTPs, 25000 µM $MgCl_2$, 5U/ µM Dream Tag Polymerase, 10 µM of the primer ITS-7R and 3 µM of ITS-4 Tag was prepared for 24 DNA samples, including one negative control and five extra samples. The PCR reaction with total a volume of 50 µL (25 µl of master mix and 25 µL of DNA template) was initiated with a denaturation step at 95^0 C for 5 min, then 33 cycles of denaturation at 95^0 C for 30 sec, annealing at 56^0 C for 30 sec and extension at 72^0 C for 30 sec, followed by a final extension step at 72^0 C for 7 min.
The PCR performance was such that for each category of samples (Damaged, Undamaged and Suspect) different cycle numbers (23, 25, 27, 29, 31 and 33) were assigned.
From the results of the cycle test, the following cycle numbers were selected for each category: damaged (31), undamaged (30) and suspect (30).

2.1.8. PCR final products for 454 sequencing

A master mix with the same reagents (excluding ITS-4 Tag) was prepared and the PCR reaction was performed following the same protocol as mentioned above. Each PCR tube contained 20 µL of master mix, 25 µL of DNA template and 5 µL of ITS-4 Tag primer. Different Tag primers were assigned to each sample, i.e. one applied Tag primer was dedicated only for one sample.

PCR products were separated by gel electrophoresis on 1% agarose gel and visualized under UV light. The PCR products were purified using AMPure (Agencourt) to eliminate salts and other undesirable reagents like dNTPs and primers prior to sequencing. The purified DNA was stored at -20^0 C before being sent to LGC (Germany) for 454 sequencing. The sequences were analyzed using the SCATA pyrosequencing pipeline (http://scata.mykopat.slu.se).

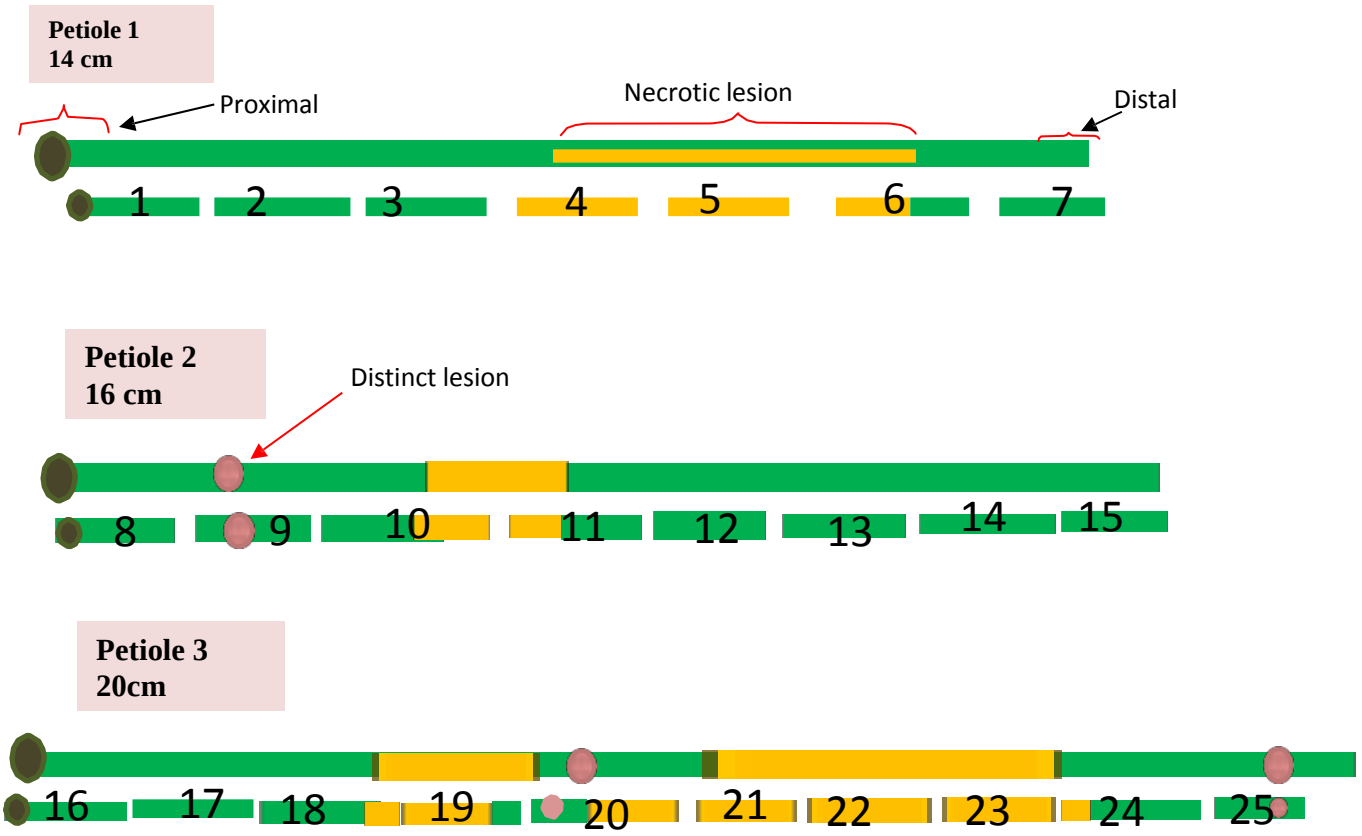
Quality filtering of sequences included removing those sequences that were too short (i.e. <200 bp) and with low read quality. About 83,000 sequences passed the quality control thresholds and were clustered into operational taxonomic units (OTUs). Singletons and doubletons were excluded from the dataset. OTUs were identified in the SCATA program by comparing them with known reference sequences at the Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences and GenBank (NCBI) by BLASTN manually. The ITS homology for delimiting taxa was set to 98-100% for presumed fungal species and 94-97% for determination at the genus level.

2.2. Study II: Population dynamics of *H. pseudoalbidus* in ash petioles

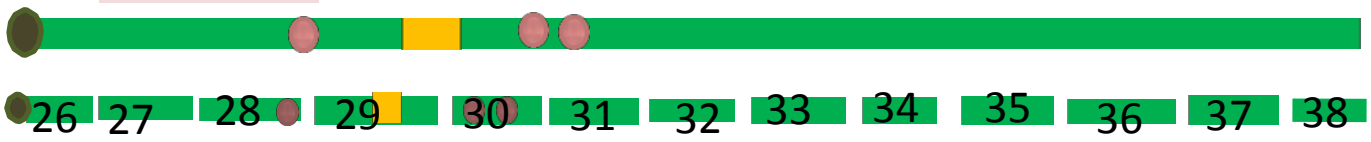
2.2.1. Sample preparation

The samples used in this study were fresh petioles collected from a single symptomatic *F. excelsior* tree in Uppsala, Sweden during autumn 2011. Nine petioles were selected for further study, eight of which showed the presence of necrotic lesions and one without any symptoms. Lesion length was measured for each petiole and then the petiole was cut into 2 cm sections (in total 82 sections from 9 petioles). Each section was surface sterilized in 70% ethanol for 50 s, washed in ddH_2O and then put in an Eppendorf tube containing one screw and two nuts. The tubes were freeze-dried overnight.

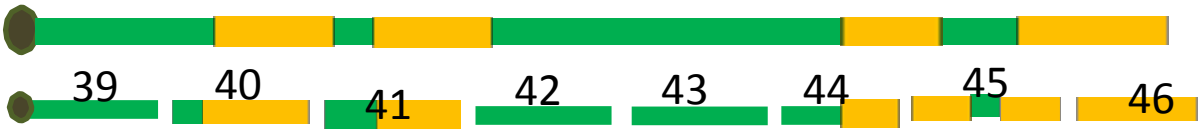
Fig. 8. shows the schematic illustration of the nine petioles selected for this study. For each petiole the distribution of lesions were shown. Necrotic lesions were those lesions with greater than 0.5 cm length on the petiole surface, while distinct lesions were considered separately from necrotic lesions as having a distinct zone of necrosis that was 0.5 cm or less.



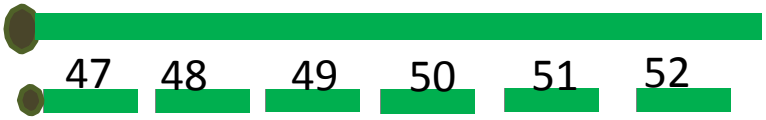
Petiole 4
26 cm



Petiole 5
16cm



Petiole 6
12cm



Petiole 7
16cm

Petiole 8
22cm

Petiole 9
22cm

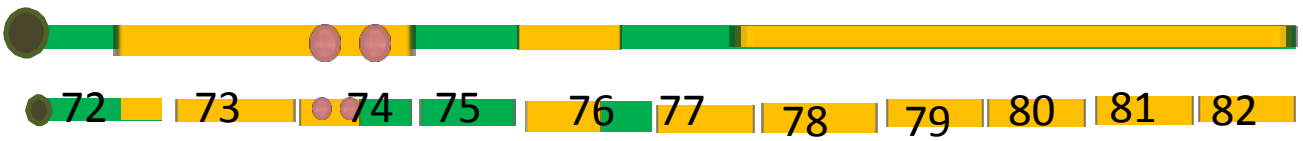


Fig.8. Illustration of the nine petioles selected for this study. On each petiole, the length of lesions is shown. Each petiole was cut into 2-cm length sections and consecutively numbered from the proximal to the distal part of the petiole. Petiole 6 had no lesions and was considered healthy. The green rectangles represent 2-cm sections of petiole with no necrosis. The yellow rectangles indicate the part of petiole with necrosis. The pink circles represent distinct lesions, which are defined as very small (less than 0.5 cm) necrosis lesions.

DNA extraction was performed on freeze-dried samples following the previous methods described in this study. The microsatellite primers *Chafra* (Bengtsson et al., 2012) and *mHp* (Gross et al. 2011) were used (Table 1). The PCR reactions containing 10× PCR buffer, 0.2 mM dNTPs, 0.75 mM $MgCl_2$, 0.05 Dream tag polymerase and 0.2 µM of each primer were performed in a Veriti 96 well Thermal cycler (Applied Biosystems). The DNA concentration used was l ng/µl. The PCR reaction was initialized with 5 min denaturation step at 95^0 C for 5 min followed by 35 amplification cycles of denaturation at 94^0 C for 30s, annealing at 56^0 C for 30s and elongation at 72^0 C for 30s. The reaction ended with a final extension step at 72^0 C for 7 min. The analysis of the fragment was performed by Uppsala Genome Center (http://www.igp.uu.se/Serviceverksamhet/Genomcenter/)

Table 1. Microsatellite loci of *H. pseudoalbidus* with their characteristics and the corresponding allele of *H. albidus* applied in this study (Gross et al., 2011, Bengtsson et al., 2012).

		H. pseudoalbidus		***H. albidus*** **(Allele size)**
Locus	**Primer**	**Primer Sequences (5'-3')**	**Fragment size**	
Chafra 03	F	GGAATTCTGGGTCAGAAAC	179-189-208	179
	R	CAATACGCCAGCACAATACG		
Chafra 04	F	TGAACCTGGCTCTTGCTTTAG	95-101-111	95
	R	AGCGGCAACAAAGAAAAC		
Chafra 09	F	ATGAGGGGATACTGCGATTG	130-136-145	130
	R	GTCAGTAGCAGCCTCGGAAG		
Chafra 13	F	CCCGTCAGATAACAACTTTGC	172-175-179	172
	R	AGCTTGAGCGCCACTTACTC		
Chafra 14	F	TTGATGCGTGATGGTCTTGT	143-151-160	np[a]
	R	CCACGAAGAATTGCCGTTAT		
mHp_060142 (A)	F	TGGCTCTCGAGAAAGAGGAG	164-170	np
	R	TTACTCGATTGATCGTCCTT		
mHp_095481 (C)	F	AGCTTGGTTGTAGCCCAGAG	138_147	128
	R	TATTCCAAGCAGCCGATTCT		
mHp_095478 (D)	F	ATTTTAAACCCCTCGAATAA	230_236	np
	R	ATTTGTGAACCTGCGACTAC		
mHp_067022 (F)	F	CGCACGAAAACGAAAGTCTA	246_250	240
	R	GCCAATGGCAATTACTCGAA		
mHp_077098 (I)	F	TTGATGCGTGATGGTCTTGT	170_173	135
	R	GTAATCCGTCCGGCGTAAA		
mHp_108810 (K)	F	CTCGACTGACTTGCACCTAT	269_279	np
	R	TCACCATAAGACCGACTGAC		
mHp_080495 (L)	F	TCAAGACGAGTTGGGTCACA	148_154	np
	R	GCTTGGCGTTATGGTGAGTT		
mHp_080497 (M)	F	CTTGTTGGACTTGCAAGAGT	245_254	227
	R	GTTGGTAGTGGTGGAGGTAA		

[a] **(No Product)**

2.2.2. Statistics

The Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) was performed to determine genetic distances among populations and subpopulations. The analytical process derived from a squared-matrix compares haplotypes (genotype of a haploid organism) in pair. AMOVA was carried out in anEXCEL-add-inGeneALEx6.2 (http://biology.anu.edu.au/GenAlEx/Welcome.html). The petioles were considered as a population and the sections of petioles were considered as a subpopulation.

The Principal Coordinate Analysis (PCA) was calculated with GeneALEx6.2 software to find the genetic distances among populations (considered as petioles). The procedure of this technique is derived from a distance matrix with multivariate dataset (dimensional) that clusters data on the axes (X, Y) of a graph (http://biology.anu.edu.au/GenAlEx/Welcome.html)

3.0. Results

3.1. Study I:

3.1.2. Incidence of damage in seeds

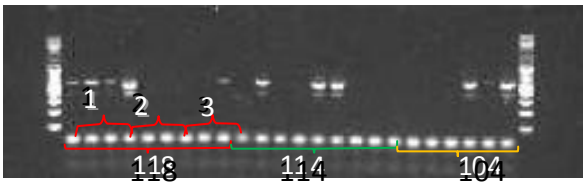
Table 2 shows the incidence of seeds parasitized by the unidentified insect for all susceptible and more resistant clones. The level of parasitization ranged between 6-19% for more resistant clones and between 11-38% for susceptible clones. A BLAST search of the DNA sequence revealed that the larvae of the insect were the ash shoot moths *P. conwagana.* *H. pseudoalbidus* was not associated with any larvae of *P. conwagana.*

Table 2. Level of insect damage in more resistant and susceptible clones of declining *F. excelsior*

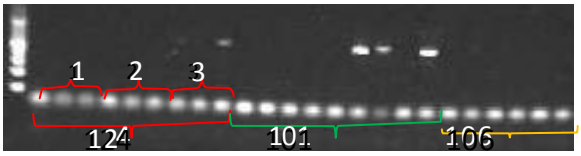
Clone No.	Resistant (R) Susceptible (S)	% Parasitized
S21×1060**104**	R	12
S21×1060**114**	R	10
S21×1060**118**	R	6
S21×1060**122**	R	18
S21×1060**115**	R	19
S21×1060**121**	R	13
S21×1060**108**	S	15
S21×1060**110**	S	38
S21×1060**101**	S	11
S21×1060**106**	S	15
S21×1060**124**	S	20
S21×1060**125**	S	11

3.1.3. *H. pseudoalbidus* specific primer on the seeds

PCR test using the *H. pseudoalbidus* -specific primer resulted in the amplification of a ~ 500bp fragment from both more resistant and susceptible seed clones of *F. excelsior* (Fig. 9).



(a)



(b)

Fig. 9. Gel picture of amplification products visualized under UV light generated by applying *H. pseudoalbidus* -specific primer in seeds of (a) three more resistant clones (118, 114, and 104) and, (b) three susceptible clones (124, 101 and 106). For each clone, three replicates from each category of (1) Undamaged, (2) Damaged and (3) Suspect are shown.

The frequency of seed samples that detected *H. pseudoalbidus* is shown in Table 3. *H. pseudoalbidus* was detected in three of the six more resistant clones and in four of the six susceptible clones. Within a clone, the fungus was also detected in seeds of all damaged classes. The incidence of *H. pseudoalbidus in* more resistant and susceptible clones by damaged class is shown in Table 4.

Table 3. Number of seed samples by clone, which detected *H. pseudoalbidus* using the *H. pseudoalbidus* specific primer

Clone No.	Categories			More Resistant (R) Susceptible (S)
	Undamaged (n=3)	Damaged (n=3)	Suspect (n=3)	
S21×1060**104**	0	3	0	R
S21×1060**114**	1	2	0	R
S21×1060**118**	3	1	1	R
S21×1060**122**	0	0	0	R
S21×1060**115**	0	0	0	R
S21×1060**121**	0	0	0	R
S21×1060**108**	0	0	0	S
S21×1060**110**	1	0	0	S
S21×1060**101**	0	1	2	S
S21×1060**106**	0	0	0	S
S21×1060**124**	0	0	2	S
S21×1060**125**	0	1	0	S

Table 4. Mean incidence of *H. pseudoalbidus* in seed from more resistant and susceptible *F. excelsior* by damaged class using *H. pseudoalbidus*-specific primer

Damage Class	Resistant (n=18)	Susceptible (n=18)
Undamaged	22	6
Damaged	33	11
Suspect	6	22

3.1.4. Fungal community obtained from 454 sequencing analyses

The fungal community detected from seed of susceptible and more resistant ash clones is shown in Table 5. Of the 108 seeds tested, 46 fungal taxa were identified. Of those, 22 were identified to species level, 22 to genus level and 2 remained unidentified.

The most frequently detected fungal species were *Alternaria infectoria* (93%), *Cryptococcus sp* (93%), *Cladosporium sp.* (74%), *Coniothyrium fuckeli* (72%), *Didymella fabae* (72%), *Phomopsis sp* (80%). and *Phoma sp* (72%).

Interestingly, *H. pseudoalbidus* was detected in both the more resistant and susceptible clones at equal frequency (30%). With the exception clone no. 108, all other clones had *H. pseudoalbidus* and the fungus was detected in seed from all damage categories (Table 6).
The incidence of *H. pseudoalbidus* ranged between 11-56% for more resistant clones and 0-56% for the susceptible clones (Table 7). However, there was no clear difference in the incidence of *H. pseudoalbidus* detected among different categories of damage (Table 8).

Table 5. Frequencies of fungal taxa associated with seeds of susceptible and more resistant clones of *F. excelsior*

Fungal taxa	Frequency of detection (%) from more resistant and susceptible genotypes: Resistant (n=54)	Susceptible (n=54)
Ascomycete and anamorphic fungi		
Acremonium strictum	2	4
Alternaria infectoria	93	87
Alternaria sp.	35	28
Aureobasidium sp.	30	35
Bionectria compactiuscula	6	4
Botryotinia fuckeliana	4	0

Chalara fraxinea	30	30
Cladosporium sp.	74	69
Coniothyrium fuckeli	72	65
Coniozyma leucospermi	4	2
Cryptodiaporthe sp.	7	0
Cyclaneusma minus	6	15
Didymella fabae	72	65
Diplodia sp.	6	13
Epicoccum sp.	6	2
Fusarium sp.	24	33
Helotiales sp.	6	0
Leptosphaeria sp.	2	4
Lophodermium pinastri	6	4
Naemacyclus minor	6	15
Neurospora sp.	11	4
Penicillium sp.	2	0
Phoma sp.	72	65
Phomopsis sp.	76	80
Ramularia coleosporii	2	0
Ramularia sp.	13	6
Septoria sp.	43	11
Sphaeropsis sapinea	6	9
Stemphylium sp.	6	2
Sydowia polyspora	9	11
Taphrina sp.	2	4
Unidentified	9	4
Basidiomycetes		
Cryptococcus foliicola	17	35
Cryptococcus laurentii	4	2
Cryptococcus sp.	91	93
Cryptococcus victoriae	4	2
Cryptococcus wieringae	9	22

Dioszegia sp.	7	2
Malassezia globosa	2	13
Malassezia sp.	22	41
Pyrofomes demidoffii	11	9
Resinicium bicolor	0	9
Rhodotorula sp.	20	22
Sporobolomyces roseus	4	6
Sporobolomyces sp.	6	7
Unidentified	2	7
Other species		
Fraxinus excelsior	87	80
Juglans regia	4	0
Pinus sp.	7	7
Trebouxia sp.	4	2
Triticum sp.	13	9

Table 6. Number of seed samples which detected *H. pseudoalbidus* using 454 sequencing

Clone No.	Categories			Resistant(R)/Susceptible (S)
	Undamaged (n=3)	Damaged (n=3)	Suspect (n=3)	
S21×1060**104**	0	3	2	R
S21×1060**114**	2	2	1	R
S21×1060**118**	0	0	1	R
S21×1060**122**	0	1	0	R
S21×1060**115**	0	1	1	R
S21×1060**121**	0	1	1	R
S21×1060**108**	0	0	0	S
S21×1060**110**	2	1	1	S
S21×1060**101**	1	2	2	S
S21×1060**106**	2	0	0	S
S21×1060**124**	1	0	2	S
S21×1060**125**	1	1	0	S

Table 7. Incidence (%) of *H. pseudoalbidus* detected in seed from more resistant and susceptible clones of *F. excelsior* using 454 sequencing

Clone No.	Incidence (%) of *H. pseudoalbidus*	Resistant (R)/ Susceptible (S) (n=9)
S21×1060**104**	56	R
S21×1060**114**	56	R
S21×1060**118**	11	R
S21×1060**122**	11	R
S21×1060**115**	22	R
S21×1060**121**	22	R
S21×1060**108**	0	S
S21×1060**110**	44	S
S21 ×1060**101**	56	S
S21× 1060**106**	22	S
S21×1060**124**	33	S
S21×1060**125**	22	S

Table 8. Mean incidence (%) of *H. pseudoalbidus* in more resistant and susceptible clones of *F. excelsior* seeds by damaged class using 454 sequencing

Damage Class	Resistant (n=18)	Susceptible (n=18)
Undamaged	11	39
Damaged	44	22
Suspect	33	28

Of the 46 detected fungal taxa, 40species were common to both more resistant and susceptible clones (Fig. 10.). Five species were uniquely detected in more resistant clones, which included *Botryotinia fuckeliana, Cryptodiaporthe sp., Helotiales sp., Penicillium sp.,* and *Ramularia coleosporii. ,* while one species (*Resinicium bicolor)* was uniquely associated with susceptible clones of Damaged and Undamaged seeds.

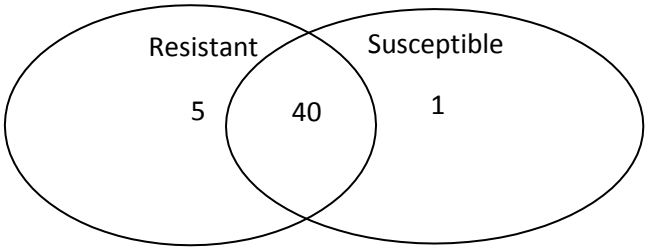


Fig.10. Comparison of number of all taxa detected in more resistant and susceptible *F. excelsior* clones

Forty- five fungal taxa were detected in the more resistant clones (Fig. 11a). Of those, 37 were detected on Undamaged seed, 32 on Damaged seed and 31 on Suspect seed. Twenty-one species were common among the three categories. Seven species were uniquely detected from the Undamaged category including *Cryptococcus laurentii, Dioszegia sp., Helotiales sp., Malassezia globosa, Ramularia coleosporii, Taphrina sp.* and an unknown Basidiomycete. Two species were uniquely associated with the Damaged category (*Penicillium sp* and *Stemphylium sp*.), and two species with the Suspect category (*Acremonium strictum* and *Leptosphaeria sp*) (Fig.11a).

In Susceptible clones (Fig. 11b), a total of 41 species were detected. Of those, 32 were detected from Undamaged seed, 32 from Damaged seed and 32 from Suspect seed. *Dioszegia sp* was uniquely associated with Undamaged seeds. Four species were exclusively associated with the Damaged category including *Coniozyma leucospermi, Cryptococcus laurentii, Epicoccum sp*. and *Pyrofomes demidoffii* and four with the Suspect category (*Acremonium strictum, Cryptococcus victoriae, Lophodermium pinastri* and *Stemphylium sp*.)

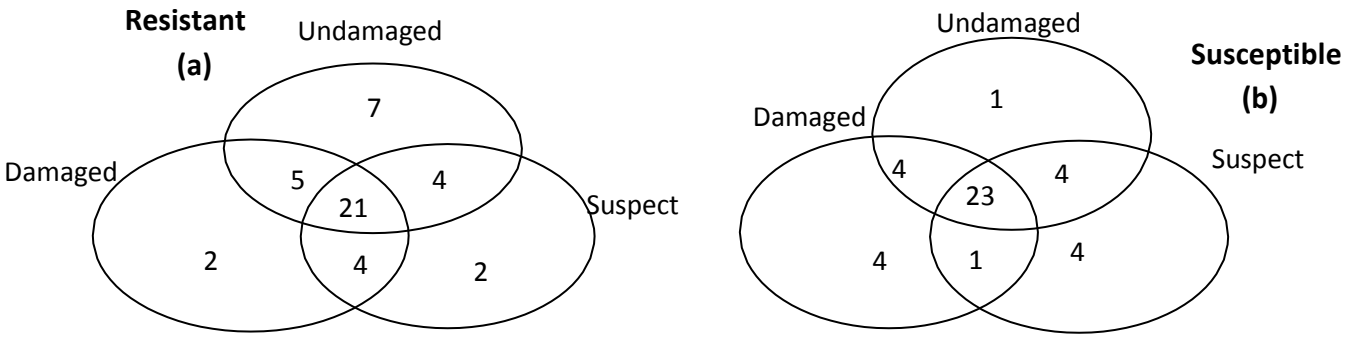


Fig.11. Comparison of number of species detected from seeds of Undamaged, Damaged and Suspect categories of (a) more resistant and (b) susceptible *F. excelsior* clones using 454 sequencing

3.2. Study II

3.2.1. Microsatellite analyses

Petiole 1 was selected as an example to demonstrate results of microsatellite (MS) analysis showing the allele size of each locus amplified by MS primers (Table 9). The top row of the table represents the 13 MS loci used in this study. The amplified fragments (allele size of each locus) are shown by different numbers in each cell. The cells colored red are considered to be mixed samples having double alleles amplified at one locus. The cells colored yellow with -99 are given by default in the GeneAlEx program indicating lack of sufficient data. The fragments (allele size) amplified in each row of the table correspond to the haplotype identified in one sample and the allele frequency (pattern of fragments size) in the entire rows of the table show the number of haplotypes in the petiole. In the given example, petiole 1 was 14 cm in length and was cut into seven 2-cm length sections (samples). It had a necrotic lesion that was 5 cm long within which, three haplotypes (rows 1, 5 and 6) and three mix samples (rows 2, 3 and 4) were found. In this sample, *H. pseudoalbidus* was also found in green sections (i.e. seemingly healthy tissues).

Table 9. The fragments amplified using 13 MS loci applied for one petiole from an infected *F. excelsior* tree

MS Loci →	3	14	13	4	9	A	D	C	I	F	K	M	L
Petiole1 2 cm	208	-99	-99	-99	-99	164	235	146	166	253	269	256	143
	208	143	175,179	111	136,145	164,170	235	-99	166	253	278	256	143
	208	143,151	175,179	111,101	136,145	164,170	235,252	137,146	166	-99	278	256	143
	208	143,151	175,179	111,101	136,145	164,170	235,252	137,146	166	253	278	256	143
	208	151	179	101	136	170	252	137	166	253	278	256	143
	208	143	179	111	136	-99	252	146	166	253	278	256	150
	208	151	179	101	136	170	252	137	166	253	278	256	143

A summary of the microsatellite analysis from all nine petioles is shown in Table 10. Petioles varied considerably with respect to lesion length and haplotype frequency.

Table 10. Length and type of lesions and number of haplotype and mixed samples in petioles

Petiole no.	Length of petioles (cm)	Type of lesions		Total lesion length (cm)	No. of haplotypes	No. of mixed samples
		Necrotic	Distinct[a]			
1	14	1	0	5	3	3
2	16	1	1	1.5	2	0
3	20	2	2	11	3	3
4	26	1	3	2	4	0
5	16	4	0	7	4	3
6	12	0	0	0	0	0
7	16	1	0	3	2	0
8	22	3	0	6	2	6
9	22	3	2	18	2	6

[a] **(Lesions less than 0.5 cm)**

Figure 12 shows the relationship between lesion length and the frequency of genotypes (number of haplotypes and mixed samples) on petioles. It appears that there is a positive correlation between lesion length and number of haplotypes; i.e. longer lesion suggests higher frequency of genotypes and mix samples.

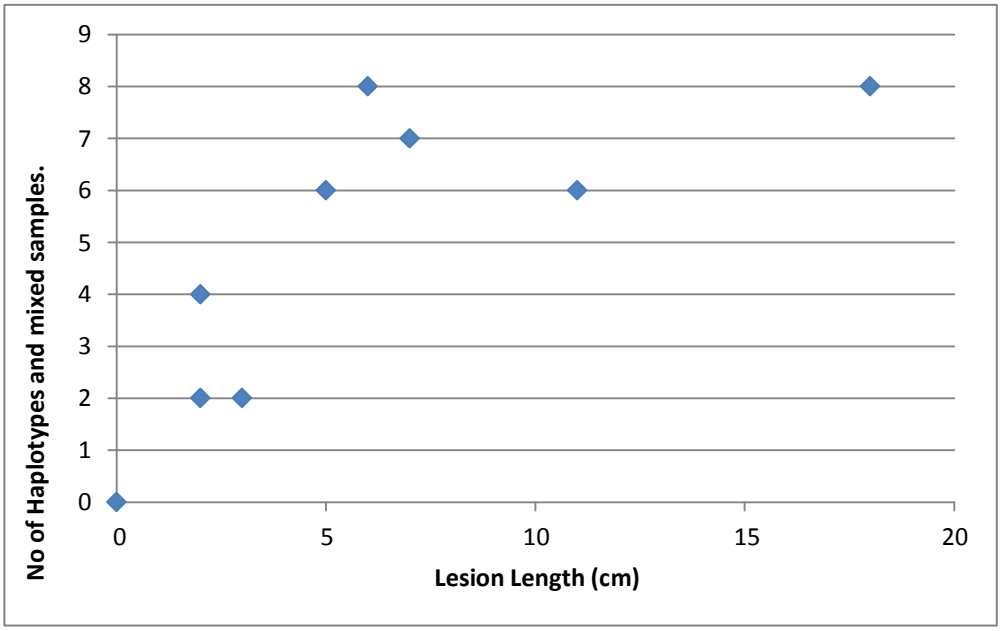


Fig.12 Relationship between lesion length and number of haplotypes plus mixed samples on petioles.

The molecular variance among individual petioles (i.e. among populations in the GeneAlEx program) was 19% while that occurring within an individual petiole, i.e. among the 2-cm length sections (i.e. within population) was 81% (Fig. 13). Consequently, it appears that there is a close genetic structure among petioles, in contrast to high genetic variations occurring within individual petioles.

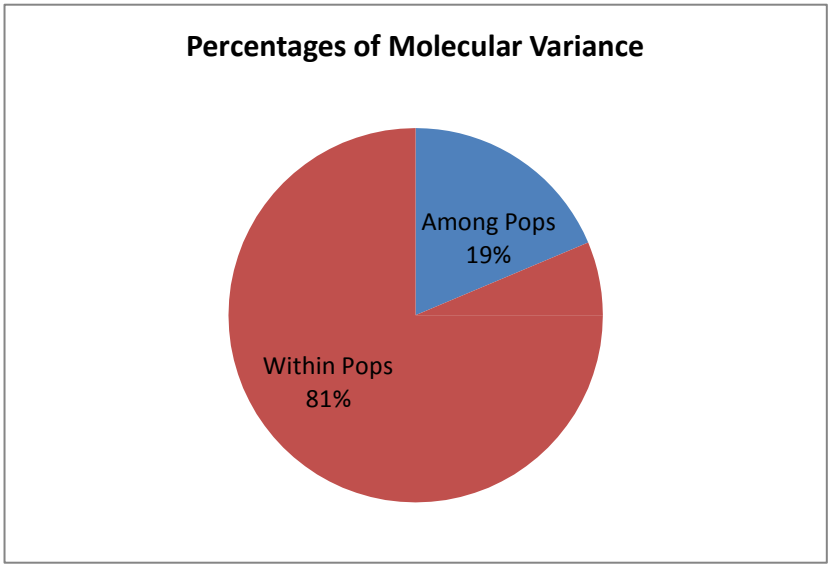


Fig.13 AMOVA test of petioles

The Principal Coordinate Analysis (PCA) test shows the genetic distance patterns of the petioles (considered as population in the GeneAlEx program) (Fig. 14). This figure shows that genetic distance among petioles (populations) is close. The mixed samples obtained from microsatellite analysis are not present in the PCA, thus only considers samples with single haplotypes.
Petiole 6, which is free from *H. pseudoalbidus*, is excluded from the analysis and is not presented in the figure.
Poor DNA quality of some samples (i.e. petiole 2) led to lack of sufficient data in the Gene Alex program, which affected the clustering patterns of genotypes in Fig. 14.

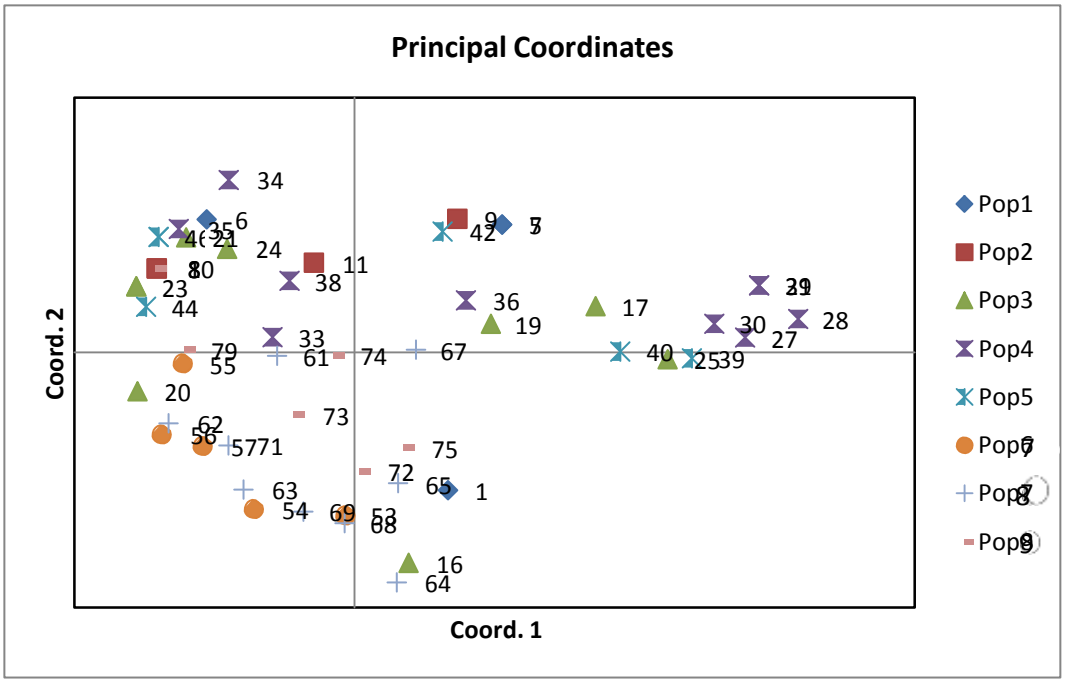


Fig.14. Principal Coordinate Analysis (PCA) of microsatellite products of *F. excelsior* petiole. Petiole number 6 is excluded from the analysis. Petioles 1-9 are shown as populations (pop) 1-9 in the Gene Alex program.

4. Discussion

4.1. Study I

The pathogenicity of the ascomycete fungus *H. pseudoalbidus*, as the major causal agent of ash dieback disease has been frequently confirmed on *Fraxinus* spp. throughout Europe (van Opstal, 2011). Leaves and petioles of *F. excelsior* are known to have a vital role in the life cycle and epidemiology of the disease (Timmermann, 2011). Besides the fundamental role of ash petioles, movement of propagating materials such as infected ash seeds may lead to a high potential risk for introducing the disease to new areas. The main objective of this study was to identify *H. pseudoalbidus* in the seeds considering the intra- and intercontinental trade purposes to non-infested areas such as USA, Canada and Australia. Interestingly, the results of this study confirmed the existence of *H. pseudoalbidus* in the seeds of *F. excelsior* for the first time, using both methods *H. pseudoalbidus*-specific primer and 454-sequencing. The fungus was detected in the seed of both more resistant and susceptible clones. The seeds in this study were not surface sterilized but the recent investigation by Cleary et al. (2012) detected *H. pseudoalbidus* also in surface sterilized seeds. There are different possibilities for infection of *F. excelsior* seeds by the fungus *H. pseudoalbidus*. The first scenario is that the airborne ascospores dispersed from July to August will reside the external tissues and the seed coat within the different phases of seed production and the second scenario is as follows: first penetration will be followed by systemic spread of the fungus into the inner tissues of branches (phloem and xylem) and will continue with movement of the fungus to the petioles, buds, internal contents and embryo of the seeds. In contrast to the expectation and the finding by McKinney et al. (2012b) that perhaps less *H. pseudoalbidus* would be detected less frequently in more resistant clones, the results actually showed the fungus to be detected at higher frequencies in some of the more resistant clones (e.g. clones 104 and 114). Accordingly, the second hypothesis describing the systematic infection of seeds is eliminated and this possibility will remain that such clones with higher incidence of *H. pseudoalbidus* were more exposed to the local spread of ascospores. However, this should be also taken into the consideration that seeds collected from the different clones were not carefully selected from healthy or seemingly healthy branches, since no one really recognized the importance of this. Further studies are needed to fully demonstrate risk for disease transmission via seeds or to prove the existence of the fungus inside the embryo or to show if this is a seed born pathogen. One way in which this could be done would be to germinate the seed and investigate the vertical transmission of the fungus from seed to germinating seedling or to isolate viable propagules of the fungus via isolation. Since the results of this study confirmed the existence of *H. pseudoalbidus* in the seeds of European ash trees regardless of their susceptibility levels to the ash dieback disease, this should be taken into consideration for informing strategies to preserve biodiversity and conservation genetics purposes. Both methods used in this study (i.e., specific primer and 454 sequencing) successfully detected *H. pseudoalbidus* in all seed clones, although the different methods resulted in dissimilar frequency of detection. A higher percentage of the fungus was detected in samples (i.e., clones 106 and 110 of susceptible clones) using 454 sequencing. Though two molecular methods were used, it was not the intent to evaluate their relative efficiency. It is conceivable that 454 sequencing is more sensitive and analyses the communities more deeply with having a high ability to sequence 800,000-1,000,000 fragments with read lengths of 400-500 nucleotides in one run (van der Heijden et al., 2012). For a successful PCR result, there are different prerequisites needed including, a proper binding process of specific primers (forward and

reverse), good DNA concentration and also more amounts of DNA are needed to be observed in the gel document.

The results confirmed that *P. conwagana* is the causal agent parasitizing seeds. This insect belongs to the family Tortricidae and is mostly reported throughout Britain. The adult insect flies during May to July and utilizes *Fraxinus* .spp seeds as their main food source (http://ukmoths.org.uk/show.php? bf=1011). The first morphological observation of the damaged seeds led to a question as to whether the insect could serve as a vector of the fungus. However, the DNA of the insect larvae showed no amplification when tested with the *H. pseudoalbidus* specific primer. The highest frequency of the fungus was detected in Damaged seeds of the more resistant clones using both methods, but this was not the case for Damaged seeds of susceptible clones, thus the presence of *P. conwagana* does not result in higher incidence of *H. pseudoalbidus* in the seeds. It is assumed that it happens by coincidence in such seeds. The higher levels of parasitism by *P. conwagana* and lower incidence of *H. pseudoalbidus* in the Damaged seeds of susceptible clones suggests that occurrence of the disease in the seeds is not correlated with the insect infestation. There are other factors explaining the presence of *H. pseudoalbidus* in seed such as ascospore infection.

The most frequently detected fungi using 454 sequencing were *Alternaria sp, Cladosporium sp., Coniothyrium fuckeli, Didymella fabae, Phomopsis sp., H. pseudoalbidus and Phoma sp.* These species were also common among the fungi isolated from shoots of declining *F. excelsior* in Sweden (Bakys et al., 2009b). Though in the study by Bakys et al. (2009b), pathogenicity tests demonstrated that *H. pseudoalbidus* was the most pathogenic and induced similar necrotic symptoms on stems of *F. excelsior.* The present study though identified the Basidiomycete *Cryptococcus sp*., which is a yeast (Chang et al., 2013) at a high frequency in more resistant clones (91%) and susceptible clones (93%) that was not included in Bakys et al. (2009b). *Cryptococcus sp*., *Aureobasidium sp*, a black yeast-like fungus (Chi et al., 2009), *Diplodia sp.*, *Fusarium sp., Phomopsis sp.*, which are pathogen (Guerrero and Perez, 2013, Li et al., 2013, Sun et al., 2013) and *Phoma sp*., an endophyte (Zhang et al., 2013), detected in this study were common among the fungi detected from the seeds of *F. excelsior* using Sanger sequencing in the study by Cleary et. al. (2012) and the fungal taxa detected from wood, bark and bud samples of *F. excelsior* in New Zealand (Chen 2011). *H. pseudoalbidus* and *Septoria sp*. detected in the seeds of present study were also detected in ash seeds in the study by Cleary et al. (2012). *Resinicium bicolor*, a common white rot Basidiomycete (Holmer, 1997), was uniquely detected in the Damaged and Suspect category of susceptible clones. It is assumed that airborne spores of the fungus caused contamination of the seeds, since the seeds were collected from the tree. It is likely that inoculum of *R. bicolor* was present in the surrounding forest. *Acremonium strictum*, belongs to the group of white or pink moulds (Tsutsui et al., 2013) , was the only species detected in the Suspect category of both more resistant and susceptible clones, which could possibly explain the existence of black discoloration on such seeds. *Botryotinia fuckeliana, Cryptodiaporthe sp., Helotiales sp., Penicillium sp.* and *Ramularia coleosporii* were uniquely identified in more resistant clones. *Botryotinia fuckeliana, Cryptodiaporthe sp* and *Penicillium sp.* were isolated in the study by Bakys et al. (2009b). *Ramularia sp.* and *Helotiales sp.* were also isolated consistently from different tissues of *F. excelsior* in the study by Bakys et al. (2009a). The Basidiomycete *Dioszegia sp.* detected in the present study has also been previously detected from the buds of *F. excelsior* (Chen 2011). Detection of plant taxa, e.g. *Juglans regia*, *Pinus* sp. in the seeds may likely occur from pollen of neighboring trees. The findings from the present study in comparison to the results of

other studies (Cleary et al. 2012, Bakys et al. 2009 a,b, Chen 2011) suggest that the most detected fungal species in this study, including *Botryotinia fuckeliana, Cryptodiaporthe sp., Helotiales sp., Penicillium sp., Ramularia coleosporii, Cryptococcus sp., Alternaria sp, Cladosporium sp., Coniothyrium fuckeli, Didymella fabae, Phomopsis sp., H. pseudoalbidus and Phoma sp.,* are consistently dominating *F. excelsior* tissues (Bakys et al. 2009 a, b). More studies are needed to analyze all other fungal species detected in the seeds of the present study and more investigations should be considered to determine their interaction with *H. pseudoalbidus.* For other taxa uniquely detected in this study i.e. *Acremonium strictum, Cyclaneusma minus, Lophodermium pinastri*, which is an endophyte specific to pine trees (Sieber, 2007), *Naemacyclus minor* and *Rhodotorula sp.* it is probable that infection on such species neighboring *F. excelsior* trees caused infection of ash seeds.

4.2. Study II

Previous population studies of *H. pseudoalbidus*, isolated from fresh cultures and one-year-old leaf petioles of *F. excelsior*, confirmed the outcrossing heterothallic life cycle and a high gene flow of the fungus (Gross et al., 2012, Bengtsson et al., 2012). The present study investigated the genetic structure of *H. pseudoalbidus* in nine fresh petioles of a single symptomatic *F. excelsior* tree. Eight of selected petioles showed the presence of necrotic lesions and one without any symptoms. The result of microsatellite analysis revealed that there is a positive correlation between lesion length and the number of haplotypes and mixed samples. There was no *H. pseudoalbidus* detected in the only asymptomatic petiole of this study (petiole 6). In both petiole 7 and petiole 2 with 3 cm and 1.5 cm lesion lengths, respectively, two haplotypes were amplified. Petiole 4, with similar lesion length as petiole 7, instead had four haplotypes amplified; thought in this example, the petiole had three distinct lesions (lesions with less than 0.5 cm length) on its surface (Table. 9). Necrotic lesions approximately 0.5 cm in length were considered distinct lesions in this study. For such samples containing distinct lesions; it was assumed amplification of only one allele per locus. This assumption was proven in that none of the sections that contained a distinct lesion produced double alleles per loci (mixed samples). This was the case for petiole number 4 whereby the three distinct lesions did not produce mixed samples (genotypes). Amplification of one allele in distinct lesions can be described as mycelium infections in distinct lesions. The other sections of this petiole, which appeared healthy (e.g. green tissue), however, produced two haplotypes. This finding indicates existence of several genotypes in necrotic lesions and also in green tissues of symptomatic petioles and confirms sexual outcrossing of the fungus and ascospore infections in such parts. The remaining petioles investigated in this study, i.e. petioles 1, 3, 5, 8 and 9 comprised of longer lesion lengths and few distinct lesions tended to produce more haplotypes and mix samples. The results of this study did not identify any fragments of *H. albidus*, which may imply that this saprotroph did not inhabit the fresh petioles applied in this study. The other possibility for the absence of *H. albidus* is that the detection of this species has become a rare occurrence these days due to being displaced by the rapid expansion of the *H. pseudoalbidus* (McKinney et al., 2012a) . The AMOVA test (Fig. 13) revealed a considerable genetic variation of the fungus within petioles (i.e. among the 2-cm length sections of individual petioles) and low genetic variation among petioles. Considering the recent investigation by Gross et al. (2012), multiple infections through dispersed ascospores of the fungus lead to the occurrence of multiple genotypes. Low genetic variation among petioles applied in this study despite to the small sample size agrees with the results of other studies indicating that *H. pseudoalbidus* is an invasive pathogen that has a low geographical genetic structure and existence of a high genetic variation among sub-populations

(within petioles in this study) (Rytkonen et al., 2011, Gross et al., 2011, Bengtsson et al., 2012).The PCA results (Fig. 14) showed poor allelic structure of *H. pseudoalbidus* among populations (i.e. petioles). Poor DNA quality of some samples resulted in lack of sufficient data in GeneAlEx program for PCA analysis affected the clustering patterns of some genotypes. Consequently, amplified genotypes should show a closer genetically distance from each other, whereas they produced more distance. This finding will confirm the fact that *H. pseudoalbidus* has a poor allelic structure geographically.

As observed during the recent decade, the disease spread very rapidly across Europe causing decline and death of *F. excelsior* trees. The poor allelic diversity of European populations of *H. pseudoalbidus* among populations and its rapid spread suggest that the fungus is an introduced pathogen (Husson et al., 2011, Bengtsson et al., 2012). Where is the origin of this invasive pathogen? The answer to this question could prevent additional introductions of this pathogen to currently non-affected areas and possibly aid in some control options (Pautasso et al., 2013). Furthermore, the results of study I, confirming the existence of *H. pseudoalbidus* in the seeds of ash trees should be taken into advantage to inhibit more spread of the disease. Recent evidence now suggests that this fungus originates from Asia (Zhao et al., in press). More studies are needed in the future to determine the true origin of the disease. Moreover, selection of more resistant populations of *F. excelsior* should be taken into consideration for future breeding purposes. There are aspects of the life cycle of the disease that are still unknown; however the recent study by Gross et al. (2012) aimed to clarify some of these. According to Gross et al. (2012), the disease starts with infections of the leaves in summer by means of dispersed ascospores produced in fruiting bodies of petioles from the previous year in the leaf litter. After colonization and growth of the fungus in the leaves, it will enter the petioles. After leaf fall in autumn, the fungus will form a pseudosclerotial layer on the surface of the petiole where it will overwinter. Conidia produced in autumn at low temperatures are responsible for fertilization of the fungus and they act as spermatia. In the following summer new apothecia will develop to initiate new infections.

The sample size and investigation of only one asymptomatic petiole were the most important limitation factors of this study, since the samples had been collected from a single tree. For further researches it should be taken into consideration that samples are collected from several trees and from different locations. In addition, more healthy looking petioles (completely green petioles) should be investigated. Although there are several limitations in this study (considering the small number of samples), the results nonetheless show high genetic diversity of *H. pseudoalbidus* within a single tree. This finding also agrees with previous studies (Kraj et al., 2012, Rytkonen et al., 2011, Bengtsson et al., 2012) showing high gene flow and low geographic structure of the fungus. This fact is such a rare incident for an invasive pathogen which is characterized mostly by having low genetic structure and low gene flow (Rytkonen et al., 2011).

5. Conclusion

This Study confirmed the occurrence of the fungus *H. pseudoalbidus* in the seeds of different *F. excelsior* clones regardless of their different levels of susceptibility to ash dieback and in three categories of seed (Undamaged, Damaged and Suspect) using different molecular methods (specific primer and 454 sequencing). A large number of fungal taxa were detected in seeds of both more resistant and susceptible clones, but in general, more were detected in more resistant

clones than in susceptible clones. This may impose a risk of introducing the disease to non-infested areas through the importation of infected seed. Import and movement of *F. excelsior* seeds should be certainly taken into consideration for phytosanitary purposes. It is recommended that further research be undertaken to investigate the risk of *H. pseudoalbidus* in seeds as a potential pathway for dissemination of the disease to new areas. The close genetic structure of the fungus among the nine petioles from one symptomatic tree applied in this study confirms the results of previous investigations that *H. pseudoalbidus* has a poor geographic structure and supports the fact that the fungus is an introduced pathogen. High genetic variation within petioles suggests that the fungus has a sexual outcrossing and sexual reproduction of the fungus could increase the virulence of the fungus towards the host.

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