Dieback of *Fraxinus excelsior*

Biology of Ash Dieback and Genetic Variation of the Fungus *Hymenoscyphus pseudoalbidus*

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Abstract

Ash dieback caused by *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*) is a disease that has emerged during the past twenty years. It was first observed in Poland and has expanded over most of the distribution area of European ash (*Fraxinus excelsior)* in Europe.

This thesis comprises four scientific papers. The first reports the production of a phytotoxin, viridiol, by the fungus, and shows that it causes necrotic spots on ash cotyledons.

The second paper describes a working tool, a species-specific DNA primer situated in the internal transcribed spacer (ITS) region of the ribosomal DNA, which can be used to detect the fungus in diseased host material and confidently identify fungal cultures.

In the third paper, microsatellites and arbitrary primed PCR were used to investigate the genetic population structure of the fungus. The genetic variation was evenly distributed in the whole European population, implying a high level of gene flow. Double alleles in apothecia compared with single alleles in mycelia cultures strongly indicated sexual reproduction. *H. pseudoalbidus* was clearly distinct from the native closely related *Hymenoscyphus albidus* and it is unlikely that the disease is derived from a *H. albidus* ancestry. The low number of microsatellite alleles per locus indicated a recent founder effect in *H. pseudoalbidus*.

In the fourth paper disease development was surveyed in 261 naturally infected ash trees over a 32 month period. A clear seasonal pattern was demonstrated, with lesion activity and growth rate peaking during the summer. A substantial proportion of the lesions ceased to develop, often when the lesion reached a branch base; however the rate at which new lesions emerged was greater than the rate at which lesions entered a resting phase. During the course of the survey a third of the trees died and only a few seedlings remained healthy. In addition, the lower temperature limit for the fungus in culture was estimated to be 0.5°C.

Keywords: Chalara fraxinea, phytotoxin, viridiol, ITS, genetic marker, microsatellite, AP-PCR, population, season, lesion.

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Dedication

To the Universe

En ask vet jag stånda, den Yggdrasil heter, ett väldigt träd, överöst av vita sanden. Därifrån kommer daggen, som i dalarna faller, den står evigt grön över Urdarbrunnen. (...) Då skälver Yggdrasils ask, där den står, urträdet jämrar sig, jätten blir lös

Valans spådom

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I P. F. Andersson, S. B. K. Johansson[1], J. Stenlid and A. Broberg (2010). Isolation, identification and necrotic activity of viridiol from *Chalara fraxinea*, the fungus responsible for dieback of ash. *Forest Pathology* 40, 43–46.

II S. B. K. Johansson[1], R. Vasaitis, K. Irhmark, P. Barklund and J. Stenlid (2010). Detection of *Chalara fraxinea* from tissue of *Fraxinus excelsior* using species-specific ITS primers. *Forest Pathology* 40, 111–115.

III S. B. K. Bengtsson, R. Vasaitis, T. Kirisits, H. Solheim and J. Stenlid (2012). Population structure of *Hymenoscyphus pseudoalbidus* and its genetic relationship to *Hymenoscyphus albidus*. *Fungal Ecology* 5, 147–153.

IV S. B. K. Bengtsson, P. Barklund, C. von Brömssen and J. Stenlid. Seasonal pattern of lesion development in *Fraxinus excelsior* diseased by *Hymenoscyphus pseudoalbidus* (Manuscript).

Papers I-III are reproduced with the permission of the publishers.

[1] Stina Bengtsson previously Stina Johansson.

The contribution of Stina Bengtsson to the papers included in this thesis was as follows:

I Preparation of fungal material, performance of toxicity test, shared experimental design and part of the writing.

II Laboratory work, shared development of primers, shared writing.

III Writing, analysis, responsible for laboratory work, shared experimental design.

IV Field and laboratory work, writing, experimental design in discussion with supervisors, shared analysis.

1 Introduction

Why do research? To understand the world we live in, and sometimes to alter it a bit. Research gives a comforting impression of control. It all arrives from the basic need to observe, draw conclusions and make up plans for survival. However, as with most actions needed for survival, the need to do research drives itself, if given the possibility. We just have to look under that rock… and who knows when something amazing will appear? Or something else…

One year in the early 1990s in north eastern Poland, ash trees began to wither, leaves and shoots died off and bark turned brown in rapidly extending lesions. The following summer tiny fruit-bodies started to emerge on the fallen ash leaves, releasing clouds of infectious spores into the air. The exact time and place will remain unknown, but by the year 1992 the diseased ash trees were common enough to get noticed amidst the noise of other conditions that a tree can exhibit (Timmermann et al. 2011).

Ash dieback is a fungal disease of European ash (*Fraxinus excelsior*) trees that causes severe damage and death. Since the first symptoms were recorded in Poland and soon after in Lithuania, the disease has been reported from more and more countries and now covers most parts of the European continent. The disease extends from the northern limit of ash in Sweden, Finland and Norway to north Italy, Slovenia and Croatia and from the west coast in Belgium and France to an undefined border in the east, at least as far as Ukraine. Recently it was also introduced to the British Isles. (Figure 1)

In the early stages of ash dieback research, symptoms on ash were thought to be caused by frost, or a complex of abiotic and biotic factors (Przybył 2002). However, by investigating the fungal community in ash, a few pathogens and many saprotrophic and endophytic species were identified (Przybył, 2002, Bakys et al. 2009a).

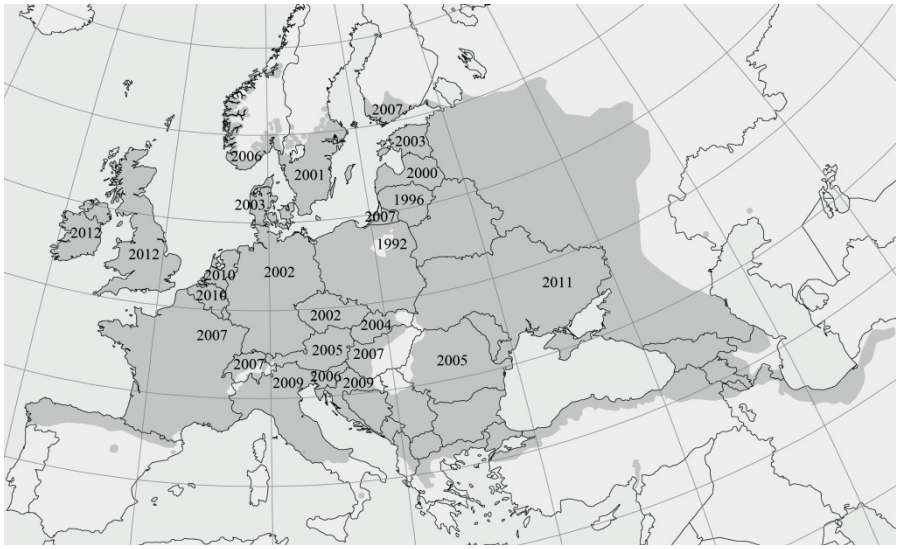

Figure 1. Distribution map of *Fraxinus excelsior*, including year for first report of ash dieback. (Timmermann et al. 2011, EUFORGEN 2009, personal communication[2])

The pathogenic agent responsible for the disease was recognized in 2006 and named Chalara fraxinea (Kowalski 2006), based on the morphology of the conidiospores and mycelia. However, with the aid of molecular methods the fungus was later ascribed to the phylogenetic genera Hymenoscyphus. For a short while it was confused with the morphologically similar Hymenoscyphus albidus (Kowalski & Holdenrieder 2009), which is known to be a saprotroph, decomposing ash leaves. However, molecular differences led to the recognition that this was a new species, and hence a new name: Hymenoscyphus pseudoalbidus (Queloz et al. 2011). Even more recently, the species was shown to be identical to Lambertella albida (Zhao et al. in press), a fungus found on ash petioles in Japan described by Hosoya et al. in 1993.

This enigmatic fungal species and the extensive, rapidly spreading disease have presented European researchers with many challenges. Over the few years that I have worked on my PhD project, much knowledge has been acquired by the research community concerning ash dieback. However, the questions have multiplied since ash dieback research first began. In the following section I will present the ash dieback situation as well as discuss my work in the context of forest diseases, invasiveness and how fungal pathogens and tree hosts interact.

[2] Fraxback COST action, meeting in Vilnius 2012

2 Background

2.1 What is a fungal disease?

The definition of a disease on plants is when the normal function is reduced or altered (Agrios 2004). In phytopathology, a figure describing the prerequisites for a disease was created by Stevens (1960): three components pathogen, host and environment, create a triangle, within which is the resulting disease.

Numerous fungal species parasitizes living trees, meaning that they take nutrient resources from the host. Many of these fungi are beneficial for the tree because they provide the tree with water and minerals by forming mycorrhizae. Other fungi do little or no harm, sometimes acting as saprotrophs after the death of the tree. A fungus that causes disease is named a pathogen. Besides taking tree´s resources, it can also produce substances such as toxins and enzymes that are harmful to the tree (Anderson et al. 2010). Although the death of parts of a tree is normally considered as a disease state, it is not always clear if the condition is harmful for the tree as a whole or not. One example is natural pruning or self-pruning where a fungus kills energy-sink branches shaded by the main crown (Long 1924).

Regardless of the effect on the exposed tree, on a wider scale, diseases affect the degree of biodiversity because they alter the fitness of competing species. A pathogen that is specialized on a single host tends to promote coexistence between species because it limits the dominance of that species (Hudson et al. 2006). By contrast, a generalist may have the opposite effect due to, for example, pathogen spill over to more susceptible host species (Mordecai 2011). Furthermore, species dependent on a specific host for survival are simultaneously threatened because their host population is reduced, as has been shown for lichens living on ash (Jönsson & Thor 2012).

From the point of view of a pathogen specialist, it is never beneficial to eradicate its only host. However, for an introduced species that succeeds by

establishing in a host, no natural regulation between pathogen virulence and plant resistance has been established. This may result in what is called an invasive pathogen, an introduced species that causes major harm towards a host.

Ash dieback is clearly a disease and, based on molecular data (Bengtsson et al. 2012, Gross et al. 2012a) and on the major impact it has had on the European ash population, it is likely to be caused by an introduced invasive pathogen. What are the biological processes that determine the impact of *H. pseudoalbidus* on ash? The answers lie in the interaction between fungus, environment and tree.

2.2 Symptoms of ash dieback

Symptoms are, strictly speaking, only a measurable trail of the interaction between pathogen, host and environment. A more detailed understanding would require studies involving, for example, microscopy, chemical analysis and molecular methods. However, observing symptoms combined with knowledge of the specific disease gives a good measure of the level of disease in the plant (Agrios 2004).

A typical symptom of ash dieback is as the name suggests, the death of shoots (Figure 2a). This activates growth of new shoots in still living parts of the tree, replacing the lost branches and shoots (Figure 2b and 2c). When the top shoot dies, its apical dominance is broken, inducing other shoots to take over the vertical lead. Together, these two mechanisms create a bushy mix of dead and living shoots, with several leaders (Figure 2c). Other symptoms emerge when the inner-bark and wood dies in lesions, which are often centred around a shoot. Warm-brown coloured, smooth lesions emerge on the stem, branches and even on the roots if disease is severe (Figure 2d). With time the lesion border will become ruptured as the surrounding tree tissue continues to grow. These old lesions often seem to be slightly sunken into the stem or branch thanks to the continued growth of the surrounding tree tissue combined with dehydration of the dead wood and bark (Figure 2d).

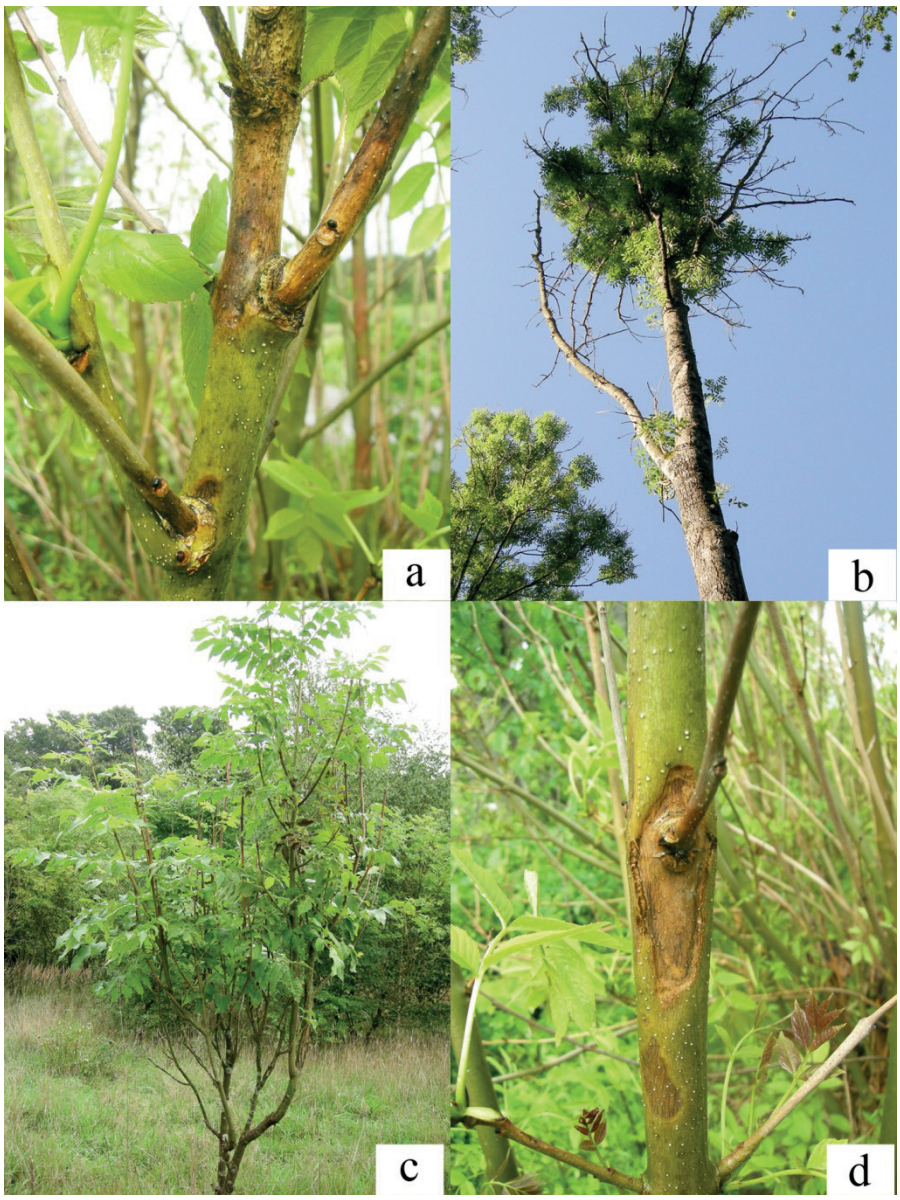

Figure 2. Typical symptoms of ash dieback: a, fresh lesions and dead shoots; b, secondary leaves and shoots on a heavily diseased tree; c, a young tree with a mixture of dead and living leader shoots; d, stem lesion partly fresh, partly with cracked bark and sunken into the stem. (Photo Stina Bengtsson)

In addition to the symptoms shown by the tree, the fungus can also be detected; apothecia (fruiting structures) form on the previous year´s leave petioles in the forest litter (Figure 3). These white, millimetre-sized cup shaped spore

producers emerge from the petioles beneath infected ash trees from bud-burst to leaf-fall with a peak in midsummer, (Timmermann et al. 2011). Typically, the petioles from which apothecia emerge are black owing to pseudosclerotia formed by the fungus (Gross et al. 2012b).

Figure 3. Apothecia of *H.pseudoalbidus* emerging on ash petioles. (Photo Michelle Cleary)

2.3 Challenges for *H. pseudoalbidus*

The ash dieback fungal pathogen *H. pseudoalbidus* spreads by producing light-weight ascospores that carry with the wind (figure 4). As with all wind-dispersed fungi, *H. pseudoalbidus* is dependent on the air-currents, the survival rate of the spores and the spatial availability of its host to find its substrate.

When attached to its host, *H. pseudoalbidus* forms an appresorium on the leaf surface before penetrating an epidermal cell (Cleary et al. in press). Once inside, the fungus is able to spread into all cell types and to all parts of the tree (Cleary et al. in press, Schumacher et al. 2010). Many fungal species take advantage of existing injuries in the bark or the lenticels to enter through the tough bark tissue. Other fungal species are able to directly penetrate the bark of roots, stem or shoots by chemical and physical forces. To date, no evidence of *H. pseudoalbidus* entering through the bark has been reported, although single

cases of lesions emerging on the bark, with no other likely originating point than through the bark, was observed (paper IV).

Once established in the host tissue, pathogenic fungi have different life-strategies. For an obligate pathogen, or biotroph, it is crucial to remain unnoticed, to not provoke the host´s defence mechanisms and to absorb nutrients from the living tissue, causing only limited damage to the host. Non-obligate pathogens are able to live part of their life in the living host tissue and part of it in dead tissue (hemibiotrophs). Alternatively, host cells are killed directly after pathogen entry (necrotrophs) (Agrios 2004, Oliver & Solomon 2010). The non-obligate pathogen produces enzymes, which, together with physical force enables the fungus to penetrate the host cells as well as to degrade the tree tissue. Toxins – unspecific or specifically developed in interaction with the host – can be produced and kill the host tissue. The toxin may also inhibit the plant´s ability to defend itself (Oliver & Solomon 2010).

If the host is able to detect the pathogen, either by the pathogen-associated molecular pattern (PAMP) or effectors such as toxins, or by substances produced by the host as a result of infection (Ferreira et al. 2006), the host will respond with an oxidative burst, phenolics, salicylic acid, pathogenesis-related proteins and by "burning the field before the enemy" with the aid of the suicidal hypersensitive response, and by building physical barriers around the invader (Ferreira et al. 2006, Agrios 2004). The tree may also inhibit further infection of the pathogen by activating systemic acquired resistance (Christiansen et al. 1999).

In ash dieback, the host tissue dies directly after pathogen entry and, hence, *H. pseudoalbidus* is classified as a necrotroph (Cleary et al. in press). The chemical processes involved in this have yet to be determined. However, investigations have been initiated and one phytotoxin, viridiol, has been shown to cause necrotic spots on ash cotyledons (Andersson et al. 2010, Grad & Kowalski 2009). The steroid viridiol is grouped as a secondary metabolite, a group of small molecular compounds often involved in communication between individuals and species. Some of these are phytotoxins, meaning that they are either produced by a plant or are harmful to plants. Additional steroids and steroid relatives have been reported to be produced by *H. pseudoalbidus*, however, their possible toxic effects have not been tested (Andersson et al. 2012, Andersson et al. 2013). There is also a need to further investigate the possible role of viridiol during infection in the natural system.

Several studies have shown a strong variation in the inherited resistance of ash trees to ash dieback, although none of the clones or progenies assessed in these studies were completely resistant (Pliūra et al. 2011, McKinney et al. 2011, Stener 2012).

The mode of reproduction is highly relevant with regard to the ability of the pathogen to sustain and enhance its virulence towards a host: By recombining, new variants with new combinations of virulent traits are continually created (Raffaele & Kamoun 2012). The frequencies of those different variants will be affected by their fitness through natural selection as well as by genetic drift combined with the size of the population. Furthermore, gene flow dictates how well distributed the genotypes will be in the population. (McDonald & Linde 2002).

The European population of *H. pseudoalbidus* does indeed have the prerequisites for effective development of more virulent genotypes. Although a founder effect has reduced its genetic variation in the past, it still carries considerable genetic variation (Rytkönen et al. 2011, Bengtsson et al. 2012, Kraj et al. 2012), and gene flow has been shown to be high (Bengtsson et al. 2012). Moreover, the dominating mode of reproduction is sexual recombination (Gross et al. 2012b) (figure 4).

Figure 4. Disease cycle of ash dieback, read clockwise. Windborne ascospores infects ash leaves, the fungus spreads toward the shoot and further into the tree. Leaves fall in autumn, and the fungus develops in the petioles. Different strains mate, hypothetically by conidia. Apothecia are produced during summer, releasing ascospores to the air. (Drawing Stina Bengtsson)

Today it is clear that *H. pseudoalbidus* is an introduced species and that the species has a higher genetic variation in Japan (Zhao et al. in press). Alternative theories of pathogen emergence have been discussed, and it cannot be excluded that the introduction of *H. pseudoalbidus* has been preceded or coincided with additional events. For example, mutations may occasionally lead to more aggressive variants of a pathogen, for example, by deletion or reduced expression of effector genes, reducing the host's ability to detect the pathogen (Raffaele & Kamoun 2012, Oliver & Solomon 2010). These genetic changes are sometimes enhanced by various stressors such as chemicals, UV-light and climatic factors (Lamb et al. 2008, Somers et al. 2002). Hybridization has repeatedly resulted in enhanced disease severity and host jumps (Fisher et al. 2012). Furthermore, horizontal gene-transfer between fungal species facilitated by viruses may lead to unexpected outcomes (Yoder & Turgeon 2001), such as increased virulence or emergence of new pathogens (Anderson et al. 2010).

While genes give the potential, environment affects which traits that are expressed and, hence, the phenotype of the organism (Pichancourt & Van Klinken 2012). Environment may also affect the organism's fitness: when stressed by unfavourable environmental conditions, the tree will alter the allocation of resources, potentially leading to a reduction in the active defence against pathogenic attack (Matyssek et al. 2005). The ash tree for example is sensitive to frost, low pH and compact soil as well as water logging (Dobrowolska et al. 2011). Certain environmental conditions have been associated with the severity of ash dieback, for example, the disease is more severe where trees are growing in wet soils, thinned stands or are suppressed (Kenigsvalde et al. 2010, Skovsgaard et al. 2010). *H. pseudoalbidus* shows a certain degree of variation with regard to growth rate and phenotype under different temperatures, with an average optimum temperature of 20°C in culture (Kowalski & Bartnik 2010).

2.4 Impact of ash dieback

A multitude of interactions occur in nature, some of which humans notice and some that severely alters the environment we live in. One example is Dutch elm disease, which led to the death of most mature elms worldwide in about a century. It is thought that the pathogenic fungus *Ophiostoma ulmi* was introduced from Asia to Europe and North America, from where it was later reintroduced into Europe in its more aggressive form *Ophiostoma novo ulmi* (Brasier & Buck 2001). A number of invasive species of the fungus-like *Phytophthora* organism have spread through Europe, causing disease on oaks

(Jung et al. 1996), larch (Brasier & Webber 2010), alder (Gibbs et al. 1999) and beech (Nechwatal et al. 2011). In North America, Chestnut blight caused by the introduced *Cryphonectria parasitica*, which also originated in Asia, almost eradicated the Chestnut population (Sinclair & Lyon 2005).

In some European countries ash has been an important forest crop: the timber is used, for example, in the floor and furniture industries, and to make tool handles. In Sweden, conifers dominate the forest and ash plays a marginal economical role. The timber volume of ash (*F. excelsior*) is 6.5 million m^3, or 0.2 % of the total timber volume in Swedish forests (Swedish National Forest Inventory, http://www.slu.se/riksskogstaxeringen). However, the ecological and the cultural values are more important. As well as *F. excelsior*, about 60 species associated with ash have been put on the Swedish red-list of threatened species, most of them insects and lichens (Gärdenfors, 2010). A Swedish modelling study has predicted that a substantial amount of lichen species will become extinct and that species composition will change as a result of ash dieback (Jönsson & Thor 2012).

The ash tree also has a cultural value in Sweden. For example, ash is one of the major tree species traditionally used for pollarding. Even today a lot of these environments are still sustained and support an important source of biodiversity (Jönsson et al. 2011).

The surrounding environment is very important for people's well-being (Sjölander 2006). Urban trees have been shown to have high values regarding human physical health as well as aesthetic, social and psychological factors (Sommer 2003). Ash is a tree that many people feel an affection for, often seen as a "vårdträd", an ornamental tree with a special status in the garden. In Nordic mythology the whole world is held up by an enormous ash tree that is continuously threatened by various forces, a sign of the ash tree´s impact on human beings.

2.5 The invasive pathogen *H. pseudoalbidus*

An invasive species can be defined as a non-native species that harms or threatens the environment biologically or ecologically (Santini et al. 2013). However, only a fraction of the species that are introduced will be able to find their niche and become invasive.

Species either spread naturally or they are transmitted by humans. Natural spread is predominantly gradual, for example, latitudinal spread following global warming. Natural single-step introduction on a continental scale is a rare and unpredictable event, where airborne spores are carried long distances by the wind-current. The chances of surviving and being deposited on a suitable

host are small and has only occasionally been reported, mainly for rust fungi (Brown & Hovmøller 2002).

Natural single-step spore spread is far outnumbered by the single-step introductions facilitated by human activity.

Santini et al. (2013) estimate that there has been an exponential increase of introduced forest pathogens to Europe over the past 200 years, with more than 40 introduced invasive species in the past 30 years. They found that the origins of the species introduced into European countries have changed over the years, from being mainly of European origin to being predominantly from other parts of the world. The most common source of the introduced species is thought to be living plants followed by wood, but transmission has also occurred via soil, cuttings, bark and seeds. The mechanisms behind the rising number of introductions are most likely to be the increase in both the amount and the speed of transport worldwide (Santini et al. 2013).

Even though it is obvious that the number of invasive species is increasing, some of the apparent increase in recent years might be due to better laboratory techniques for detection as well as a greater focus on invasive species in recent years. It is problematic to label a disease as 'invasive' if there is no knowledge of its previous occurrence. For many introductions in the past the evidence might have been lost and therefore, the diseases are classified as native. However, by comparing the population genetics of the species in different parts of the world the origin may be revealed (Estoup & Guillemaud 2010).

The ash dieback fungus has light-weight ascospores that can potentially travel far with the wind. However, as mentioned by Hietala & Solheim (2010) the spores lack pigment, thereby exposing them to harmful UV-light (Hietala & Solheim 2010), and their survival is limited by the small nutrient reserves. It is not known how far a *H. pseudoalbidus* spore can travel and still be viable. What is better known is that frequent trade of ash seeds and seedlings has occurred across Europe; however, the exact trading route needs to be mapped.

Recently, ash dieback was found in nurseries in the British Isles, and soon after in the wild. British nurseries have frequently imported ash seedlings from the European mainland, and did so as late as 2012 (personal communication[3]). This may be a good example on how human transportation of plant material facilitates the introduction of an invasive species into a region and how the species subsequently disperses and establishes itself in the area.

In addition to seedlings, ash seeds may also act as a potential inoculum source of the ash dieback pathogen given that the seeds has been found to contain *H. pseudoalbidus* (Cleary et al. 2013). Furthermore, it has been shown that conidia are produced on diseased ash logs (Husson et al. 2012). However,

[3] Fraxback COST action, meeting in Vilnius 2012

so far, germination attempts show that these spores are not able to germinate in culture or on ash seedlings (Zhao et al. in press, Kirisits et al. 2009).

3 Objectives

The objective of this thesis was to contribute with new knowledge on dieback of *Fraxinus excelsior* (common ash) focusing on the pathogenic agent, *Hymenoscyphus pseudoalbidus*. The detailed objectives were to:

1. Initiate an understanding of the chemical crosstalk between *H. pseudoalbidus* and its host by exploring the fungal metabolome (paper I).
2. Provide a method for detecting *H. pseudoalbidus* in diseased ash material and in mycelia culture (paper II).
3. Investigate the origin of the fungus by studying the genetic variation of *H. pseudoalbidus* and evaluate its relatedness to *H. albidus* (paper III).
4. Describe the development of ash dieback symptoms throughout the year and distinguish important factors important for lesion activity (paper IV).

Regarding pathogen and host interaction, it was hypothesized that the fungus produces secondary metabolites affecting tree health and resistance.

Two possible scenarios were hypothesized to explain the outbreak of ash dieback. First, that the fungus existed within the geographic area now afflicted by ash dieback, but that its interaction with ash changed for some reason, such as a genetic change of the fungus or an environmental change possibly enhancing tree sensitivity. Second, that the fungus was introduced from another geographic area, for example, by trade of plant material or by natural spread, and is now acting as an invasive species standing outside the natural control system that an established ecosystem provides.

In addition, a small genetic difference between *H. pseudoalbidus* and *H. albidus* was hypothesized, based on the morphological similarities. A close

relationship could explain the origin of *H. pseudoalbidus* as a descendant or hybrid of *H. albidus*.

I hypothesized that lesions caused by *H. pseudoalbidus* in ash trees would develop more during certain times of the year due to the lifecycles of the tree and fungus as well as the influence of temperature.

4 Material and Methods

4.1 Fungal material and cultivation

The growth substrate for *H. pseudoalbidus* in nature is everything from ash wood, bark, roots, leaves and leafstalks (petioles). In the laboratory, mycelia development occurred on agar-plates or in liquid media with 1% malt-extract and on the nutrient mix provided by Basal Norkrans media.

Many of the cultures used in my projects were provided by research colleagues throughout Europe. However, Swedish, Lithuanian and Danish isolates were isolated in our lab. The collected shoot or piece of wood was surface sterilized using 70% ethanol and 4% sodium hypochlorite and rinsed in sterile water. Small pieces (0.5 cm) were cut from the shoot in the border of healthy and diseased tissue, and placed on a malt agar-plate. During incubation at 20°C fungal cultures were examined and mycelia resembling reference cultures of *H. pseudoalbidus* were transferred to new malt agar-plates. This was repeated a few times to exclude possible contaminants. Pure cultures were stored in darkness at 4°C.

For metabolite production (paper I), a number of *H. pseudoalbidus* isolates were cultivated. One isolate originating from Sweden that produced particularly high levels of various substances was used for further studies. Liquid malt media and Basal Norkrans medium were used. In an attempt to mimic nature somewhat, pieces of ash-shoots were mixed into the malt medium. However, because this did not enhance or visibly change metabolite production, the ash-shoot medium was not used in the continued work. Incubating the bottles on a mechanical shaker was found to increase metabolite production, possibly because shaking increased the nutrient and oxygen availability for the fungus. It is also possible that the fungus was stressed by the treatment, responding with increased metabolite production. The duration of cultivation was found to affect the metabolite content in the media: the

optimal cultivation period was six weeks in most cases. Cultures were incubated at room temperature (22°C) and in shaded day-light.

A vegetative incompatibility test was performed with 41 of the *H. pseudoalbidus* isolates (paper III). Isolates from Denmark, Poland, Sweden, Lithuania, Czech Republic and Germany were paired in all combinations including self-pairings. An inoculum plug from each culture was placed on either side of an autoclaved piece of ash shoot on a 1% malt agar-plate. While being incubated at 20°C for six months and then at 4°C for 18 months in darkness the interaction zone was regularly assessed. The plates were evaluated to determine whether an interaction zone of dead cell had formed, or if the two cultures had fused, or if a zone of no mycelia persisted between the cultures.

To determine the optimum temperature for *H. pseudoalbidus* growth in the lab, seventeen randomly chosen isolates were cultivated at 0.5°C, 4°C, 12°C, 20°C and 25°C in darkness, with three replicates of each. The isolates originated from Sweden, Lithuania, Austria, Denmark and Åland.

4.2 Identification and evaluation of secondary metabolites

In paper I, strains of *H. pseudoalbidus* were cultivated and the chemical components produced were extracted, focusing on the low molecular weight secondary metabolites. One of the identified compounds was tested for necrotic effects on ash cotyledons.

The first step after fungal cultivation was to remove the mycelia from the liquid growth medium by filtering. Hydrophilic compounds were rinsed away by Solid Phase Extraction (SPE), and the lipophilic compounds were washed out by 95% acetonitrile and analysed further. The next step was to perform reversed-phase high-performance liquid chromatography, RP-HPLC. By letting the compounds interact with the silica material (C-18) in a column, they were more or less retained in the column based on their polarity. Smaller parts of these fractions were analysed by liquid chromatography-mass spectrometry (LC-MS). The mass spectrometer ionises the eluting molecules and measures the ratio between mass and charge of the ions; this information in conjunction with the literature is sometimes sufficient to make an assumption about the identity of some of the compounds. However, in this study one additional step was needed: nuclear magnetic resonance (NMR), which is a technique that measures the resonance of nuclei in a strong magnetic field. By using this technique in combination with literature data, it was possible to determine the chemical structure of some of the molecules.

The detected compound viridiol had previously been found to have a toxic effect on plants and, therefore, it was hypothesized that it could be involved in

symptom development in ash dieback. To determine whether viridiol could cause necrosis on ash, an additional experiment was prepared. Seeds from a diseased tree were grown under sterile conditions, until they had developed cotyledon leaves. Viridiol was dissolved in 50% aqueous methanol in four different concentrations. Four replicates were used for each treatment, as well as for the control, which was only treated with 50% aqueous methanol. One drop of the solution was applied to each of the two cotyledon leaves. The cotyledons were assessed one and two days after the treatment, noting if brown spots were produced on the leaves.

4.3 DNA analysis

After the development of the polymerase chain reaction (PCR), studying species at the DNA level became more time-efficient and there was less need of expert skills than in the past. Depending on the research question and the organism of interest, different sequences are used for analysis.

In the majority of fungal species, the internal transcribed spacer (ITS) situated in the ribosomal RNA has a limited variation within species but sufficient variation among species to allow for species identification. To develop species-specific primers for *H. pseudoalbidus* (paper II), the ITS sequences and adjacent 18S gene of *H. pseudoalbidus* were compared with the related species *Hymenoscyphus scutula.* Previous comparison revealed an intron in the 18S gene of *H. pseudoalbidus* that was absent in the related species (Bakys et al. 2009b). Based on this, the forward primer sequence from within the intron was chosen and the reverse primer was picked from the ITS region. Primer specificity was tested by substituting one primer at a time with the universal fungal ITS primers ITS 1F and ITS4 (White et al. 1990). In addition, amplification was tested at different concentrations of DNA-extracted fungal mycelia, DNA from diseased inner-bark of ash as well as pure culture isolates mixed with healthy inner-bark. Amplification was also attempted on the related species *Hymenoscyphus fructigenous* and *Hymenoscyphus caudatus* as well as on 55 different fungal species previously found in healthy, diseased and dead ash shoots (Bakys et al. 2009a).

In the following projects, these newly constructed primers were used to verify culture identity and to identify *H. pseudoalbidus* in my surveyed lesions. At that time *H. albidus* was not considered an ecological and genetically similar species, and to my knowledge, no ITS sequences of this species were available. Therefore, the primers were not developed to distinguish between these related species.

To investigate population structure and genetic variation of *H. pseudoalbidus* (paper III), as well as to separate between fungal genotypes (paper IV), markers with higher resolution were needed. Microsatellites are repeated non-coding regions in the genome that are made up of short, repeated sequences. The number of repeats changes at a high rate due to mutational mechanisms, independently of selection pressures (Selkoe & Toonen 2006). In paper III, seven bi-allelic microsatellites in the *H. pseudoalbidus* genome were identified and primed. The amplified DNA fragments were analysed using Genemarker®.

Similar to microsatellites, minisatellites are non-coding regions that consist of repeated sequences. However, the repeated sequence is longer, between 10 to 100 nucleotides. Different lengths of the minisatellite sequence together with its conserved flanking regions exist in numerous copies along the DNA chain. This is utilized in so called arbitrary primed PCR: one single primer amplifies minisatellite sequences all over the genome. When the PCR product is visualized by electrophoresis on an agarose gel, a banding pattern appears, providing a fingerprint for individual fungal strains. In paper III, the M13 minisatellite core sequence (Stenlid & Vasiliauskas 1998) was used to provide additional information regarding genetic variation of *H. pseudoalbidus*.

Using microsatellites combined with AP-PCR, I analysed 181 *H. pseudoalbidus* isolates collected in ten European countries, mainly Lithuania, Sweden, Denmark and Austria, but also a few from Hungary, Germany, Poland, Czech Republic, Norway and Åland (paper III).

In paper IV, the microsatellite primers were used to distinguish individual genotypes of *H. pseudoalbidus* in the planted seedlings. DNA was extracted and analysed from a one centimetre piece of inner-bark cut from the lesion border from 25 lesions in nine trees.

4.4 A field survey

To investigate seasonal patterns of lesion activity (paper IV), naturally infected trees at four locations were surveyed from 2008 until 2010. Three of the sites, Gnesta, Österbybruk and Åkersberga were situated within a 60-km radius south, north and east, respectively, of Uppsala, Sweden (hereafter these sites are collectively referred to as 'the three sites'. In addition, seedlings of approx. 1.3 m height were planted in the vicinity of our lab at Ultuna and treated as a replicate and as a source of additional information. At two of 'the three sites' the trees were planted in 1992 or 1994, and at the third site naturally regenerated trees 1 – 10 m high were surveyed.

In total, 261 trees were chosen in a random fashion at 'the three sites' so as to include a variety of trees from each stand. One lesion was chosen from each tree surveyed at 'the three sites'. Often, there was only one lesion that looked fresh and active within 1.8 m of the ground, but when given a choice, I aimed for an even distribution between lesions on shoots, branches and stems.

For the 52 planted seedlings at Ultuna, all emerging lesions were surveyed as they emerged. By doing so, I could collect data on the time-point and the location of new lesions. Therefore, lesion age at Ultuna was known, and the set-up resulted in a higher and more even distribution of young, developing lesions in the planted seedlings compared with 'the three sites'.

Around the 20th of each month, 'the three sites' and the planted seedlings at Ultuna were visited and each lesion measured (Figure 5). Notes were made regarding 1) whether the lesions had been active or not; 2) how much each active lesion spread measured in cm^2; and 3) the shortest and the longest distance from the previous month's lesion border. In addition, notes were taken regarding whether the lesion had reached a branch base or a junction of three or more shoots of equal size, because this was hypothesized to be an obstacle to further lesion spread. Additional measurements recorded at 'the three sites' were height, diameter at 1.3 m and the health status of the tree, which was based on estimate of the percentage of healthy leaf-mass.

Figure 5. Examples of lesions that were surveyed. (Photo Stina Bengtsson)

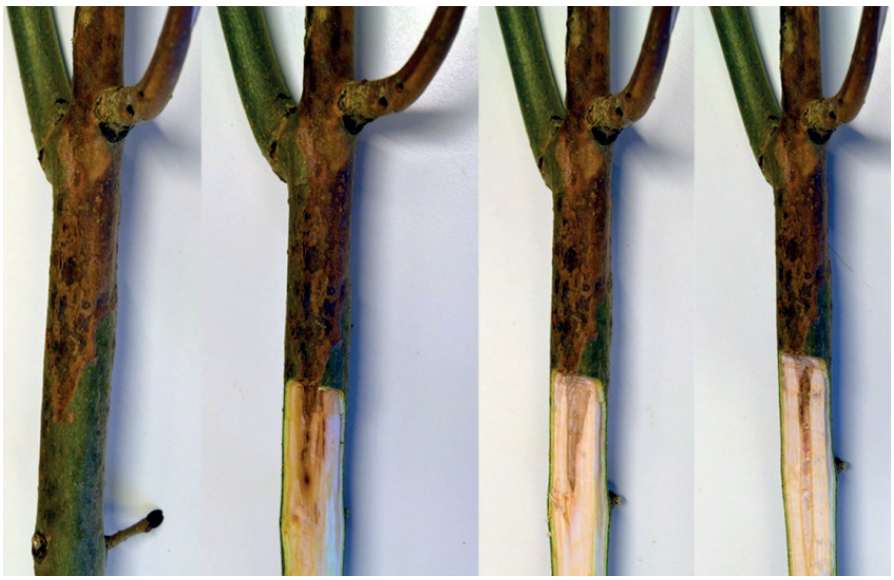

Figure 6. Dissection of a lesion step by step, inner-bark to wood and core wood. (Photo Stina Bengtsson)

In September 2010, the survey was ended by cutting off the bark and layers of wood around the lesions (57 lesions on the planted seedlings and 88 lesions on the trees at 'the three sites') to reveal whether wood discoloration corresponded with the bark lesion (Figure 6). DNA extraction was performed on 78 lesions from the planted seedlings and 95 lesions from 'the three sites' followed by amplification with the species-specific primers as well as fungal isolation. Moreover, the DNA from the planted seedlings were analysed with the microsatellite primers.

4.5 Statistical analysis

To analyse the population structure of *H. pseudoalbidus* (paper III), a few different methods were used, including both microsatellite data and AP-PCR data.

The data were organized in subpopulations according to the country of origin, and F_{ST} values were calculated for each subpopulation. A low F_{ST} value indicated a high level of gene flow between groups. The same sub-populations were used in analysis of molecular variance (AMOVA). The allele frequencies between and within sub-populations were compared to give an estimate to the degree of genetic variation that existed within the sub-population or between the different sub-populations. For the above calculations, the excel-add-in GeneAlEx 6.2 (Excoffier et al. 1992) was used.

In addition, the software Structure 2.3.1 (Pritchard et al. 2000) was used to assign the different individuals to different sub-populations based on allele-frequencies.

A good visual complement to these methods was Principal Coordinate Analysis (PCA), which created a graph in which the isolates were distributed according to their allelic similarity to each other (GeneAlEx 6.2, Excoffier et al. 1992).

The possibility of linkage disequilibrium between microsatellite markers and between AP-PCR products was investigated with the software Genepop 4.0.10 (Genepop on the web, http://genepop.curtin.edu.au/genepop_op1.html) (Guo & Thompson 1992, De Meeus et al. 1998). When two loci are in linkage disequilibrium they coexist in a non-random fashion in the genome. Loci that are physically close to each other in the genome tend to be in linkage disequilibrium.

For paper IV, a logistic regression model was used to compare a variety of factors affecting lesion activity, such as the effect of month, locality and year. To fit the model, PROC GLIMMIC in the SAS statistical software was used (V9.3., SAS Institute, Cary, NC, USA). Confidence intervals, correlations and linear regressions were calculated in Minitab.

5 Results and Discussion

5.1 A toxic metabolite (paper I)

By extracting and analysing chemical compounds dissolved in the growth medium of *H. pseudoalbidus*, the two related metabolites viridiol and viridin were detected. Viridiol has previously been shown to act as a phytotoxin, harming pigweed and cotton seedlings (Howell & Stipanovic 1984), whereas viridin has been shown to have a fungi-static effect (Brian & McGovan 1945).

Ash cotyledon leaves treated with viridiol showed brown spots after one day. A higher concentration generally resulted in necrotic spots on more replicates than with the lower concentrations: all but one of the cotyledons treated with the two highest concentrations had necrotic spots (7/8), whereas at the two lowest concentrations only one cotyledon had necrotic spots (1/8). One of the four control cotyledons had a small brown spot.

Necrotic spots on ash leaves are seen in nature as an early symptom of ash dieback (Figure 7). Viridiol might be involved in the development of ash dieback, for example, by killing tree tissue or immobilizing the tree´s defence mechanisms. Further studies are needed to evaluate the role of viridiol in the disease process.

As an attempt to further investigate the role of viridiol, a few samples of wood and bark in the border of diseased and healthy tissue were collected and analysed. No viridiol were detected (not published). The samples were taken during the summer when lesions are likely to develop (paper IV). A large sample size is recommended to increase the chance of including an active lesion, as well as to lower the risk of too low a concentration of viridiol in the sample.

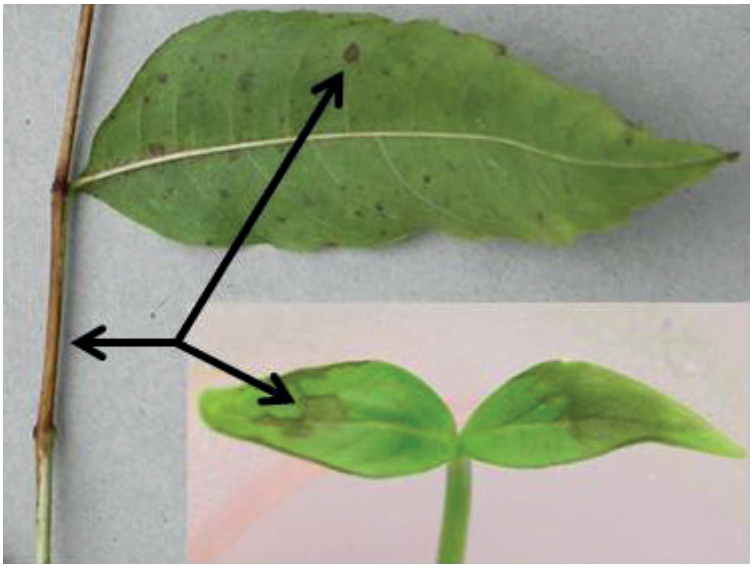

Figure 7. A symptomatic ash leaf and an ash cotyledon treated with viridiol. Arrows point out the necrotic parts. (Photo Stina Bengtsson)

5.2 Detection of the fungus (paper II)

The aim was to develop species-specific DNA primers for *H. pseudoalbidus*. The constructed primers amplified *H. pseudoalbidus*, both from pure culture and from diseased ash material. DNA from *H. albidus* was equally amplified, at the same product length. Using the protocol described by McKinney et al. (2012), the primers can be used to distinguish between *H. pseudoalbidus* and *H. albidus*. Weak products were produced for some of the fungal species that live in ash wood, but at different lengths than for *H. pseudoalbidus*. Therefore it is necessary to include a positive control (Figure 8).

Healthy ash bark in concentrations of between 0.5 and 0.05 ng/µl did not inhibit the PCR reaction, and fungal DNA concentrations between 0.25 and 0.0025 ng/µl were easily amplified. The conclusion is that by using the primers it is possible to detect small amounts of DNA in asymptomatic tissue: for example, at early infection stages. This is valuable for revealing disease in infected but healthy–looking diseased ash material, or to study the epidemiology of the disease.

Using the primers, *H. pseudoalbidus* was detected in all 20 samples taken from the inner-bark in lesion borders of ash shoots. By contrast, *H. pseudoalbidus* was only isolated from 14 of these samples. In fungal isolation attempts there is always a risk of competing fungi growing over the fungus of interest, especially for a comparatively slow grower such as *H. pseudoalbidus*.

However, DNA techniques may detect dead mycelium, over-estimating the actual pathogen presence.

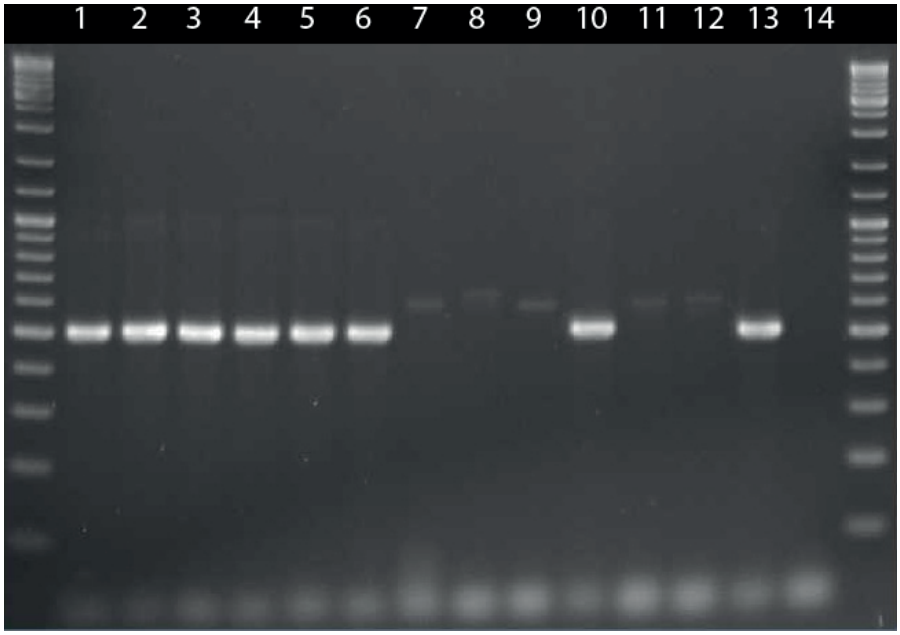

Figure 8. PCR products amplified with the species specific primers. Band 1–5 represents samples from symptomatic *F. excelsior* bark; band 6, 10 and 13 are *H. pseudoalbidus* mycelia and bands 7–9, 11, 12 are species previously found in ash shoots: *Cladosporium tenuissimum, Apiospora montagnei, Botryotinia fuckeliana, Phoma herbarum* and *Fusarium lateritium* respectively. In the last well, a negative control was loaded.

Attempts were made to detect the fungus in old, less active lesions (data not shown). The detection rate was much lower in these samples compared with fresh lesions. This may be an effect of inhibitory compounds in degraded wood, or reflect that *H. pseudoalbidus* was extinct in the lesion, possibly a result of competing secondary fungi. As a primary pathogen it is likely that *H. pseudoalbidus* is effective in the first stages of the disease, killing and degrading tree cells, but is not able to successfully compete with secondary fungi invading the dead wood.

5.3 Origin of *Hymenoscyphus pseudoalbidus* (paper III)

When exploring the population structure of *H. pseudoalbidus*, I found some interesting clues regarding the disease break-out.

First, a few additional alleles were detected (Figure 9), all from samples with a different origin. To find additional alleles during the analysis was not

surprising, because only a limited number of *H. pseudoalbidus* isolates were used for primer development. More interesting though were the big gaps between the alleles. The number of repeats in a microsatellite changes mainly by slippage in the copying process followed by a fail in the mismatch repair-system (Ellegren 2004). Normally, the errors only add or subtract one or sometimes a few nucleotides at a time (Tautz & Schlötterer 1994). However, my data reveal long distances, up to 19 bp between two alleles. This implies that some alleles are missing and that the included samples only represent a part of the total population.

This is intriguing because the fungal isolates originated from all over Europe. It appears that some part of the population is simply not there. A possible cause could be a bottle neck effect where a substantial part of the population has been wiped out in the past. However, this seems unlikely: *H. pseudoalbidus* had not been recorded in Europe before the onset of the disease and the population (detection of *H. pseudoalbidus* apothecia) has only increased since then. Instead, I assumed that a small part of a bigger population arrived in Europe from an unknown location resulting in a so called founder effect. This is in concordance with recent findings of an *H. pseudoalbidus* population in Japan that has a much higher genetic variation compared with that found in the European population (Zhao et al. in press).

One might argue that the genetic diversity is still too high to support a founder effect, because it is more likely that an introduction involved a few individuals rather than many individuals. However, it has been shown that as many as eight *H. pseudoalbidus* genotypes exist within a single leaf petiole (Gross et al. 2012b), and that several genotypes exist within a single ash seedling and even in a specific lesion (paper IV). This, in combination with sexual recombination, allows a high genetic variation to be present after a founder event. Therefore, a few seedlings or leaves imported to Europe prior to the disease outbreak would have been enough to initiate the present European *H. pseudoalbidus* population.

The even geographic distribution of the present genetic variation and the high level of gene flow between sub-populations are consistent with the rapid spread of disease through Europe. In fact, there was no proof for existing sub-populations in my analysis, but rather a single, continuously intermingling European population. The vegetative incompatibility test indicated a similar pattern because all isolates easily fused, with no difference between self-pairings and pairings between strains.

All the mycelial samples included in the study only amplified one allele per microsatellite. By contrast, when using DNA from apothecia, more than one allele appeared. Given that DNA was extracted from whole apothecia and not from single spores, recombination could not be proved. However, it was clear that more than one genotype existed within single apothecia, providing the prerequisite for sexual recombination in heterothallic fungi.

Later it was confirmed that *H. pseudoalbidus* exhibits a heterothallic life-cycle and that spores produced by sexual recombination are the dominant form of spread (Gross et al. 2012b).

A few recent studies, including paper III in this thesis, have shown substantial molecular differences between *H. pseudoalbidus* and *H. albidus* (Queloz et al. 2011, Bengtsson et al. 2012, Gross et al. 2012a). I showed that only four of seven microsatellite markers were amplified by *H. albidus* and no alleles were shared between the species (Figure 9). Furthermore, only three of eight AP-PCR products were of similar weight in the two species.

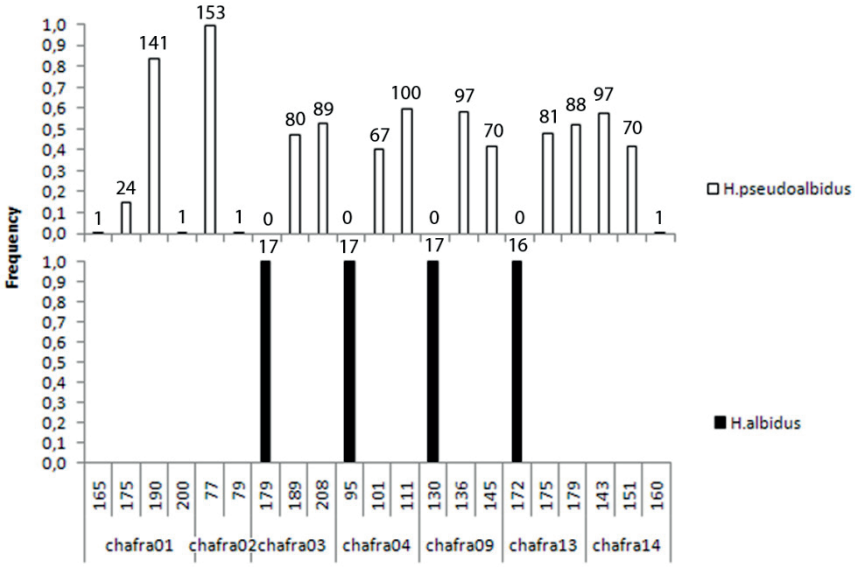

Figure 9. Frequencies of microsatellite alleles for *H. pseudoalbidus* and *H. albidus*.

When comparing *H. pseudoalbidus* with *H. albidus* I detected a significant difference in the degree of genetic variation. Whereas a substantial genetic variation within the *H. pseudoalbidus* population was observed, isolates of *H. albidus* lacked all variation regarding both microsatellites (Figure 9) and AP-PCR (Figure 10). To confirm this, a bigger sample size for *H. albidus* is needed. For this objective the AP-PCR is more informative then the microsatellites: it amplifies regions all across the genome, providing a general picture of the genetic variation. Microsatellites, developed for a specific species, do not always show the true genetic variation if used on a related species.

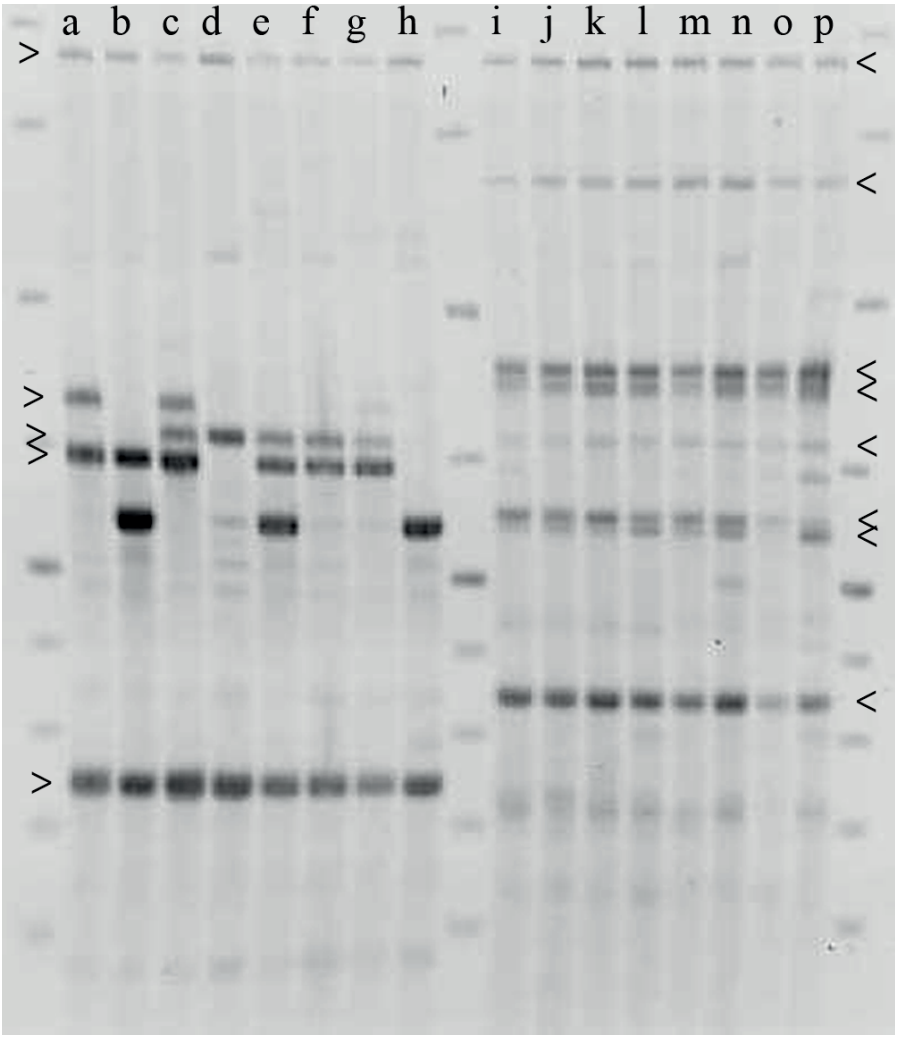

Figure 10. Eight isolates of *H. pseudoalbidus* (a–h) and *H. albidus* (i–p) amplified by AP-PCR and visualized on an agarose gel. Reproducible bands used in the study are marked. The ladder shows fragment sizes of 500 bp to 2500 bp.

5.4 Lesions in time and space (paper IV)

It was hypothesized that the outcome of ash dieback in individual trees would be affected by the phenology of the tree and the life-cycle of the fungus as well as by environmental factors such as temperature, creating a seasonal pattern of lesion activity. The present survey confirms such a pattern and highlights additional important factors. I showed that most of the lesions were active in June (Figure 11). Furthermore, the growth rate of active lesions at 'the three sites' peaked the following month. Hypothetically, this might result from

differences in tree defence in early summer when leaves are sprouting compared with later in the season when the tree has gathered new resources allowing a more active defence. The lesion development in the seedlings at Ultuna lacked this disparity. This might be because of differences in the defence mechanisms of the young seedlings compared with the trees at 'the three sites'.

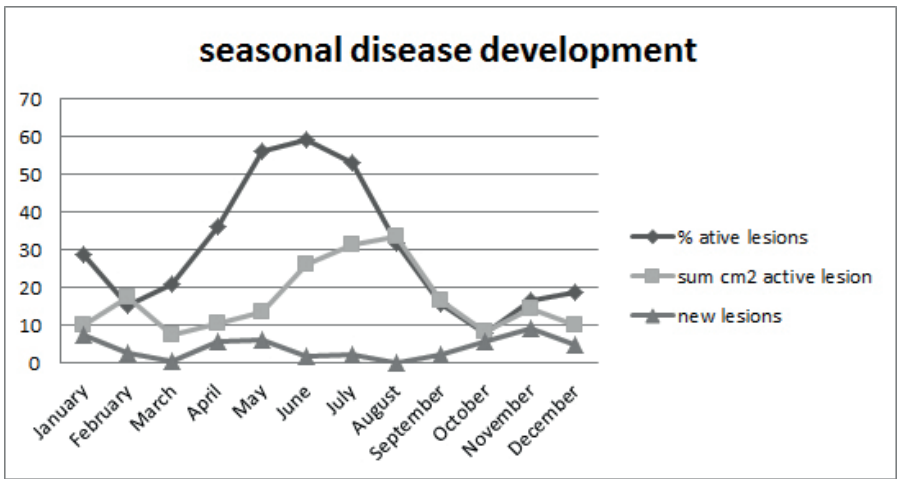

Figure 11. Percentage of active lesions and the summed areal spread for active lesions (cm^2), per month, as well as new lesions emerging on the planted seedlings. Average values for the total surveyed period.

I observed that most lesions that had reached a branch base entered a resting phase. An important proportion of the lesions, although not as many, also ceased activity at the junction of three or more shoots. This implies that the physical structure of the tree may provide an inactive defence. Many lesions also stopped developing for no visible reason. However, when examining the bark and wood at the end of the survey, it was revealed that these lesions had considerable wood discoloration exceeding the lesion borders. There was a significant difference between the extents of exceeding wood discoloration between both non-resting (more) and resting (less) lesions as well as between lesions at a branch-base (less) and lesions that stopped for unknown reasons (more). By contrast, it was not possible to distinguish between non-resting lesions and lesions that became resting for unknown reasons. The sample size for the latter was limited, but if this is true, the lesions that entered a resting phase for no obvious reason were likely to be active under the surface. Other reasons for a lesion to stop developing might be the influence of secondary fungi, outcompeting *H. pseudoalbidus* but unable to spread in the healthy tree tissue.

Temperature could have explained a small proportion of the seasonal pattern in the study. However, temperature is strongly correlated with the

season. To be able to distinguish between temperature and the developmental stages of the tree and the fungus, respectively, additional studies would be needed. One step was to establish the temperature optimum of *H. pseudoalbidus*, which was done by Kowalski & Bartnik (2010). In this study, I confirmed and complemented these findings by showing that the lower limit of *H. pseudoalbidus* growth in culture was between 0.5°C and 4°C. However, in the survey, lesion spread continued to occur in trees even though the temperature did not exceed 0°C for two months in 2010. However, the conditions within a tree are different from those outside because, for example, sugars in the tree increase the frost resistance of the tree (Sauter et al. 1996), which may allow fungal activity at a lower temperature. Moreover, healthy tree tissue bordering the dead tissue of the lesion is likely to be more sensitive to frost damage, causing extension of the lesion.

There was a weak but significant negative correlation between tree health and lesion spread. The correlation may derive from the tree's inherited ability to defend itself, resulting in low overall health as well as rapid and frequent lesion development for susceptible tree genotypes. Alternatively, a tree with low health status (possibly enhanced by the local site conditions) has fewer resources to allocate to active defence.

Tree mortality correlated negatively with tree health but to a low degree. This suggests that reasonably healthy trees could die as a result of, for example, girdling by a rapidly spreading lesion (which was observed in some cases). Tree health was only assessed on two occasions during the survey, which might partly explain the weak correlation observed. A positive correlation between tree size and tree health was observed. This is in agreement with previous findings (Skovsgaard et al. 2010, McKinney et al. 2011). In the present survey, the correlation was weak, probably because no trees exceeded ten metres or were more than 18 years old.

The intention of using 'the three sites', together with the seedlings at Ultuna, was that they should act as replicates of each other, and the general pattern was consistent at all localities. However, the odds for a lesion to be active were significantly higher at Gnesta compared with Österbybruk. The reasons for this were not apparent. Temperature was very similar between the localities (data not shown), even though Österbybruk is the farthest north of the sites. Other site conditions not recorded in this study, for example, soil properties might also influence lesion activity. Tree genetics are known to have an effect on disease development, and might explain part of the variation.

New lesions on the Ultuna seedlings were detected on the stem, on branches and shoots, with or without connection to a leaf-scar. Previous research (Cleary et al. in press) has described the fungus entering seedlings through the leaf-cells, but did not report entry through the bark of shoots or stem. If new lesions are the result of new infections, it is likely that they would appear during or shortly after spore release, which was not the case for many new lesions in my study: new lesions appeared throughout the year, peaking in spring and autumn (although a bigger sample size is needed to confirm this

pattern) (Figure 11). It is known that the fungus can spread under the bark causing wood discoloration, and then emerge on the bark surface far from the original lesion (Schumacher et al. 2010). Based on this, the most likely scenario is that the new lesions detected in this study did arrive from older infections, spreading under the bark; however, single lesions did emerge without a potential originating lesion, free from leaf-scars. Further microscopy is needed to reveal whether fungal penetration through the bark does occur.

Different lesions in a tree were found to contain different genotypes of *H. pseudoalbidus*, and some lesions contained multiple genotypes. Gross et al. (2012b) have shown that several genotypes can share the space of a petiole. These findings highlight the high numbers of spores successfully infecting the tree. A mix of genotypes enables sexual recombination in this heterothallic species (Gross et al. 2012b). However, the apothecia have only been observed in petioles and occasionally in shoots. The consequence for the fungus spreading in the wood is not yet understood, although the outcome for the tree is clear enough.

This study shows that the rate of new lesions emerging on the bark greatly exceeds the loss of old lesions due to permanent rest at, for example, a branch-base. This will result in an ever increasing disease pressure for the average tree, even though tree genotype susceptibility and site conditions will alter the outcome for individual trees.

I estimated that 17 % of the 1-10 m high trees would die per year in a location were ash dieback has been present for about eight years. The death rate is likely to change as susceptible trees disappear and more resistant ash trees remain.

6 A few answers and further suggestions

A plausible scenario for the origin of the ash dieback outbreak can now be drawn: an introduced pathogenic species has established in the *Fraxinus excelsior* population, an ash variant that is apparently unable to sufficiently restrict the fungal growth.

European forest pathologists have been investigating this disease from various angles for almost two decades, but there is still much more that needs to be understood. For example how the fungus and tree interact. I approached this specific question in two ways: by investigating the fungal metabolites and their effect on ash (paper I), and by studying symptom development in diseased ash stands during the year (paper IV). The identified phytotoxin viridiol was shown to cause harm to ash cotyledons. Future research needs to investigate the role of viridiol and other substances in the interaction zone between tree and fungus. The observed effect of season on lesion activity in paper IV should be studied with more controlled experiments, elucidating the detailed mechanisms. My predictions of tree death and emergence of new lesions versus extinction of old lesions provides a picture of the future for ash trees and as a result, of the many species associated with this tree. This type of information provides the basis for decision making with regard to trade regulations and other possible approaches to halt the disease spread to new areas.

The population study in paper III supported a hypothesis of a recent introduction of *H. pseudoalbidus*, a hypothesis that is strongly supported by recent findings (Gross et al. 2012a, Zhao et al. in press). The high level of gene flow and the indications of sexual recombination recently confirmed by Gross et al. (2012)b are important predictors for the pathogenic population's ability to develop and spread more virulent genotypes. Further research should involve more comprehensive population studies comparing the European and the Japanese populations, as well as alternative still unknown populations, to elucidate the exact origin of *H. pseudoalbidus*. The dispersal of *H.*

pseudoalbidus within Europe needs to be studied by mapping trading pathways as well as modelling spore spread.

In the surveys, I focused on the interaction between the host and the pathogen, as well as touching on environmental effects. However, interactions in nature are rarely so simple: even though *H. pseudoalbidus* is the major pathogen of ash dieback, other species living in ash do potentially affect the disease in some way. For example, root rot caused by *Armillaria* species has been shown to enhance the disease severity of ash dieback (Bakys et al. 2011).

Furthermore, it has been observed that as identification rates of *H. pseudoalbidus* in lesions decreased with lesion age, the isolation of other fungal species increased. Even though detection of *H. pseudoalbidus* often failed, the sampled lesion had been active just a few months before (paper IV). Can some of the secondary fungi establishing in the dead tissue continue lesion spread after *H. pseudoalbidus* has gone, aggravating the effect of ash dieback? Are these secondary fungi involved in eradicating the primary pathogen?

In a third of all lesions that permanently stopped developing in the survey, no visible cause was seen. Dissection data of the wood underneath suggests that these lesions might still have been active. However, it cannot be excluded that the lesion in some cases did stop developing, and that this might have been due to a competing species. If such a species were identified it could be used as a biocontrol agent, as is the case with *Phlebiopsis gigantea*, which is used to restrict infection of stumps of, for example Norway spruce, by the pathogen *Heterobasidion* spp. (Vasiliauskas et al. 2004).

A frequent question from journalists and interested people in general concerning ash dieback is: how many ash trees will die? Given that all living things die eventually the question might be better specified as “how many will survive until they have fulfilled their life-cycle and spread their genetic material to new saplings?” Or, “how will the landscape picture change, affecting people’s experience of their surroundings?” Moreover, “how will the loss in substrate affect communities living in and on ash?” A relevant question for some may be “will my favourite ash tree die or live for another hundred years?”

I will try to answer some of these questions. For trees between one and ten metres high, in a region where ash dieback has been reported for eight years, it is estimated that about 17% of ash seedling and trees will die each year (paper IV). Other estimates have been given by various authors: 34% including dead and dying ash trees (Jönsson & Thor 2012); 30% in a Polish ash stand ten years after first symptoms (Dmyterko et al. 2003); 23% of superficially inoculated

seedlings after six weeks (Bakys et al. 2009b); or 17% per year calculated from inventories in Swedish forests between 2008–2010 (Wulff & Hansson 2011). Before any conclusions are drawn from these estimates, it is important to consider the effect of tree size on tree survival, which has a positive effect on tree survival (paper IV, Skovsgaard et al. 2010, McKinney et al. 2011). Furthermore, the mortality rates are likely to change as susceptible trees die leaving a higher proportion of more resistant tree genotypes.

Symptoms develop at a greater rate than they cease to develop (paper IV). However, to predict the outcome for an individual tree one would need to know about the inherited resistance of the tree combined with the environmental conditions that might enhance disease development at the specific site.

Everything that can delay disease development in the tree increases its chances of living until it can reproduce. The inherited resistance of the tree is one part of this, evidently helped by the physical structures of the tree (paper IV). In addition, as the tree grows and becomes bigger the disease pressure is reduced, therefore enabling a somewhat more stable landscape picture to be sustained.

The death rate of diseased ash trees is apparently low enough to ascertain survival of the ash species. However, the reduction in population size of *F. excelsior* will lead to an increased risk of extinction for species associated with ash.

From the first reports of the disease in Poland in 1992, there has been a gradual pattern of expansion of ash dieback into new countries in the north, west and south of Europe.

It took 18 years for the disease to reach Belgium, about 1000 km from the starting point in North East Poland. If this was simply the result of spore spread, one spore would have to travel and successfully establish about 55 km from the source for eighteen years in a row. Timmermann et al. (2011) have reported that the disease front has spread 30 km in one year in Norway.

The spores of *H. pseudoalbidus* do indeed have the prerequisites for such a rapid spread, considering their small size and the high levels of spores released under diseased ash stands. However, spore survival is limited by small nutrient reserves and the lack of a protective pigment (Hietala & Solheim 2010).

Brown & Hovmøller (2002) have described how single-step spread to new continents is often caused by human transport, whereas the intercontinental spread may be facilitated by windborne spores. In the case of ash dieback, recent findings suggest that the pathogen may have been introduced from North East Asia (Zhao et al. in press). Visibly healthy seedlings of less

susceptible ash variants such as *Fraxinus mandshurica* (Drenkhan & Hanso 2010) may have transported the fungus. It is likely that this was followed by a combination of trade and spore dispersal within Europe.

To distinguish between the role of spores and trade in the spread of the disease, trading routes within Europe need to be mapped and spore survival investigated. The gradual spread of the disease through Europe might argue for a spore-facilitated spread, but could as well be explained by the trade of seedlings between neighbouring countries. The high level of gene flow shown by *H. pseudoalbidus* (paper III) could be a result of repeated far-distance spore-spread or trade considering the high number of genotypes within petioles and seedlings (Gross et al. 2012b and paper IV) enabling a few seedlings to contain most of the genetic variation of *H. pseudoalbidus* within Europe. Alternatively, with just 20-years of history, the lack of genetic structure could be the result of the limited time for genetic diversification to occur.

Ash dieback disease is overwhelming; hosts are dying, which is not a good long-term strategy for the pathogen. An obvious sign of the erratic 'strategy' of *H. pseudoalbidus* is its ability to grow in all the different parts of the tree. According to the present knowledge, there is no life-sustaining purpose for fungal growth other than in the leaves, given that it produces its reproductive structure on the leaf-petiole, and conidia are probably unable to grow in culture or infect seedlings (Zhao et al. in press, Kirisits et al. 2009). Furthermore, it is not likely that the fungus can effectively infect leaves from the shoot, given that the infected shoot tends to die before new leaves can sprout (personal observation).

The near future for *H. pseudoalbidus* in *F. excelsior* could be described as a glutton's party. However, eating food, plate and table alike will not let the party last long. As highly susceptible trees die off and new seedlings are generated from trees that succeed to survive long enough to produce seeds, the effect of ash dieback is likely to decline with time. However, the outcome will also be affected by the ability of the fungus to develop more virulent genotypes, facilitated by its genetic variation and sexual recombination as well as gene flow in the pathogenic population. Given that evolutionary mechanisms only work in the present and do not plan for the future, the fungus takes what it can as long as it can. When the ash population is reduced the fungal population will follow, but neither of them will be completely extinguished. With time, the resistance of the tree and the virulence of the fungus may co-evolve and result in a somewhat balanced situation. Along the way, the reduced population of ash is likely to result in major losses of biodiversity, both by reducing the genetic variation of ash, as well as the

extinction of species living in and on ash. The survival of the remaining ash population will depend on future threats, such as the emerald ash borer (*Agrilus planipennis*), which is rapidly moving towards Europe from the east.

After reading this thesis, a few pieces of practical advice for disease management might be expected. Ash dieback is caused by a wind-dispersed fungus, potentially capable of spreading long distances. However, it was most likely brought to Europe by human transport, and obviously helped to establish by the lively trade of plant material within Europe. It is probably not possible to completely stop dispersal of pathogens. Everything moves and spreads out, if it is allowed to. Realistically, the only thing that can be done is to slow the spread of pathogens down, for example, by political laws and regulations.

Similarly, it might not be possible to stop the development of ash dieback within the tree. Even though treatments may be developed, they are likely to be limited to a select number of trees. At present, the most reliable method is to slow the disease development down by choosing more resistant genotypes for breeding and planting, to plant the trees in favourable environmental conditions, and to reduce infection pressure by removing (and burning) fallen ash leaves under the tree.

The results presented in this thesis provide a broad understanding of how the ash dieback disease works. One outcome of research is practical implementation, such as the development of control methods and guidelines in forest practices and transport regulations. Another, maybe more important outcome, is the increase in overall knowledge, affecting how human beings look upon the world and all therein. With this in mind, I dedicate my thesis, as a fraction of the total knowledge about this world, to the universe, and look forward to continue learning.

Photo Stina Bengtsson

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