Heterobasidion root rot

Genetic mapping of virulence and evolutionary history

Kerstin Dalman
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Pathology
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2010

Acta Universitatis Agriculturae Sueciae
2010:81

ISSN 1652-6880
ISBN 978-91-576-7526-2

Print: SLU Service/Repro, Uppsala 2010
2

Heterobasidion root rot. Genetic mapping of virulence and evolutionary history

Abstract

Heterobasidion annosum (Fr.) Bref. *sensu lato* (*s.l.*) is a necrotrophic pathogen causing damage to conifers in the Northern Hemisphere. *H. annosum s.l.* consists of five species: three European [*H. annosum sensu stricto* (*s.s.*), *H. parviporum* and *H. abietinum*] and two North American (*H. irregulare* and *H. occidentale*); all with different but partially overlapping host preferences. A multilocus phylogenetic tree was built and the divergence times were estimated. Plate tectonics is likely to have been the main factor influencing *Heterobasidion* speciation and biogeography. Along with the geographical separation, the *Heterobasidion* species have specialized on different host genera. The *H. annosum* species complex originated in Laurasia and the *H. annosum s.s./H. irregulare* and *H. parviporum/H. abietinum/H. occidentale* ancestral species emerged between 45 million–60 million years ago in the Palaearctic. The data imply that *H. irregulare* and *H. occidentale* colonized North America via different routes: *H. irregulare* colonizing from the east via Trans Atlantic land bridges and *H. occidentale* colonizing from the west via the Bering Land Bridge. Alternatively *H. occidentale* originated from North America.

Identification of virulence factors is important for understanding the *Heterobasidion*–conifer pathosystem. Two studies of genetic mapping of virulence were performed. Virulence traits were measured as lesion length in the phloem and fungal growth in the sapwood of pine and spruce. Quantitative trait loci (QTL) were identified and positioned on a genetic linkage map for virulence of 102 progeny isolates from a cross between *H. irregulare* and *H. occidentale*. Both virulence traits in *Picea abies* identified significant QTLs on linkage group (LG) 15. Another QTL was positioned on LG 15 for the lesion length measurement in *Pinus sylvestris*. Moreover, QTLs on two separate smaller LGs were identified for fungal growth in sapwood and lesion length, respectively. The QTLs probably represent loci important for specific as well as general aspects of virulence on *P. sylvestris* and *P. abies*.

A genome-wide association study was performed for virulence on 23 *H. annosum s.s.* isolates. Twelve SNP markers distributed on seven contigs were significantly associated with virulence. From these, three regions were characterized, two with one marker each with the lowest *p*-values and one region containing six markers. The linkage disequilibrium blocks in these regions ranged between 1.2 and 31.2 kb. Seven genes were identified as candidate virulence determinants encoding calcineurin, acetylglutamate kinase/synthase, cytochrome P450 monooxygenase, serine carboxypeptidase, quinone oxidoreductase (ToxD) and two flavin-containing monooxygenases.

Keywords: *Heterobasidion annosum s.l.*, virulence, host specificity, conifer, genome-wide association study, QTL, SNP, phylogeny, divergence times.

Author's address: Kerstin Dalman, SLU, Department of Forest Mycology and Pathology, P.O. Box 7026, 750 07 Uppsala, Sweden

Email: Kerstin.Dalman@mykopat.slu.se

Dedication

This, the first and maybe only book I will ever write, is dedicated:

To my mother, Lena, who, in spite of being a single parent of three, invested time and effort to give me the best education possible.

To my partner, Per, for all the love and support throughout our time together.

To Per's son, Elliot, who added so much joy, wrestling matches and new perspectives.

To my daughter, Iris, for keeping me sane.

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 K. Dalman, Å. Olson and J. Stenlid (2010). Evolutionary history of the conifer root rot fungus *Heterobasidion annosum sensu lato*. *Molecular Ecology* 19, 4979-4993.

II M. Lind, K. Dalman, J. Stenlid, B. Karlsson and Å. Olson (2007). Identification of quantitative trait loci affecting virulence in the basidiomycete *Heterobasidion annosum s.l. Current Genetics* 52, 35–44.

III K. Dalman, K. Himmelstrand, Å. Olson, M. Lind, M. Brandström-Durling and J. Stenlid. A genome-wide association study identifies genomic regions for virulence in *Heterobasidion annosum s.s.* (manuscript).

Papers **I** and **II** are reproduced with the permission of the publishers.

The contribution of Kerstin Dalman to the papers included in this thesis was as follows:

I Planned and conducted the experiments and wrote the paper in collaboration with the co-authors.

II Conducted parts of the virulence analysis and reviewed the paper during the writing process.

III Planned and conducted most of the experiments (the virulence analysis in collaboration with Mårten Lind, performed the statistical tests for the virulence assay, extracted the DNA, performed the association mapping and LD analysis) and wrote the paper in collaboration with the co-authors.

Abbreviations

AFLP	amplified fragment-length polymorphism
Avr	avirulence
BEAST	Bayesian evolutionary analysis sampling trees, a cross platform program for Bayesian Markov Chain Monte Carlo analysis of molecular sequences
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
EFA	elongation factor 1-α
EST	expressed sequence tag
G3P	glyceraldehyde 3-phosphate dehydrogenase
GST1	glutathione-*S*-transferase 1
GTR	general time reversible substitution model
HKY	Hasegawa-Kishino-Yano substitution model
HR	hypersensitive response
ISG	intersterility group
ITS	internal transcribed spacer
LG	linkage group
LD	linkage disequilibrium
LOD	logarithm of odds, a likelihood ratio statistic used in linkage mapping and QTL analysis
MEGA4	Molecular Evolutionary Genetics Analysis software version 4.0.
NAD5	nicotinamide adenine dinucleotide 5, a mitochondrial gene
NB-LRR proteins	nucleotide binding proteins with leucine-rich repeat domains
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction

PEG	polyethylene glycol
PFG	fungal growth in pine sapwood
PLL	lesion length in pine
R-genes	resistance genes
SFG	fungal growth in spruce sapwood
SLL	lesion length in spruce
SNP	single nucleotide polymorphism
T-DNA	transferred DNA
TF	transcription factor
QTLs	quantitative trait loci

1 Introduction

1.1 Interactions between pathogens and their hosts

1.1.1 Interactions between trees and fungi during evolutionary time

Plants have been the main nutrient source for fungi throughout their evolutionary history and fungi have evolved different strategies to take advantage of the plant resource: as mutualist, parasite and saprobe. Plant parasites can be divided into biotrophs, hemibiotrophs and necrotrophs. Biotrophs enter a host and live as a parasite on living cells of the host. A biotroph may enter by penetration of the cell wall or through natural openings such as stomata or wounds (Deacon, 1997). Hemibiotrophs parasitize living tissue for some time and, later in the life cycle, feed on dead tissue. By contrast, necrotrophs are pathogens that attack living host cells, kill them and feed from degrading the cells. They can often extend their life cycle to a saprotrophic stage where they feed on the dead host.

To get an understanding of interactions between pathogens and their respective hosts during evolutionary time their present interactions have to be studied. Vacher et al. (2008) uncovered a network of 51 tree taxa and 157 parasitic fungal species in France in a long-term survey. Compartmentalization of a network is characterized by subsets of interacting species, with species more likely to be linked within than across subsets. They revealed that the compartmentalization of host–parasite interactions reflected major phylogenetic splits, but only in host phylogeny. This finding suggests that the deep evolutionary history of fungal species is not mapped by their present parasitic interactions with host trees, but can be explained by the very early divergence of Ascomycota and Basidiomycota (400 million–1500 million years ago) (Taylor and Berbee, 2006). The first fossil evidence of land plants is from 460 million years ago (Berbee and Taylor,

2001). Both Ascomycota and Basidiomycota had probably developed parasitic associations with land plants before the divergence between Magnoliophyta (flowering plants, angiosperms) and Coniferophyta (gymnosperms), which occurred 140 million–180 million years ago (Bell et al., 2005). Therefore both Magnoliophyta and Coniferophyta were colonized by both Ascomycota and Basidiomycota. The very early divergence of major fungal phyla may account for the asymmetrical influence of past evolutionary history between trees and fungi (Vacher et al., 2008).

1.1.2 Plant–pathogen interactions at the molecular level

The plant immune system consists of two branches: one outer branch using transmembrane pattern recognition receptors that recognizes and responds to pathogen-associated molecular patterns (PAMPs) and one acting inside the cell, using nucleotide binding proteins with leucine-rich repeat domains (NB-LRR proteins) that are encoded by resistance genes (R genes) (Jones and Dangl, 2006). When a pathogen comes in contact with a host cell it delivers effector molecules (virulence factors) (Jones and Dangl, 2006). NB-LRR proteins recognize pathogen effectors and activate the defence response of the host. In addition to recognizing the infectious non-self molecules included in various PAMPs, plants are able to recognize infectious self molecules (i.e. their own degraded polymer molecules) as danger-associated molecular patterns (DAMPs) (Matzinger, 2007).

Jones and Dangl (2006) proposed a four phased 'zigzag' model for the interaction between the plant immune system and pathogens. (1) PAMPs are recognized by pattern recognition receptors. The host develops PAMP-triggered immunity that can interfere with further colonization. (2) The pathogen then generates effectors contributing to its virulence and interfering with pathogen-triggered immunity, which results in effector-triggered susceptibility. (3) The host recognizes the effectors by NB-LRR proteins, which in turn lead to an activation of avirulence (*Avr*) genes in the pathogen and production of new effectors that induce effector-triggered susceptibility. Thereafter the plant deploys NB-LRR proteins that recognize the new effectors and new effector-triggered immunity occurs, which leads to disease resistance and often a hypersensitive cell death response. (4) Pathogens avoid effector-triggered immunity either by alternating or getting rid of the effector gene, or by producing new effectors driven by natural selection. Natural selection then drives the host to generate new *R* genes to trigger effector-triggered immunity again. Following recognition of a

pathogen, a hypersensitive response is induced in the host, which includes cell death associated with the infection. This is a strategy to prevent the pathogen from spreading further. This process is controlled by the gene-for-gene system where *Avr* genes in the pathogen are recognized by *R* genes in the host (Dangl and Jones, 2001; Flor, 1971). In this way pathogen Avr proteins (e.g. effectors) encoded by *Avr* genes have corresponding R proteins (e.g. NB-LRR proteins) encoded by *R* genes in their respective host. Successful NB-LRR-mediated disease resistance results in an activated hypersensitive response and cell death. This is effective against pathogens that require living host cells to parasitize (i.e. biotrophs and hemibiotrophs), but not against necrotrophs; indeed, because necrotrophs require dead plant cells to feed on, they might in fact benefit from an induced cell death response (Jones and Dangl, 2006).

Several genetic factors in pathogens that are important for host specificity and general virulence have been found. These include effectors (Dangl and Jones, 2001; Flor, 1971), toxins (Knoche and Duvick, 1987; Wolpert et al., 2002), host defence detoxifying agents, ABC transporters and many degrading enzymes (Asiegbu et al., 1998). Effectors are virulence factors that are recognized by and bind to R-proteins in the host, whereby they alter the host to enable infection, for example, by suppression of the immune system. Host selective toxins produced by some fungal pathogens have been studied as model systems for effectors essential for conditioning host susceptibility (compatibility) (Wolpert et al., 2002; Ciuffetti et al., 2010). The *Pyrenophora tritici-repentis* ToxA and ToxB are examples of dominant genetic factors for such an interaction (Ciuffetti et al., 2010). de Jonge et al. (2010) showed that the fungal LysM effector Ecp6 from *Cladosporium fulvum* mediates virulence through perturbation of chitin-triggered host immunity. Esp6 was shown to compete specifically for chitin binding with the rice chitin receptor and, thereby, inhibit the defence responses. ABC transporters are membrane proteins that are active in excreting compounds toxic to fungi through cell membranes (Stergiopoulos et al., 2002). In addition, they can be determinants of virulence given that they can also excrete plant defence compounds or mediate secretion of host-specific toxins in fungal plant pathogens (Stergiopoulos et al., 2002). Both biotrophic and necrotrophic fungal pathogens produce effectors that interact with host targets. However, the effectors of necrotrophs cause lesions, which facilitates disease and for which no true gene-for-gene-based resistance has developed (de Wit et al., 2009). They may therefore be regarded as basic effectors for which there has been a low level of co-

evolution. Plants respond to this type of basic effector in a different way compared with the gene-for-gene interaction, leading to a quantitative resistance response that rarely provides the total resistance observed for the gene-for-gene interaction (McDonald and Linde, 2002).

1.1.3 Genetic factors affecting a pathogen population

The genetic structure of a pathogen population will affect its evolutionary potential and enable it to overcome host genetic resistance. Several evolutionary forces have been proposed to affect the evolutionary potential of pathogens (McDonald and Linde, 2002):

- *Mutation*: Mutations lead to DNA changes and thereby create new alleles in populations. Mutations are a fundamental source of genetic variation. This genetic variation has the potential to create new virulent strains of plant pathogens. In the gene-for-gene interaction, a mutation is needed in the avirulence gene (which encodes the elicitor recognized by the resistance gene) to create a virulent pathogen strain. These mutations are rare, but when coupled to directional selection the frequency of virulent variants increases rapidly. Pathogens with high mutation rates increase the likelihood of virulent strains being present in the population.

- *Population size and random genetic drift*: The population size affects the evolutionary potential. Large populations have a larger number of variants than small populations. The diversity of genes in a population is also influenced by random genetic drift. In small populations genetic drift is more likely to lead to a loss of alleles over time. Thus, pathogens with larger populations have greater evolutionary potential than those with small population sizes. Bottlenecks (i.e. severe population reductions) lead to a less diverse population that has more difficulties adapting than populations of larger sizes.

- *Gene flow*: Gene flow is the process where alleles of genes, and hence also virulent variants of the pathogen, are transferred between populations. A high degree of gene flow increases the effective population size by increasing the genetic neighbourhood; pathogens from such populations pose a higher risk than those with low gene flow.

- *Reproduction and mating system*: The reproduction and mating systems affect the distribution of gene diversity within and among individuals in a

population. Reproduction can be asexual, sexual or mixed, and for sexual reproduction the mating system can vary from strict inbreeding to obligate outcrossing. There is a higher degree of genotype diversity in populations that reproduce sexually. A recombining pathogen can construct new combinations of virulence alleles rapidly, which is why outcrossing pathogens pose a greater risk than inbreeding ones.

McDonald and Linde (2002) studied the literature of 34 plant pathosystems and approximated values for four evolutionary forces (reproduction/mating system, effective population size, mutation and gene flow) using their risk model. They concluded that gene flow and mutation may be the dominant forces driving pathogen evolution in the pathosystems considered.

In this thesis I have searched for genetic components that are important for virulence in the necrotrophic conifer pathogen *Heterobasidion annosum s.l.* The aim has been to obtain an unbiased screen for novel and important factors by associating virulence traits with molecular markers.

1.2 The *Heterobasidion annosum* species complex

Heterobasidion annosum (Fr.) Bref. *sensu lato* is not only interesting because of its biology but is also a serious economic problem for forestry. *H. annosum s.l.* infection causes devaluation of timber and growth reductions, and the financial losses have been estimated to be 790 million € yearly in Europe (Woodward et al., 1998).

The order Russulales is related to Agaricales, Boletales and Atheliales but represents a basal independent evolutionary line of agarics (Hibbet, 2006). Basidiospores in Russulales are generally ornamental with amyloid warts or reticulation; *Heterobasidion* is an exception (Miller et al., 2006). The family Bondarzewiaceae resembles Polyporaceae but has ornamented spores like *Russula* and *Lactarius*. *Heterobasidion* Bref. (Agaricomycotina, Russulales: Bondarzewiaceae) is a polypore genus that has perennial basidiocarps with cuticulate pilei with asperulate basidiospores. DNA studies support the placement of the genus *Heterobasidion* within Bondarzewiaceae (Hibbet and Donoghue, 1995).

The root-rot fungus *H. annosum s.l.* is a species complex consisting of five species: *Heterobasidion irregulare* Otrosina & Garbelotto, *Heterobasidion occidentale* Otrosina & Garbelotto, *Heterobasidion annosum* (Fr.) Bref. *sensu*

stricto, *Heterobasidion parviporum* Niemelä & Korhonen, and *Heterobasidion abietinum* Niemelä & Korhonen (Table 1). They will be referred to as the *H. annosum* species complex or simply *H. annosum s.l.* throughout this thesis. These five species have different but somewhat overlapping host ranges. The two North American species, *H. irregulare* and *H. occidentale*, were recently named and considered to be biological species (Otrosina and Garbelotto, 2010). The other three species, *H. annosum s.s.*, *H. parviporum* and *H. abietinum*, are European (Niemelä and Korhonen, 1998). Before being given names, the different species were grouped according to their host preferences for a long time: P (pine), S (spruce) and F (fir) (Korhonen, 1978; Chase and Ullrich, 1988; Capretti et al., 1990). *H. annosum s.s.* and *H. irregulare* were referred to as P-types, *H. parviporum* and *H. occidentale* belonged to the S-types and *H. abietinum* to the F-type. These groups were designated intersterility groups (ISGs) because they were found to be intersterile in mating experiments (Korhonen, 1978; Chase and Ullrich, 1988; Capretti et al., 1990), although intersterility is not complete (Stenlid and Karlsson, 1991). *H. annosum s.s.* is distributed almost all over Europe (Korhonen et al., 1998). *H. parviporum* occurs throughout the natural European distribution of *P. abies* and also in spruce plantations in western Europe. *H. abietinum* is found in fir forests in central and southern Europe. *H. irregulare* is found mainly in the western and south-eastern parts of North America but is less common in the central parts. *H. occidentale* occurs only in western North America, where it is common. A summary of the five species, their ISG, host preferences and distribution is given in Table 1.

In addition to the *H. annosum* species complex there are other species in the *Heterobasidion* genus. *Heterobasidion araucariae* Buchanan is a saprotroph on *Araucaria* and *Agathis* and has been found in eastern Australia and New Zealand (Niemelä and Korhonen, 1998). It was found to be distinct from *H. annosum* in mating experiments (Buchanan, 1988). A saprophytic *Heterobasidion* species complex, *Heterobasidion insulare* (Murr.) Ryv., was found in southern and eastern Asia, China and Japan (Niemelä and Korhonen, 1998). The *H. insulare* complex is now considered to include *Heterobasidion linzhiense* Y.C. Dai & Korhonen (Dai et al., 2007), *Heterobasidion orientale* Tokuda, T. Hatt & Y.C Dai, *Heterobasidion ecrustosum* Tokuda, T. Hatt & Y.C Dai (Ota et al., 2006; Tokuda et al., 2009) and *Heterobasidion australe* Y.C. Dai & Korhonen (Dai and Korhonen, 2009). Corner (1989) described another species, *Heterobasidion pahangense* Corner from Malaysia, which has ornamental spores, and Stalpers (1996) described the tropical American species *Heterobasidion rutilantiforme* (Murril) Stalpers.

Table 1. The *Heterobasidion annosum* species complex

Species name	ISG*	Host preferences	Distribution	References
H. annosum (Fr.) Bref. *sensu stricto*	P	Mostly *Pinus sylvestris*, many other tree species, including *Juniperus*, *Picea*, *Abies* and several broadleaved species	Europe	Brefeld, 1888; Niemelä and Korhonen, 1998
H. parviporum Niemelä & Korhonen sp. nov.	S	*Picea abies* and *Abies sibirica*	Europe and Asia	Niemelä and Korhonen, 1998
H. abietinum Niemelä & Korhonen sp. nov.	F	*Abies* species, occasionally *Picea*	Southern and central Europe	Niemelä and Korhonen, 1998
H. irregulare Otrosina & Garbelotto nom. nov.	P	*Pinus* species and others	North America	Otrosina and Garbelotto, 2010
H. occidentale Otrosina & Garbelotto sp. nov.	S	Many tree genera, preferably *Abies*, *Pseudotsuga* and *Tsuga*	Western North America	Otrosina and Garbelotto, 2010

* Intersterility group

1.2.1 Evolution within the *H. annosum* species complex

Over the past two decades, several studies have examined different aspects of the phylogenetic relationships of the different species within the *H. annosum* species complex. Kasuga et al. (1993) identified the European intersterility groups, S (*H. parviporum*), P (*H. annosum s.s.*) and F (*H. abietinum*), in the *H. annosum* species complex by ribosomal DNA fingerprinting. Harrington et al. (1998) separated the *H. annosum* species complex into three clades: the American P group (*H. irregulare*), the European P group (*H. annosum s.s.*) and the "fir" group (*H. occidentale*, *H. parviporum* and *H. abietinum*). Garbelotto et al. (1998) constructed a dendrogram of the European F (*H. abietinum*) and S (*H. parviporum*) intersterility groups using arbitrary-primed PCR and mitochondrial markers and concluded that they are both monophyletic and may lack substructuring in subpopulations. Johannesson and Stenlid (2003) focused on the European S and F intersterility groups and could identify three clades: European F, Eurasian S and North American S. The P group was studied by Linzer et al. (2008) who showed that the Eurasian (*H. annosum s.s.*) and North American (*H. irregulare*) lineages are monophyletic sister clades. The North American P group (*H. irregulare*) was further divided into eastern and western clades; Mexican isolates showed affinity to both clades (Linzer et al., 2008). Japanese *H. annosum s.l.* isolates

from *Abies sachalinensis* (F. Schmidt) Mast. were found to form a subclade to *H. parviporum* (Tokuda et al., 2007).

Different factors that may be important for the speciation of the *H. annosum* species complex have been put forward, including co-evolution with the host, modern forestry, geological factors and glacial periods. Otrosina et al. (1993) proposed that *H. annosum s.l.* begun evolving its host specialization during the Tertiary period along with the more or less continuous Trans-Arctic forest that existed at that time. Linzer et al. (2008) suggest that the ancestor of *H. annosum s.s.* and *H. irregulare* was located in Eurasia and that the dispersal to North America occurred over a Beringean land bridge. They also propose that Mexico may have served as a refuge during a period of glaciation. Although several hypotheses on evolutionary scenarios for the species complex have been put forward (Otrosina et al., 1993; Linzer et al., 2008), no formal estimates of divergence times have been published previously.

1.3 Evolution of *Pinaceae*

To study the co-evolution between pathogens and their respective hosts, the evolution of both counterparts has to be considered. The evolution of *Pinaceae* has been studied using a combined dataset of the three genes: the chloroplast gene *matK*, the mitochondrial gene *nad5* and the low-copy nuclear gene *4CL* (Wang et al., 2000). A well supported multilocus phylogeny was generated which showed that *Abies* is basal compared with *Picea* and *Pinus*. Only the phylogeny of the *matK* gene was dated and the divergence times were found to correlate well with the fossil record (Wang et al., 2000). *Pinus* was found to have diverged from *Picea* approximately 140 million years ago (Wang et al., 2000).

1.4 Biology of *H. annosum s.l.*

1.4.1 Infection cycle

The infection of *H. annosum s.l.* can be divided into four stages: (1) spore adhesion, germination and appressorial formation; (2) lesion, epidermal penetration and cortical invasion; (3) invasion of endodermis; and (4) vascular colonization and loss of root turgor (Aseigbu et al., 2005). In the first stage, spores adhere to wounds or the freshly cut stump surfaces of the host, whereby they germinate and may form appressoria. *H. annosum s.l.* has the ability to grow in wood without appressoria, but when growing through

living cells in the bark appressoria are formed. In nature, the primary infection is mainly mediated by basidiospores but conidiospores have been shown to infect non-suberized seedling roots successfully under laboratory conditions. In fine root infections studied in the laboratory, the hyphae penetrated and invaded the cortical region after 3–6 days and formed a lesion (Asiegbu et al., 1993). Invasion of the endodermis occurred 7–8 days after infection. Vascular colonization and disintegration occurred within 9–15 days.

1.4.2 Virulence

Before the details of pathogenicity and virulence of *H. annosum* can be addressed specifically these concepts need to be defined. Virulence is defined as the degree of pathogenicity of a given pathogen, whereas pathogenicity is the capability of a pathogen to cause disease (Agrios, 1997).

H. annosum s.l. is a necrotrophic pathogen that secretes many extracellular compounds in order to degrade the constituents of the host, such as pectin, cellulose, lignin, sugars, starch and various phenolic compounds (Asiegbu et al., 1998; Nord and Hata, 1969). The enzymes that are secreted by *H. annosum s.l.* include amylase, catalase, cellulase, esterase, glucosidase, hemicellulase, manganese peroxidase, laccase, pectinase, phosphatase and proteases (Asiegbu et al., 2004; Hüttermann, 1980; Johansson, 1988; Karlsson and Stenlid, 1991; Korhonen and Stenlid, 1998; Maijala et al., 1995, 2003). Only a few of these enzymes have been studied extensively and their roles in virulence are fairly unknown. However, laccase is presumably involved in lignin degradation and detoxification, and has been correlated to the wood degrading ability of *H. annosum s.l.* The *H. annosum s.s.* species is known to have a greater wood degrading ability than *H. parviporum*, and has been shown to secrete five to six times more laccase than *H. parviporum* (Daniel et al., 1998).

In addition to the wood degrading enzymes, *H. annosum s.l.* has also been shown to produce several toxins during host infection and in dual cultures with other fungi. These include fomannoxin, fomannosin and fomannoxin acid, oosponol and oospoglycol (Basset et al., 1967; Heslin and Stuart, 1983; Heslin et al., 1983; Holdenrieder, 1982; Sonnenbichler et al., 1983, 1989, 1994;). Fomannoxin was demonstrated to be toxic to seedlings of Sitka spruce [*Picea sitchensis* (Bong.) Carr.] (Heslin et al., 1983). A large amount of oxalate is also produced by the fungus, which may be important for oxidating aromatics (Hüttermann et al., 1980; Volger et al., 1982). It may

also chelate calcium, enabling pectinases to degrade the host's pectin (Johansson, 1988).

Karlsson et al. (2003) studied the genes that were up-regulated during the early stage of infection of Scots pine (*Pinus sylvestris* L.) seedlings. They found genes encoding hydrophobins, cytochrome P450 monooxygenase, arabinase (polysaccharide degradation), farnesyl-pyrophosphate synthetase (secondary metabolite synthesis) and genes involved in handling oxidative stress (superoxide dismutase). Karlsson et al. (2005) identified the *SOD1* gene, which encodes a manganese-type superoxide dismutase from an early infection stage between *H. irregulare* and *P. sylvestris* seedlings. Putative glutathione-*S*-transferases, laccase, cellulase, cytochrome P450 and superoxide dismutase genes were found to be expressed by *H. parviporum* during infection of Norway spruce [*Picea abies* (L.) Karst] tissue cultures (Karlsson et al., 2007). A cytochrome P450 monooxygenase gene was expressed in the bark of Norway spruce during infection (Karlsson et al., 2008).

The virulence of hybrid isolates of *H. irregulare* (North American P-type) and *H. occidentale* (North American S-type) was shown to be influenced by their mitochondrial inheritance (Olson and Stenlid, 2001). Hybrids with the P-type mitochondria were highly efficient at killing pine seedlings (83%–90% mortality after 20 days) compared with hybrids with the S-type mitochondria (28%–55% mortality) (Olson and Stenlid, 2001). Nuclear factors have also been shown to contribute to virulence. Olson et al. (2005) showed that hybrid progeny isolates from a cross between *H. occidentale* and *H. irregulare* (carrying the S-type mitochondria from *H. occidentale*) displayed a continuous variation of virulence suggesting a multigenic control of virulence. Several nuclear factors were found in quantitative trait loci (QTLs) contributing to virulence of the same progeny isolates used by Olson et al. (2005) (study II). Association mapping between virulence traits and SNPs in *H. annosum s.s.* isolates generated several candidates that could be nuclear virulence factors (study III).

Putative virulence factors in the genome of H. irregulare

The *H. annosum* species complex has the capability to produce phytotoxic secondary metabolites. From the available genome there are genes for secondary metabolites, some of which have not yet been identified (JGI Heterobasidion annosum v2.0: http://genome.jgi-psf.org/Hetan2/Hetan2.home.html). There are, for example, genes for

terpene cyclase (fomannosin) and DMATS (fomannoxin), three polyketide synthase cyclases, 13 nonribosomal peptide synthetases, and three terpene cyclases. Therefore the *H. annosum* species complex has the capacity to produce more secondary metabolites than had previously been found, but the correct growth conditions for the production of these metabolites have yet to be determined. In addition, the genome contains several genes encoding carbohydrate degrading enzymes, for example, five enzymes that were similar to copper radical oxidases that may be involved in degrading lignin. The genome also contains seven genes encoding class II peroxidases with a putative involvement in lignin oxidation.

1.5 Genetic mapping of virulence

In genetic mapping of fungal virulence the aim is to link a phenotypic trait that is associated with virulence to a genotype. Two different approaches can be applied: QTL mapping and association mapping. The difference between the two methods is that in QTL mapping, progeny isolates from a cross between two unrelated isolates are used whereas in association mapping, the mapping population is unrelated (Balding, 2006). The purpose of these methods is to identify novel virulence factors. The advantages of performing association mapping in fungi compared with other organisms are the relatively small genome sizes and the possibility to work with haploid individuals.

To perform QTL mapping a map consisting of genetic markers is essential. A map of physical markers was previously constructed for *H. annosum s.l.* based on the segregation of 358 AFLP markers distributed on 19 larger and 20 smaller linkage groups (Lind et al. 2005). The mapping population consisted of 102 progeny isolates from a cross between the two homokaryotic strains TC_122-12 (*H. occidentale*) and TC_32-1 (*H. irregulare*), which had been tested for compatible mating and genotyped (Chase and Ullrich, 1990).

1.5.1 QTL mapping

QTL mapping is a tool to investigate the genetic basis for quantitative traits. Quantitative traits are controlled by several genes (polygenic) and their corresponding phenotypes often vary along a continuous gradient within or among populations (Falconer and Mackay, 1995). Several QTLs for virulence on pumpkin were found for *Nectria haematococca* MPI (Hawthorne et al., 1997). A study on *Gibberella zeae*, which causes Fusarium head blight,

found a QTL associated with pathogenicity on wheat (Cumagun et al. 2004). Lind et al. (2007) showed that somatic incompatibility in *H. annosum s.l.* is controlled by four loci. The disadvantage of QTL studies is that only a few generations are included in the study and hence only a few recombination events will have occurred, which reduces the resolution of the map. Although this resolution can be increased by testing a greater number of individuals, a higher resolution can be obtained with the greater number of generations and meioses reflected in association mapping, as described below.

In study II, we have identified QTLs for virulence on 1-year-old *P. sylvestris* and 2-year-old *P. abies* seedlings and positioned them on a genetic linkage map of *H. annosum s.l.*

1.5.2 Association mapping

The aim with population association studies is to identify patterns of polymorphisms that vary systematically between individuals with different phenotypes, for example, virulence. By finding markers such as single nucleotide polymorphisms (SNPs) that are closely linked to the trait or phenotype of interest it is then possible to determine what genes are associated with the trait. Logistic regression or Bayesian statistics can be used to deduce these associations (Balding, 2006; Stephens and Balding, 2009). The SNPs found to be associated with virulence can also be related to blocks of linkage disequilibrium (LD). LD is a condition where alleles at two linked loci are non-randomly associated with each other, which occurs if the region is under selective pressure or simply because these alleles are physically close. Generally the resolution potential is higher in association mapping compared with QTL mapping because the distance is shorter between the recombination events that have occurred over the longer evolutionary history seen in a natural population. This longer evolutionary history also leads to smaller linkage blocks than in QTL mapping, enabling more detailed mapping because more markers can be used before the population is saturated (Hall et al., 2010).

In study III, SNP markers were linked to virulence in 23 fully sequenced *H. annosum s.s.* isolates by genome-wide association mapping.

1.6 Transformation methods in fungi

The term *reverse genetics* is applied to studies conducted to discover what phenotypes arise as a result of the specific gene of interest. By contrast, *forward geneticists* investigate the genetic basis of a phenotype or trait. It has been essential to develop gene-specific transformation systems for fungi to be able to manipulate the genome of these organisms so as to be able to correlate *in vitro* studies of DNA with biological consequences *in vivo*. For Basidiomycetes, it has been unexpectedly difficult to obtain reproducible transformation systems that generate a high frequency of transformants. As an alternative, a forward genetics approach can be applied where mutants can be produced using, for example, radiation, followed by investigation of the genetic basis for the resulting phenotypes.

When transforming *Saccharomyces cerevisiae* nearly all plasmids carrying selectable markers are integrated, primarily by homologous recombination, in the genome (Struhl, 1983). Homologous recombination is necessary to effectively analyse gene function. For filamentous fungi, high frequency transformation often results from non-homologous (ectopic) integration of DNA into the genome (Ruiz-Díez, 2002; Kück and Hoff, 2010). Furthermore, the integration is driven mainly by non-homologous end joining whereas site-specific recombination occurs at low frequencies (less than 1%) (Kück and Hoff, 2010). In contrast to homologous recombination, non-homologous end joining does not require a homologous sister chromatid. Double-strand breaks are brought together by protein Ku and sealed by the DNA ligase Lig4 in eukaryotes (Shuman and Glickman, 2007).

Several types of transformants can be distinguished, for example: (a) *knock-in transformants* where a foreign DNA is inserted into the target gene and truncates the transcripts, (b) *knock-out transformants* where the target gene is substituted by a marker (e.g. a selectable marker gene) and the target gene cannot be expressed, and (c) *knock-down transformants* where the expression of the target is reduced by RNAi but the transformants are still carrying the target gene (Kück and Hoff, 2010).

1.6.1 Polyethylene glycol transformation of protoplasts

The most common method of preparing fungal cells for transformation is to prepare the protoplasts using cell-wall degrading enzymes (Ruiz-Díez, 2002). Young mycelial fragments, germinated asexual spores or basidiospores are digested with lytic enzymes, mostly enzyme mixtures containing 1, 3-glucanases and chitinase, for example, Helicase, Glusulase

and Zymolase 100T (Ruiz-Díez, 2002). Uptake of DNA is performed in the presence of calcium ions and the addition of polyethylene glycol (PEG) (Ruiz-Díez, 2002). To regenerate the protoplasts, an osmotically buffered selection medium is added to allow growth of transformed cells only.

1.6.2 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is able to transfer a part of its DNA, T-DNA (transferred DNA), to a wide range of fungi during co-cultivation (Michielse et al., 2005). The T-DNA is integrated randomly and mostly as a single copy and therefore *Agrobacterium*-mediated transformation is suited for insertional mutagenesis in fungi. It is also useful for targeted mutagenesis given that it has been shown to generate high homologous recombination frequencies (Michielse et al., 2005). *Agrobacterium*-mediated transformation has been successfully applied to *H. irregulare* (Samils et al., 2006).

2 Aims

The overall goal of the work presented in this thesis was to identify virulence determinants of *H. annosum s.l.*, to functionally analyse candidate genes and to elucidate the evolutionary history of the species complex. More specifically, the aims were to:

- Date the divergence of species in the *H. annosum* species complex and test whether there has been a co-evolution with the host trees.

- Identify and locate QTLs linked to virulence in *H. annosum s.l.* using infection systems on *P. abies* and *P. sylvestris*.

- Identify and locate SNPs associated with virulence in *H. annosum s.s.* using infection systems on *P. abies* and *P. sylvestris*.

3 Materials and Methods

3.1 Biological material

3.1.1 Fungal isolates

Various isolates of *Heterobasidion* sp. have been used in the different studies and they are representative for their habitats. *H. irregulare* isolates originate from western and eastern North America from sites ranging across its habitats, which also applies to the *H. occidentale* isolates sampled from North America, Canada and Mexico. *H. annosum s.s.*, *H. parviporum* and *H. abietinum* isolates are well represented from habitats across Europe, although there are only a few *H. parviporum* isolates from Asia. The only *H. insulare s.l.* isolate was collected in China and *H. araucariae* isolates came from Australasia; both species were used as outgroups in study I. A few isolates have been used in other studies (Asiegbu et al., 2004; Johannesson and Stenlid, 2003 and Harrington et al., 1998). For study I it was important to have a representative spread across habitats and at least ten individuals from each species in the *H. annosum* species complex. In addition, in study II, progeny isolates from a hybrid strain between TC_32-1 (*H. irregulare*) and TC_122-12 (*H. occidentale*) named AO8 were used (Olson et al., 2005). They were originally chosen because they were compatible for mating and they have a gene set that allows segregation of the IS-P and IS-S genes (Chase and Ullrich, 1990). In addition, the advantage of having unrelated isolates for QTL mapping is that more genetic and phenotypic differences can be detected compared with the number detected when using related isolates. All isolates were grown on Hagem-medium at 21°C in darkness prior to DNA extraction and virulence experiments (Stenlid, 1985).

3.1.2 Plant material

For the infection experiments in study II, 2-year-old *Picea abies* plants (provenance Vitebsk Polotsk and Saleby) and 1-year-old *Pinus sylvestris* plants (provenance Almnäs and Sollerön) were used. In study III, 2-year-old *P. abies* plants (provenance Latvia) and *P. sylvestris* (provenance Gotthardsberg) were used. All the plants were washed and planted in 2 L of fertilized peat and placed in the green house for one month prior to infection.

3.2 Phylogenetic analysis and estimation of divergence times

A multilocus approach was used to study the evolution of the *H. annosum* species complex. DNA was extracted from 10 to 30 isolates per species and *H. insulare* and *H. araucariae* were used as outgroups (Table 2). Several isolates per species were selected to enable detection of within species diversity. Six different genes, transcription factor (*TF*), glutathione-*S*-transferase 1 (*GST1*), elongation factor 1-α (*EFA*), internal transcribed spacer (*ITS*), NADH dehydrogenase subunit 5 (*NAD5*) and glyceraldehyde 3-phosphate dehydrogenase (*G3P*), were amplified by PCR and sequenced. The different genes were selected as representatives of different types of evolution. The genes *TF*, *GST1* and *EFA* are all nuclear genes, *ITS* is a piece of non-functional RNA and *NAD5* is a mitochondrial gene. Single-locus genealogies were constructed for *TF*, *EFA*, *G3P*, *GST1* and *ITS* using the neighbour-joining method implemented in MEGA4. Bayesian methods were used for the multilocus phylogeny of *GST1*, *TF*, *EFA* and *G3P* by using BEAST (Bayesian evolutionary analysis sampling trees) version 1.5.3. This software was also used to estimate the divergence times from the intronic regions by using the upper and lower end of the neutral mutation rates proposed by Kasuga et al. (2002): 0.9×10^{-9} and 16.7×10^{-9} substitutions per site per year, respectively. When estimating the divergence times two different substitution models were tested, the general time reversible (GTR) model and the Hasegawa-Kishino-Yano (HKY) model, and three different clock models: one strict clock model and two relaxed clock models with uncorrelated, branch-specific rates following lognormal or exponential distribution.

There are various tree priors that can be used to model population size changes through time in BEAST. The Bayesian skyline plot calculates the effective breeding population size through time (Drummond and Rambaut, 2007). The HKY model and the site heterogeneity model Gamma with six

gamma categories and a proportion of invariant sites were applied. All three different clock models mentioned were tested for comparison. The estimated mean population sizes varied between 1 million and 40 million individuals. To estimate the divergence times by using a coalescence model, the tree prior "Coalescent: Constant size" was applied in BEAST. In this analysis, the limits to the estimated population sizes were used as a guideline, namely 1 million to 100 million individuals. These upper and lower limits were included as a tree prior and the following MCMC (Markov Chain Monte Carlo) analysis was run as before. In addition, haplotype networks were drawn for each gene using the variable sites. The Median-Joining method was used to construct the networks by using the software Network 4.2.0.1 (Fluxus Technology Ltd, Kiel, Germany).

3.3 Virulence analysis

Virulence assays were performed to measure the ability of the fungus to grow in the living host and to induce lesions. The fungal isolates that were chosen for virulence testing were grown in Petri plates on Hagem medium (Stenlid, 1985) for one week before placing autoclaved wood blocks of *P. sylvestris* (5 × 5 × 5 mm) on the culture and incubating the plates for 4 weeks to allow the blocks to be colonized. The pine and spruce plants were inoculated with the colonized wood through a small opening in the bark, 5 × 10 mm, which was cut in between two nodes (Figure 1A). The infections were replicated in blocks five or ten times, depending on the study, and were run for four weeks. At harvest the lesion length was measured upstem and downstem from the wound (Figure 1B). The stem was then cut into 5-mm pieces and placed on wet filter paper (Figure 1C). After one week, fungal growth in sapwood was measured as the number of stem pieces with conidiophores (Figure 1D, E). Where no lesion, no sapwood growth and no conidiophores in the wound could be seen, inoculations were judged unsuccessful and the stems discarded.

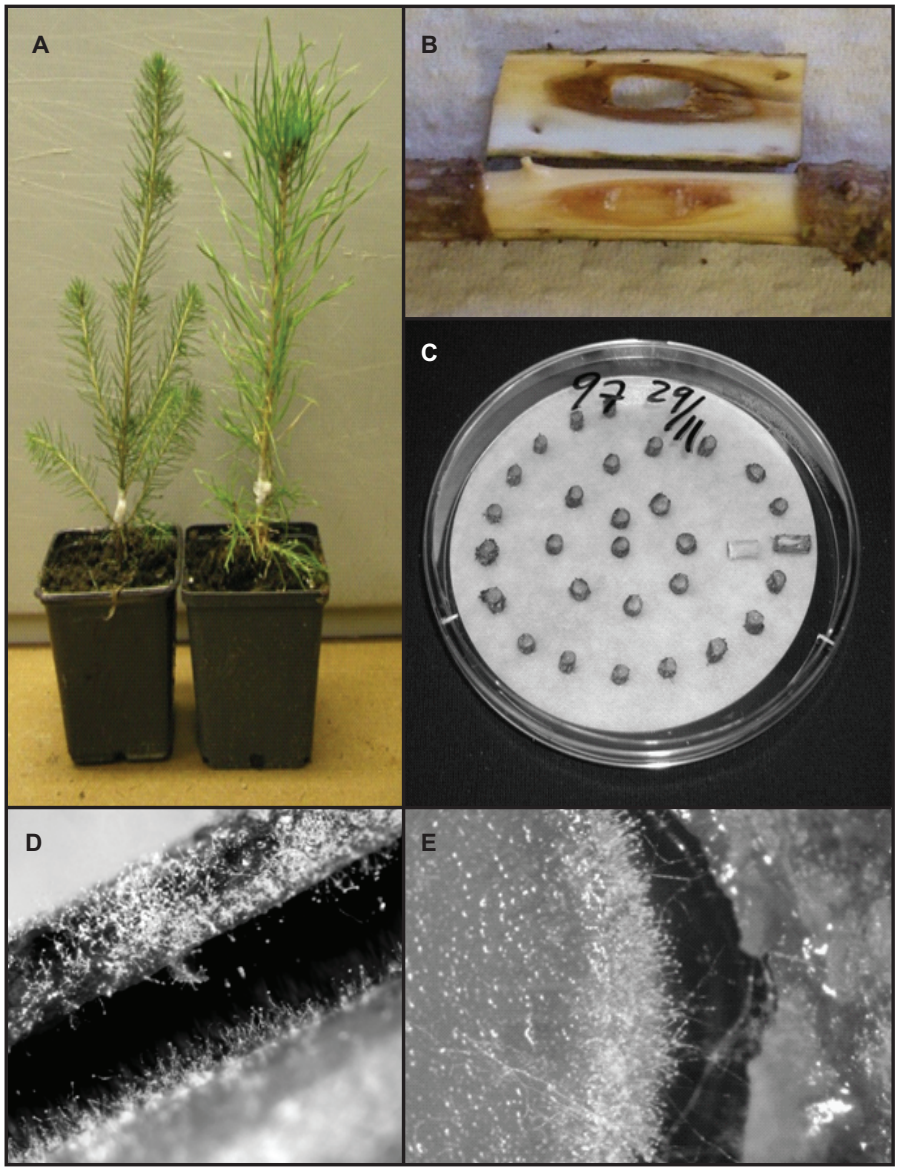

Figure 1. Infection system for *Heterobasidion annosum s.l.* (A) Inoculation with colonized wood block in a wound on the stem. (B) Visible lesion under the bark of pine or spruce. Lesion length was measured upstem and downstem from the infection site. (C) The stem of an inoculated plant that has been cut into 5-mm pieces and placed on wet filter paper. (D) Close up of conidiophores in the wound and bark. (E) Close up of conidiophores on a piece of stem after inoculation.

3.4 Genetic mapping of virulence

3.4.1 QTL analysis

The aim of QTL analysis is to describe the genetic loci that contribute to a quantitative trait (Erickson et al., 2004) by linking a measured trait to genetic markers in a linkage map. A likelihood ratio statistic, LOD (logarithm of odds), is used to determine whether linkage between a trait and markers is significant. When a marker or several linked markers cosegregate with the trait strongly enough for a significance threshold of the LOD to be exceeded, a QTL is detected and defined at the position with the largest LOD (van Ooijen et al., 2002). The threshold level of 1% significant linkage is thus determined by the LOD value exceeded in 1% of random permutations of the trait data, using the MapQTL 4.0 software (van Ooijen et al., 2002). The method of interval mapping (Lander and Botstein, 1988) was used to estimate QTL for virulence. Here, the likelihood for the presence of a segregating QTL is determined for every 1 cM of the genetic linkage map of *H. annosum*. For the traits, lesion length and fungal growth in sapwood, least square mean values were used in the QTL analysis.

3.4.2 Association mapping of virulence

Association mapping was performed to identify and locate SNPs linked to virulence in *H. annosum s.s.* The association between SNP markers and virulence mean estimates were analysed using TASSEL (Trait analysis by association evolution and linkage) version 2.1 (Bradbury et al., 2007). At first the whole genome SNP dataset consisting of a minimum of 2 reads per SNP covering all individuals with a minimum allele frequency of one was used. In addition, for each genomic region found to be significantly associated with virulence an extended dataset consisting of a minimum of two reads per SNP covering a minimum of 15 individuals and a minimum allele frequency of two was used. LD heat maps were constructed for the genomic areas with significant association using TASSEL. The regions significantly associated with virulence were used to blast against the sequenced genome of *H. irregulare*, isolate TC_32-1 (http://genome.jgi-psf.org/Hetan2/Hetan2.home.html) and the genes were annotated and analysed for synonymous and non-synonymous substitutions, as well as for support from expressed sequence tags (ESTs).

3.5 Transformation

PEG-mediated transformation of protoplasts was developed as an alternative to the *Agrobacterium*-mediated transformation protocol (Samils et al., 2006). In addition, we aimed to generate knock-out mutants by homologous recombination by using PEG transformation.

3.5.1 Vectors

Three candidate genes for virulence were chosen from the QTL region for lesion length in pine (Table 2). In principle, the construct was designed to knock out a central part of the gene by homologous recombination and the part that is knocked out is replaced by a marker, such as a selectable marker or a gene encoding a fluorescent protein (Figure 2). The 5' element, the 3' element and the marker were individually cloned and merged in a vector using the MultiSite Gateway® Three Fragment Vector Construction Kit (Invitrogen™, Stockholm, Sweden) (Figure 3). The 5' element is located between the *att*B4 and the *att*B1 site whereas the 3' element is located between the *att*B2 and *att*B3 site (Figure 3). The three-fragment construct was linked to the promoter of GPD (glyceraldehyde 3-phosphate dehydrogenase) and the end of the CYC1-terminator (cytochrome c-1), both from *H. irregulare*. In addition, a selectable and a visual marker were tested separately: the phleomycin resistance gene (ble) and the gene encoding green fluorescent protein, GFP.

Table 2. Candidate genes for knock-out

ProteinId	Description	Location in the genome on scaffold 1	EST evidence	Significantly upregulated in bark
40847	G-protein alpha subunit	74612–75744	Yes, partial	No
58532	Flavin-containing monooxygenase	90785–96890	Yes, partial	Yes
165998	Unknown	190981–191614	No	No

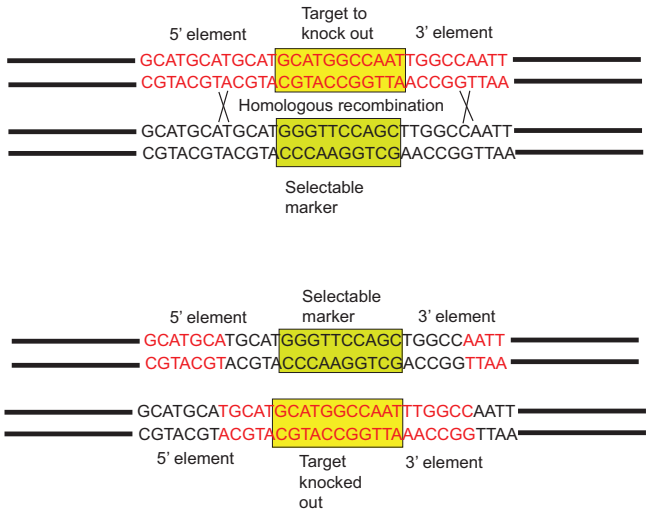

Figure 2. Principle for knock out by homologous recombination.

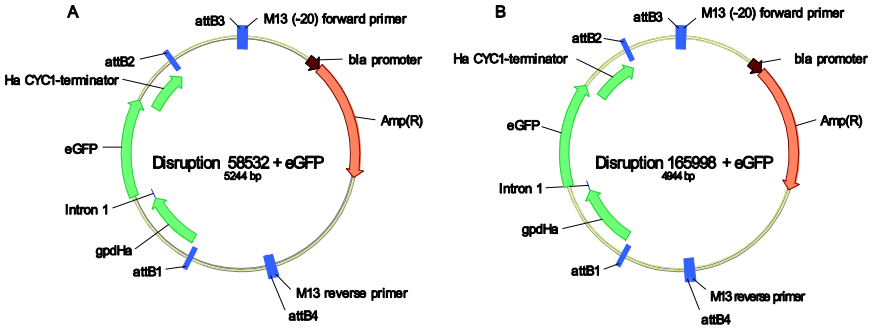

Figure 3. Vector constructs for disruption of (A) the flavin-containing monooxygenase gene (58532) and (B) the unknown gene (165998).

3.5.2 PEG-mediated transformation of protoplasts

The protoplasts were prepared from a three- to four-day-old liquid culture of *H. irregulare* isolate TC_32-1 using a mixture of degrading enzymes consisting of Glucanex, chitinase, BSA and driselase in a buffer containing 0.6 M mannitol dissolved in 0.05 M sodium maleate, pH 5.8. The culture was incubated with these enzymes for 1–2 hours at 26°C with agitation and then filtered and washed in two different buffers (0.6 M mannitol/0.05 M sodium maleate, pH 5.8 and 0.6 M mannitol/100 mM Tris-HCl, pH 8.0/100 mM $CaCl_2$). The protoplasts were visualized after harvesting using fluorescein diacetate (5 mg/mL). An aliquot, 100 µL, of the protoplasts was mixed with 10 µg of the PCR construct DNA together with a nuclease

inhibitor and 50 µL PEG-buffer and incubated on ice for 30 minutes. Thereafter 700 µL PTC was added and incubated at room temperature for 40 minutes. The transformation was regenerated on HMR (Hagem medium with 0.6 M sucrose) for two days at 25°C and then plated with MYGS (1% maltose, 0.4 % glucose, 0.4 % yeast extract, 0.6 M sucrose and 1.5% agar). The transformations were tested for two different backgrounds, TC_32-1 (*H. irregulare*) and TC_122-12 (*H. occidentale*).

4 Results

4.1 Evolutionary history of the *H. annosum* species complex

4.1.1 Phylogenetic analyses

Phylogenetic trees were constructed for the five species in the *H. annosum* species complex: *H. annosum s.s.*, *H. irregulare*, *H. occidentale*, *H. parviporum* and *H. abietinum*. In addition, one isolate from *H. insulare s.l.* and four isolates from *H. araucariae* were used as outgroups. The single-locus Neighbour-Joining genealogies for *TF*, *EFA* and *G3P* each generated a phylogeny where each species grouped into separate clades. By contrast, the phylogenies for *GST1* and *ITS* revealed a mixed clade for *H. abietinum* and *H. parviporum* isolates. However, the combined Bayesian phylogeny for the intronic regions of *GST1*, *TF*, *EFA* and *G3P* generated a tree where each species grouped into a well supported clade (Figure 4). The pine-infecting species (*H. annosum s.s.* and *H. irregulare*) grouped together as well as the pine non-infecting species (*H. abietinum, H. parviporum*, *H. occidentale*). In addition, *H. irregulare* was found to be separated into two subgroups, corresponding to the western or eastern distribution areas of the North American isolates.

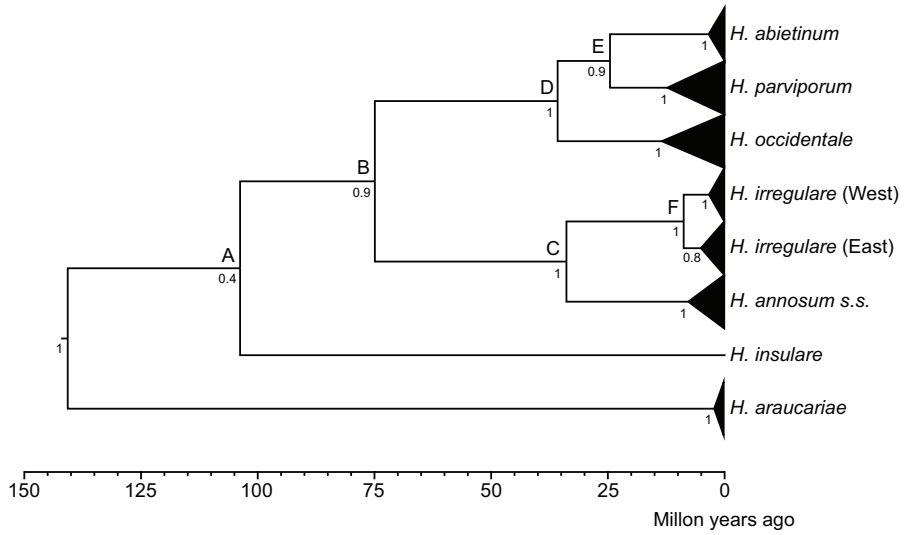

Figure 4. Bayesian phylogeny for the combined non-coding regions of *TF*, *GST1*, *G3P* and *EFA* estimated with BEAST using the HKY substitution model. Numbers correspond to the support for the nodes (posterior probabilities). Divergence times were estimated for the nodes A–F and are shown in Table 3. This tree was generated using the strict clock model and a mutation rate of 0.9×10^{-9} substitutions per site and year. Reproduced from study I.

4.1.2 Divergence times

The molecular clock hypothesis was tested using Tajima's relative rate test (Tajima, 1993). The test could not be rejected, assuming equal substitution rates among the different branches in the studied set of *Heterobasidion* spp. By using the given neutral substitution rates found, 0.9×10^{-9} and 16.7×10^{-9} substitutions per site per year, the limits for the divergence times could be estimated (Kasuga et al., 2002). The Bayesian MCMC sampling generated posterior mean estimates of the time for the most recent common ancestor (TMRCA) and corresponding 95% highest posterior density intervals shown in Table 3 for the HKY model applied in BEAST.

Table 3. Bayesian estimates of divergence times (in millions of years) for the most recent common ancestor (TMRCA). Values are posterior mean estimates of TMRCAs and 95% highest posterior density (HPD) intervals. A combined set of introns was used from the four genes *GST1*, *TF*, *EFA* and *G3P* consisting of 384 sites from 93 *Heterobasidion* spp. isolates. Reproduced from study I

	Mutation rates per site per year			
	0.9×10^{-9}		16.7×10^{-9}	
TMRCA	Mean	95% HPD	Mean	95% HPD
Clock model*				
A.** *H. annosum s.l.*, *H. insulare*	103.8	No interval	7.6	4.8–10.6
B. *H. annosum s.l.*	75.0	54.3–95.5	4.0	2.9–5.1
C. *H. annosum s.s./H. irregulare*	33.9	20.5–47.5	1.8	1.1–2.6
D. *H. parviporum/H. abietinum/H. occidentale*	35.8	23.5–48.2	1.9	1.3–2.6
E. *H. parviporum/H. abietinum*	24.5	14.6–34.5	1.3	0.8–1.9
F. *H. irregulare* (east and west)	8.8	3.2–14.3	0.5	0.2–0.8
Relaxed exponential clock model*				
A. *H. annosum s.l.*, *H. insulare*	162.4	58.1–303.7	8.6	2.9–17.0
B. *H. annosum s.l.*	85.2	34.5–142.8	4.5	1.9–8.0
C. *H. annosum s.s./H. irregulare*	41.3	13.4–71.3	2.2	0.6–4.0
D. *H. par./H. abi./H. occidentale*	42.1	17.8–73.3	2.2	0.9–3.9
E. *H. parviporum/H. abietinum*	27.7	9.8–42.5	–	–
F. *H. irregulare* (east and west)	13.2	2.7–21.0	0.6	0.1–1.2
Relaxed lognormal clock model*				
A. *H. annosum s.l.*, *H. insulare*	159.7	66.1–258.3	8.2	3.6–13.9
B. *H. annosum s.l.*	81.0	43.8–123.4	4.4	2.4–6.7
C. *H. annosum s.s./H. irregulare*	38.2	15.5–65.5	2.0	0.9–3.5
D. *H. par./H. abi./H. occidentale*	39.8	11.7–43.2	2.2	1.0–3.6
E. *H. parviporum/H. abietinum*	26.4	11.7–43.2	1.4	0.6–2.3
F. *H. irregulare* (east and west)	10.1	2.5–17.6	0.5	0.1–1.0

* The substitution model was HKY with the site heterogeneity model Gamma + Invariant sites using six gamma categories.

** The nodes are as indicated in Figure 4.

4.2 Genetic mapping of virulence

4.2.1 QTL mapping

In QTL mapping, a phenotypically observed trait is linked to a genetic marker in the genome of the organism. In study II, the phenotypically observed traits were measured as lesion length and fungal growth in

sapwood around the infection site in spruce and pine. The map of genetic markers was based on the segregation of 358 AFLP markers distributed on 19 larger and 20 smaller linkage groups (Lind et al., 2005). In this AFLP linkage map, Linkage Group (LG) 15 was found to be important for several traits: fungal growth in sapwood and lesion length in spruce and pine (Figure 5 a, b, c, e). In addition, two small LGs were found to harbour QTLs for virulence: LG 36 was linked to fungal growth in pine sapwood and LG 20 was linked to lesion length in pine (Figure 5 d, f). The parental TC_32-1 and TC_122-12 and the hybrid progeny isolates were also included in the infection experiments. The mean virulence across the infection experiments showed that the hybrid homokaryotic progeny had a significantly lower mean virulence than their homokaryotic parents. However, this effect was reversed in the winter-hardened plants.

From the genome sequence of *H. irregulare*, several gene models were found in the QTL involved in the pathogenic interaction with Norway spruce and Scots pine. For LG 20 located on Scaffold 1, 47 gene models were found, for example, genes encoding G-protein alpha, flavin-containing monooxygenase, protein kinases, transcription factors and *N*-acetylglucosaminyltransferase (unpublished data). For LG 15 located on Scaffold 12, 332 gene models were found, including genes encoding G-protein alpha, protein kinase, glutathione-*S*-transferase, cytochrome P450 and chitinase (unpublished data).

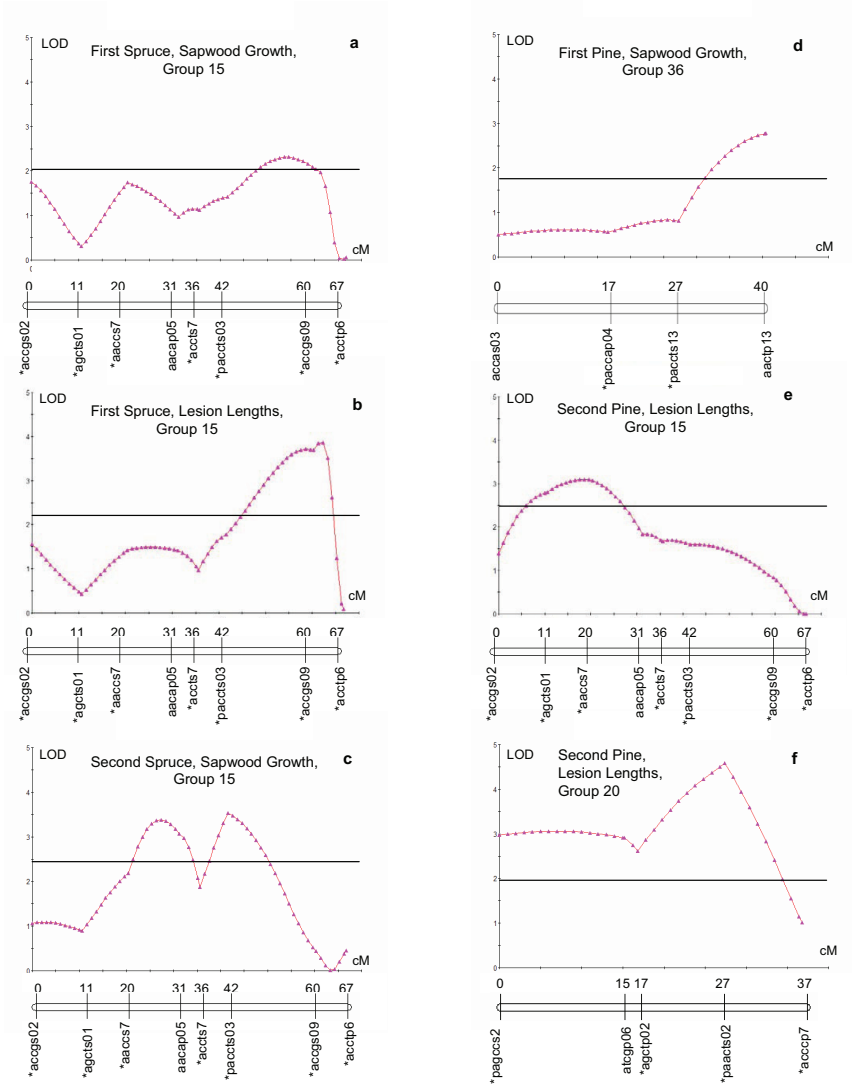

Figure 5. QTLs for virulence to *P. abies* and *P. sylvestris* in *H. annosum s.l.* (a–f) Data for virulence upstem/downstem combined are presented. The solid line denotes the LOD value for the 1% level of significance for the measurements included in each experiment. Reproduced from study II.

4.2.2 Association mapping

Twelve SNP markers out of the aligned 38,472 SNPs from the whole genome of *H. annosum s.s.* were found to be significantly associated with different virulence traits. From these twelve, eight markers that were distributed on three regions were characterized. These three regions were 31, 18, and 2.5 kb long, respectively. One significantly associated marker was found for fungal growth in spruce sapwood in contig 4128 (Figure 6). It was located between two genes, a gene encoding a serine protease and a transcriptional co-repressor. Contig 41480 contained six markers significantly associated with fungal growth in pine sapwood, which were located to genes encoding calcineurin and an acetylglutamate kinase/synthase and to an unknown gene. The significantly associated marker associated with sapwood growth in spruce located to contig 15627 but could not be assigned to any gene because it was only 2.5 kb long and there were some gaps in the data.

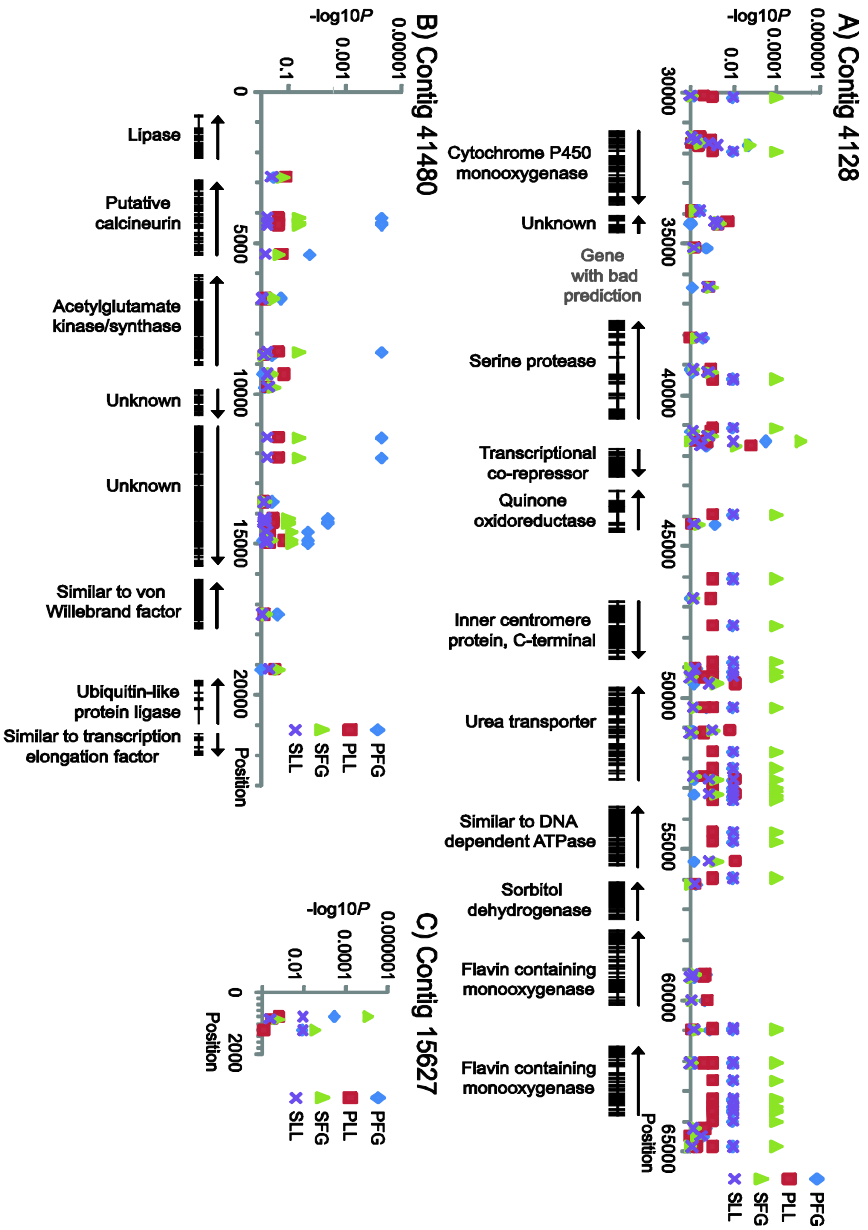

Figure 6. Overview of the three genomic regions significantly associated with *H. annosum s.s.* virulence in spruce and pine. (A) Contig 4128, (B) Contig 41480 and (C) Contig 15627. The upper part of each figure plots the *p*-values for virulence to the genomic position (up- and downstem combined). Abbreviations: PFG, fungal growth in pine sapwood; PLL, lesion length in pine; SFG, fungal growth in spruce sapwood; SLL, lesion length in spruce.

The four regions associated with virulence were located to blocks of LD. In contig 41480 there is an LD-block between 2.2 and 7.0 kb that corresponds to a region significantly associated with sapwood growth in pine. This region spans a putative calcineurin gene and the end of an acetylglutamate kinase/synthase gene. In contig 4128, five LD-blocks were found: between 5.1–6.5 kb, 6.7–7.0 kb, 32.6–33.0 kb, 34.2–34.9 kb and 34.9– 66.1 kb. The significant marker associated with fungal growth in spruce was located in the last block containing nine genes: genes encoding a serine protease, a transcriptional co-repressor, a quinone oxidoreductase, an inner centromere protein, a urea transporter, a gene encoding an enzyme similar to DNA-dependent ATPase, a sorbitol dehydrogenase and two flavin-containing monooxygenases. No LD blocks were found for contig 15627.

4.3 Transformation

Three candidate virulence factor genes were chosen from the QTL for lesion length in pine for the generation of knock-out mutants: genes that encode a G-protein alpha and a flavin-containing monooxygenase, and an unknown gene. The gene encoding flavin-containing monooxygenase and the unknown gene were successfully constructed by the MultiSite Gateway® Three Fragment Vector Construction Kit (Invitrogen™, Stockholm, Sweden). The 5' element and 3' element of these genes were successfully merged together with a selectable and a fluorescent marker respectively: the Ble gene for phleomycin resistance and the green fluorescent protein (GFP). The generation of protoplasts was successful but the number of colonies in the positive control plates varied between 0 and approximately 1000. The optimum number of protoplasts was achieved from cultures that were three days old. For the constructs containing the Ble gene there was initially some growth in a concentration ≤60 μg/mL (up to 30 colonies); however, growth was arrested after transfer onto a higher concentration (125 μg/mL). DNA was extracted from 22 putative transformants for the flavin-containing monooxygenase gene and from 20 for the unknown gene. The result from the following PCR showed that none of them carried the construct: all the putative transformants were wild type. In addition, none of them had the Ble gene integrated ectopically. Because no selection was applied to the transformants with the GFP visual marker, all colonies were examined under the microscope. In total, 203 isolates were screened from the transformation using the gene encoding the flavin-containing monooxygenase and 170 isolates were generated from the transformation using the unknown gene. None of these isolates were

carrying the GFP gene and were therefore scored unsuccessful. By contrast, when using only GFP it was possible to generate approximately 25 isolates showing GFP fluorescence.

5 Discussion

5.1 Evolutionary history of the *H. annosum* species complex

The ancestor of the *Heterobasidion* genus emerged a maximum of 162 million years ago (study I), which is in line with the appearance of gymnosperms (Chaw et al., 1997). The current distribution of the *H. annosum* species complex is consistent with a geographic origin to be located somewhere in Laurasia. This hypothesis is supported by the phylogeny and the haplotype networks. Two groups diverged from the ancestor about 75 million–85 million years ago: one group evolved into infecting pines (*H. annosum s.s./H. irregulare*) and the other group did not (*H. parviporum/H. abietinum/H. occidentale*. This split took place well after the split of the host genera *Pinus* and *Picea*, which has been estimated to have happened between 150 million and 225 million years ago (Axelrod, 1986; Millar, 1993, 1998; Eckert and Hall, 2006). In addition, the *H. insulare* species complex and the *H. annosum* species complex diverged about 104 million–162 million years ago. These two complexes probably diverged because of vicariance and may prevent each other from further spread into the distribution area of the other species complex.

The origin of the *H. parviporum/H. abietinum/H. occidentale* species was either in western North America or in eastern Asia. It could not be interpreted from the haplotype networks which one of these two was the most probable origin and the single genealogies show contradictory branching patterns (data not shown). The ancestral *H. parviporum/H. abietinum/H. occidentale* colonized eastern Asia or North America around 30 million–40 million years ago. The westward spread was probably stopped at the Turgai Sea, which existed 30 million–160 million years ago between what today is Asia and Europe. If the ancestor of *H. occidentale* spread from

Eurasia it was probably via the Trans-Bering Bridges, which were open for exchange between 100 million and 3.5 million years ago. The number of fixed differences between *H. occidentale* and *H. parviporum* is low (2–13), which indicates that these species have been in contact in recent evolutionary time. The current distribution of *H. occidentale* is consistent with the appearance of this species in western North America. There was a dispersal barrier between eastern and western North America, about 35 million years ago (Sanmartín et al., 2001), consisting of an arid region that was probably colonized by pines, which are nonhosts to this species. The current distribution of *H. parviporum* in Europe was probably a recolonization event together with its host after the last glaciation periods from refuges in southern Europe and the southern Ural mountain range. The haplotype analysis suggests that *H. abietinum* is more closely related to *H. parviporum* than to the other species in the *H. annosum* species complex; they even share the same haplotypes in three genes, which can be explained by current gene flow or recent divergence. Nucleotide diversity was lowest for *H. abietinum*, which can be related to its small effective population size on a less abundant host (Oliva et al., 2010). *H. abietinum* arose around 20 million years ago, became specialized to *Abies* species and has had a different biogeographical history to that of *H. parviporum*.

The common ancestor of *H. annosum s.s.* and *H. irregulare* probably emerged in the western part of Eurasia about 60 million years ago and was further spread across the Trans Atlantic bridge, which was a possible route of spread until about 35 million years ago. The Eurasian origin is supported by the haplotype networks. By interpreting the multilocus data from Linzer et al. (2008), the eastern *H. irregulare* population is basal to the western population, which also supports this route of spread. The split between *H. annosum s.s.* and *H. irregulare* was estimated to have occurred about 30 million years ago, which is consistent with this theory. Further spread of *H. annosum s.s.* east in Eurasia was restricted by the Ural Mountains and the Turgai Strait (30 million–160 million years ago) (Sanmartín et al., 2001) and by the *H. insulare* complex partly occupying its niche. Study I proposes a different route of spread for *H. annosum s.s./H. irregulare* compared with those previously published (Otrosina et al., 1993; Linzer et al. 2008).

5.2 Identification of QTL affecting virulence in *H. annosum s.l.*

In study II, specific chromosomal regions were identified for fungal virulence on *P. abies* and *P. sylvestris* in single spore progeny isolates of an *H.*

annosum s.l. hybrid from a cross between *H. irregulare* and *H. occidentale*. Three LGs were identified to be important for virulence. LG 15 was twice shown to be important for fungal growth in sapwood of spruce and once for lesion length in spruce, identifying similar but not identical regions. In addition, LG 15 also proved to be important for lesion lengths in pine, indicating that this region is crucial for general virulence in both *P. abies* and *P. sylvestris*. By interpreting the shape of the QTL profile it looks as though a larger region in LG 15 is important for fungal growth in sapwood of spruce. A smaller region at the end of LG 15 is important for lesion length in spruce and a different region at the beginning of LG 15 is important for lesion length in pine. The genomic region for lesion length in LG 15 seems therefore to be host specific. In addition, LG 20 and LG 36 seem to harbour pine-specific factors. Given that the data is representative for the whole genome, there may be fewer genomic regions that are important for *H. annosum s.l.* virulence in spruce than in pine.

All the LGs identified for virulence in *H. annosum s.l.* have recently been anchored to the sequenced genome of *H. irregulare* (Lind, M., unpublished) and the genes therein have been annotated. Along with the development of a good transformation system, these candidate virulence genes can be tested by gene silencing or knock-out. Attempts at knock-out transformation with three candidates from LG 20, mentioned in this thesis, have so far been unsuccessful.

5.3 A genome-wide association study for virulence in *H. annosum s.s.*

In study III, a genome-wide association study was performed for virulence using 23 *H. annosum s.s.* homokaryotic isolates. Three regions that were identified as being significantly associated with virulence were located to three different contigs. The regions associated with virulence were found to be associated with fungal growth in sapwood of either spruce or pine. For contig 41480, which is associated with fungal growth in pine sapwood, the markers are clustered in two different LD blocks. Two genes were found for this contig encoding a putative calcineurin and an acetylglutamate kinase/synthase. Calcineurin is a phosphatase regulated by Ca^{2+} and calmodulin and is heavily involved in the calcium-dependent signal transduction pathways of eukaryotes (reviewed by Sugiura et al., 2001). The gene that encodes calcineurin is a conserved fungal virulence gene. Calcineurin has been shown to be involved in the virulence of *Candida*

albicans (Karababa et al., 2006). Furthermore, Egan et al. (2009) have shown that when the catalytic subunit of calcineurin was mutated in *Ustilago maydis*, the virulence of the mutants was reduced. Induction of antisense expression of calcineurin in *Sclerotinia sclerotiorum* resulted in reduced pathogenicity (Harel et al., 2006). The calcineurin-responsive transcription factor CRZ1 was found to be required for penetration of plant surfaces in *Botrytis cinerea* (Schumacher et al., 2008). In *Magnaporthe oryzae*, deletion mutants for the genes encoding calcineurin-responsive transcription factors had a reduced virulence (Zhang et al., 2009; Choi et al., 2009). The putative calcineurin found in this study was associated with fungal growth in pine sapwood, verifying its role in fungal virulence.

N-acetylglutamate is involved in the biosynthesis of arginine in prokaryotes, lower eukaryotes and plants. Kim et al. (2007) identified an insertional mutation of the *ARG2* gene encoding an acetylglutamate synthase for pleiotropic phenotypic changes that include reduction of mycelial growth and virulence and prevention of sexual reproduction in *Gibberella zeae* (anamorph, *Fusarium graminearum*), which is an important pathogen of cereal crops.

For contig 4128 there was a 35 kb region associated with fungal growth in spruce sapwood. Eleven genes were found in this region, including genes encoding a cytochrome P450 monooxygenase, an unkown protein, a serine protease/serine carboxypeptidase, a transcriptional co-repressor, a quinone oxidoreductase, an inner centromere protein, a urea transporter, a gene similar to DNA dependent ATPase, a sorbitol dehydrogenase and two flavin-containing monooxygenases.

Cytochrome P450 monooxygenases belong to the haem-containing monooxygenases and use haem to oxidize substrates with the aid of NADH or NADPH. They are important for the biosynthesis of several compounds, such as hormones, defensive compounds and fatty acids in plants (InterPro, IPR001128). In *Botrytis cinerea*, deletion of *bcbot1* encoding cytochrome P450 monooxygenase led to a reduced virulence in one out of three strains tested (Siewers et al., 2005). Meek et al. (2003) showed that the *Fusarium sporotrichioides Tri1* gene encodes a cytochrome P450 monooxygenase that was required for the addition of the oxygen at the C-8 position during biosynthesis of the mycotoxin trichothecene. In addition, cytochrome P450 monooxygenase was found to be up-regulated during the early infection stage of *H. annosum* on *P. sylvestris* seedlings (Karlsson et al., 2003).

However, the gene in contig 4128 was not homologous to the one found by Karlsson et al. (2003).

The quinone oxidoreductase that was found is similar to zinc-binding oxidoreductase ToxD (a host-selective toxin from *Pyrenophora tritici-repentis*). It is a zinc enzyme specific for NADPH that catalyses the one-electron reduction of certain quinones. The best substrates are the orthoquinones 1,2-naphthoquinone and 9,10-phenanthrenequinone. The product is a semiquinone free-radical that can be non-enzymically reduced to the hydroquinone or oxidized back to quinone in the presence of O_2 (Rao et al., 1992).

Two flavin-containing monooxygenases (FMOs) are found at the end of contig 4128. FMOs are xenobiotic-metabolizing enzymes that catalyse the oxygenation of nucleophilic nitrogen, sulfur, phosphorous and selenium atoms using NADPH as a cofactor and FAD as a prosthetic group (InterPro, IPR020946). They have been implicated in the metabolism of several pharmaceuticals, pesticides and other toxicants. *Arabidopsis* mutants overexpressing FMO had an increased basal resistance against *Pseudomonas syringae* pv. tomato and *Hyaloperonospora parasitica*, which suggests that FMO was involved in detoxifying virulence factors produced by the pathogens (Koch et al., 2006). However, perhaps FMOs are also involved in fungal detoxification of plant toxins and, hence, could be candidates for virulence factors in *H. annosum s.s.*

Contig 15627 was a short 2.5 kb sequence that had some missing data. Despite the missing data, an association with virulence traits could be found, mainly with fungal growth in spruce sapwood. No putative function could be found for the gene in this region.

5.4 Speciation, host adaptation and virulence

The evolution of the host tree genera predates the evolution of the *H. annosum* species complex, although the *H. annosum s.l.* species have adapted to the tree species ("host-tracking") with which they co-existed. The incongruence is evident from the fact that *Abies* sp. diverged before *Picea* and *Pinus* whereas the *Abies*-infecting *H. abietinum* was the last species to diverge in the *H. annosum* species complex. Hypothetically, the ancestor of the *H. annosum* species complex co-existed with pine and spruce and

subsequently diverged into pine-infecting and pine non-infecting species. Thereafter the pine non-infecting species came in contact with *Abies* sp. and diverged into *H. abietinum*. By interpreting the host ranges and the phylogeny of the different *H. annosum s.l.* species it seems likely that there has been a loss of efficiency to infect pines along with evolution and the most recent event was the loss of efficiency to infect spruce (*H. abietinum*). Possibly this loss of efficiency to infect pines could be due to a loss of one or several pine-specific virulence factors. QTL mapping of virulence could potentially be used to find such host specific virulence factors because the mapping population comes from a cross between a North American pine-infecting species, *H. irregulare*, and a North American pine non-infecting species, *H. occidentale*. Sexual incompatibility has emerged between the two species in nature which will limit the mixing of their respective populations and thereby preserve the specificity of their virulence factors.

The hybrid progeny isolates from a cross between *H. occidentale* and *H. irregulare* used in study II were less virulent than their parental isolates as interpreted from the mean virulence estimates. Therefore hybridization may have a negative effect on virulence. There is only one record of a natural *H. occidentale–H. irregulare* hybrid (Garbelotto et al., 1996), which may support the suggestion that hybridization has a negative effect on virulence although other factors cannot be excluded. However, when comparing the mean virulence of the hybrid progeny in study II to the *H. annosum s.s.* in study III there is almost no difference. This small difference might be because they are genetically different. In addition, *H. annosum s.s.* was found to be less virulent than *H. irregulare* TC_32-1, the parental isolate with the highest mean values for virulence traits in the assay. This effect may be explained by the fact that European spruce and pine plants were used in the study, which have had time to develop resistance against *H. annosum s.s.* but not against *H. irregulare*. It would be interesting to test whether this effect was due to a developed resistance against *H. annosum s.s.* in the European plant material by comparing with virulence of the *H. annosum s.s.* isolates on North American plant material. Swedjemark et al. (1999) tested *H. annosum s.s.* and *H. parviporum* virulence on *P. abies* and *P. sylvestris*. *H. annosum s.s.* was more virulent than *H. parviporum* on pine. The virulence on spruce was not significantly different between the two species. This result may suggest that either *H. parviporum* has lost its efficency to grow on pine or that pine is more resistant against *H. parviporum*.

In study II, several QTL were found to be associated with fungal virulence on spruce and pine. The progeny used in this study was generated from a cross between *H. irregulare* and *H. occidentale*. By using an improved linkage map and the same virulence data, further QTLs have been found (M. Lind, unpublished): two are located close to the regions found in study III (21 and 115 kb apart). These QTLs were both associated with lesion length in pine and fungal growth in spruce sapwood. The closely corresponding regions found in study III were associated with FGS in pine (contig 41480) and FGS in spruce (contig 15627). Therefore these regions seem to harbour general virulence factors for the *H. annosum* species complex. Indeed, two genes (encoding calcineurin and acetylglutamate kinase/synthase) found in these regions are documented virulence factors found in two or more fungal species. In addition, one gene (encoding quinone oxidoreductase) was similar to a gene that encodes ToxD, a host-selective toxin produced by *Pyrenophora tritici-repentis*.

One factor that can affect a pathogen population (as outlined in the introduction) is population size. The population size of the different species in the *H. annosum* species complex was estimated to vary by between 10 million and 100 million individuals, where *H. abietinum* had the smallest and *H. annosum s.s.* the largest population. Therefore *H. annosum s.s.* presumably has a greater evolutionary potential. In addition, bottlenecks will lead to a less diverse population that will have more difficulties adapting than stable and large populations. All populations have experienced a bottleneck. Tajima's D values for four species, *H. parviporum*, *H. annosum s.s.*, *H. occidentale* and *H. abietinum*, gave negative mean values, indicating population size expansion or positive selection. For *H. irregulare*, Tajima's D values were around zero, indicating a stable population size or neutral evolution.

H. abietinum and *H. parviporum* were shown to share the same haplotypes in three genes in study I, which can be explained by current gene flow or recent divergence of the species. Gene flow will increase the genetic neighbourhood, which in turn leads to greater genetic diversity and thereby an increased evolutionary potential. The closer a *H. parviporum* population is located to a population of *H. abietinum* occurring in central Europe, the lower degree of sexual compatibility it shows towards *H. abietinum:* in sympatry in the Alps the compatibility is only 24% (Oliva et al. 2010) but allopatric populations of *H. parviporum* from Scandinavia, Urals and China show a high compatibility towards *H. abietinum* (73-95%) (Dai et al. 2003).

As in agreement with the phylogeny in study I, *H. abietinum* has emerged from *H. parviporum* in Europe as a specialization on its host. In addition to the loss of efficiency on spruce, *H. abietinum* possibly developed *Abies*-specific virulence factors that are still unknown. Furthermore, the size of the genetic neighbourhood can be affected by anthropogenic activities and, therefore, it is important that import and export of infected wood is prevented. *H. irregulare* has been found in Italy, probably via untreated wood brought by the US army during World War II (Gonthier et al., 2004).

6 Conclusions

On study I, the results of the multi-locus approach used to estimate the divergence times of the *H. annosum* species complex indicate that human-mediated forest practises can not be the major driving force for the divergence of the different *H. annosum s.l.* species, although adaption to the host did occur after the ancestor of *H. annosum/H. irregulare* had separated from the *H. parviporum/H. abietinum/H. occidentale* ancestor. It is likely that plate tectonics was the main factor for separating the different species in the *H. annosum* species complex. The origin of the ancestor of *H. annosum s.l.* is likely to have occurred in Laurasia. The pine infecting (*H. annosum s.s.* and *H. irregulare*) and pine non-infecting species (*H. parviporum/H. abietinum/H. occidentale*) emerged between about 45 million and 60 million years ago in the Palaearctic, well after the host genera. From the data it can be concluded that *H. irregulare* and *H. occidentale* colonized North America via different routes. *H. irregulare* originated from a western distribution in Eurasia about 60 million years ago and was spread across the Trans Atlantic land bridge to north-eastern North America (35 million–50 million years ago). The ancestral *H. parviporum/H. abietinum/H. occidentale* species colonized eastern Asia and western North America 22 million–43 million years ago. This colonization from Asia to North America was eastward, across the Trans-Bering Bridges; any further spread eastward was restricted by the Rocky Mountains. Alternatively, *H. occidentale* originated from North America. The North American population evolved into *H. occidentale*.

In study II, QTLs that control fungal growth in sapwood and induced formation of lesions on *P. sylvestris* and *P. abies* were identified. The genomic region controlling virulence in spruce that was found in this study was concentrated mainly in one region. By contrast, three unrelated regions are important for infecting pine. Two regions were indentified on LG 15

that were linked to fungal growth in sapwood and formation of lesions in spruce. These regions were also identified as important for fungal growth in sapwood of winter hardened spruce. The linkage groups have been successfully anchored to the whole genome sequence of *H. irregulare*, which is publicly available online (http://genome.jgi-psf.org/Hetan2/Hetan2.home.html) (manuscript in preparation). LG 15 corresponds to a region on scaffold 12 of the *H. irregulare* genome. The region in LG 15 is important for general host infection because it has also been linked to lesion length in pine. In addition, two linkage groups, LG 36 and LG 20, are important for virulence on pine. The end of LG 36 controls fungal growth in sapwood and the complete LG 20 is important for lesion length. LG 20 and LG 36 correspond to a region in scaffold 1 and scaffold 8, respectively, of the *H. irregulare* genome.

In Study III, a genome-wide association study was performed for four virulence traits (i.e. lesion length and fungal growth in sapwood of spruce and pine) by using 23 *H. annosum s.s.* homokaryotic haploid isolates. Three genomic regions were found to be significantly associated with fungal virulence located in LD blocks ranging in length between 1.2 and 31.2 kb. Genes found in these LD blocks are hypothetically candidates for virulence factors given that they are genetically linked to the significantly associated SNP. Seven of these genes are promising candidates and encode calcineurin, acetylglutamate kinase/synthase, cytochrome P450 monooxygenase, serine carboxypeptidase, quinone oxidoreductase and two flavin-containing monooxygenases.

7 Future prospects

The evolution of the *H. annosum* species complex has been extensively studied compared with the *H. insulare* species complex. It would be interesting to investigate the origin and the phylogenetic relationships of the *H. insulare* species complex, inferring possible routes of spread as well as dating the divergence of these species. To be able to make a comparison with the *H. annosum* species complex, the same genes should preferably be sequenced.

The AFLP markers-based genetic linkage map has been anchored to the genome of *H. irregulare* and the map has been extended with additional simple sequence repeat markers (Lind, M., unpublished). In addition, the resolution of this map could be increased by increasing the number of offspring in the original cross to strengthen and sharpen the QTL identified so far.

The *H. irregulare* genome sequencing project is about to be published. This published genome should help to facilitate many aspects of molecular research in the future, for example, evolutionary questions, genes involved in interactions such as mating type and somatic compatibility factors, and comparative and functional genomics.

For association mapping, the next step could be to adopt a two-stage strategy where a few individuals are typed genome-wide, with the remaining individuals (e.g. up to 100) only typed at SNPs that seems promising from the first phase. The decreasing costs of genome wide sequencing and the relatively small genome size of *H. annosum* mean that it should be possible to perform whole genome sequencing of more isolates. The sequences from the whole genomes of the 23 isolates sequenced so far

still contain some gaps that need to be filled, for example, by paired-end sequencing. In the future it may also be possible to improve the virulence assay.

When it comes to functional genomics, the transformation system should be improved. There are several alternatives that could be tested, for example: testing other transformation systems, such as biolistics, electroporation, *Agrobacterium*-mediated transformation, testing other selectable markers and testing RNAi as an alternative to knock-out transformation. RNAi would be a good alternative because DNA has been shown to be integrated ectopically for GFP only. For the knock-out transformation to be improved, longer flanking regions could be tested to improve the homologous recombination rate. The DNA could also be targeted to a DNA sequence present in multiple copies. The use of autonomously replicating sequences (ARS) has been used for *S. cerevisiae* to generate transformants at high frequency (Ruiz-Díez, 2002). It may also be possible to use ARS when transforming *H. annosum*. The protein Ku brings together double-strand breaks during non-homologous end joining. To prevent non-homologous end joining, and thereby increase the frequency of site-specific recombination, *ku* genes can be disrupted. This strategy has proven successful for *Neurospora crassa* (Kück and Hoff, 2010).

8 References

Agrios, G. N. (1997) Plant Pathology, 4th ed. Academic Press, San Diego, USA

Asiegbu, F.O., Daniel, G. and Johansson, M. (1993) Studies on the infection of Norway spruce roots by *Heterobasidion annosum*. *Canadian Journal of Botany* 71, 1552–1561.

Asiegbu, F.O., Johansson, M., Woodward, S., Hütterman, A. (1998) Biochemistry of the host–parasite interaction. In: *Heterobasidion annosum, Biology, Ecology, Impact and Control* (Woodward. S., Stenlid, J., Karjalainen, R., Hüttermann, A., eds), CAB International, Wallingford, Oxon, UK, pp.167–195.

Asiegbu, F.O., Abu, S., Stenlid, J., Johansson, M. (2004) Sequence polymorphism and molecular characterisation of laccase genes of the conifer pathogen *Heterobasidion annosum*. *Mycological Research* 108, 136–148.

Asiegbu, F.O., Adomas, A., Stenlid, J. (2005) Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. *s.l. Molecular Plant Pathology* 6, 395–409.

Axelrod, D.I. (1986) Cenozoic history of some western American pines. *Annals of the Missouri Botanical Garden* 73, 565–641.

Balding, D.J. (2006) A tutorial on statistical methods for population association studies. *Nature Genetics* 7, 781–791.

Basset, C., Sherwood, R.T., Kepler, J.A., Hamilton, P.B. (1967) Production and biological activity of fomannosin, a toxic sesquiterpene metabolite of *Fomes annosus*. *Phytopathology* 57, 1046–1052.

Bell, C.D., Soltis, D.E., Soltis, P.S. (2005) The age of the angiosperms: A molecular timescale without a clock. *Evolution* 59, 1245–1258.

Berbee, M.L., Taylor, J.W. (2001) Fungal molecular evolution: gene trees and geologic time. In: *The Mycota* (McLaughlin, D., McLaughlin, E., Lemke, P., eds), Springer-Verlag, Berlin, pp. 229–245.

Bradbury, P.J., Zhang, Z., Kroon, D.E., Casstevens, R.M., Ramdoss, Y., Buckler, E.S. (2007) TASSEL software for association mapping of complex traits in diverse samples. *Bioinformatics* 23, 2633–2635.

Brefeld, O. (1888) Untersuchungen aus dem Gesamtgebiet der Mykologie. Basidiomyceten III. Autobasidiomyceten und die Begründung des natürlichen Systems der Pilze. Heft 8, 305 pp. Leipzig (1888–1889).

Buchanan, P.K. (1988) A new species of *Heterobasidion* (Polyporaceae) from Australasia. *Mycotaxon* 32, 325–337.

Capretti, P., Korhonen, K., Mugnai, L., Romagnioli, C. (1990) An intersterility group of *Heterobasidion annosum*, specialized to *Abies alba*. *European Journal of Forest Pathology* 20, 231–240.

Chase, T.E., Ullrich, R.C. (1988) *Heterobasidion annosum*, root- and butt rot of trees. *Advances in Plant Pathology* 6, 501–510.

Chase, T.E., Ullrich, R.C. (1990) Five genes determining intersterility in *Heterobasidion annosum*. *Mycologia* 82, 73–81.

Chaw, M.S., Zharkikh, A., Sung, H.-M., Lau, T.-C., Li, W.-H. (1997) Molecular phylogeny of extant gymnosperms and seed plant evolution: analysis of nuclear 18S rRNA sequences. *Molecular Biology and Evolution* 14, 56–68.

Choi, J., Kim, Y., Kim, S., Park, J., Lee, Y.-H. (2009) *MoCRZ1*, a gene encoding a calcineurin-responsive transcription factor, regulates fungal growth and pathogenicity of *Magnaporthe oryzae*. *Fungal Genetics and Biology* 46, 243–254.

Ciuffetti, L.M., Manning, V.A., Pandelova, I., Betts, M.F., Martinez, J.P. (2010) Host-selective toxins, Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the *Pyrenophora tritici-repentis*–wheat interaction. *New Phytologist* 187, 911–919.

Corner, E.J.H. (1989) Ad Polyporaceas V. *Beihefte zur Nova Hedwigia* 96, 219 pp.

Cumagun, C.J.R., Bowden, R., Jurgenson, J., Leslie, J., Miedaner, T. (2004) Genetic mapping of pathogenicity and aggressiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. *Phytopathology* 94, 520–526.

Dangl, J.L., Jones, J.D. (2001) Plant pathogens and integrated defence responses to infection. *Nature* 411, 826–833.

Daniel, G., Asiegbu, F.O., Johansson, M. (1998) The saprotrophic wood degrading abilities of *Heterobasidion annosum* intersterility groups P and S. *Mycological Research* 102, 991–997.

Dai, Y.-C., Vainio, E. J., Hantula, J., Niemelä, T., Korhonen, K. (2003) Investigations on *Heterobasidion annosum s.lat.* in central and eastern Asia with the aid of mating tests and DNA fingerprinting. *Forest Pathology* 33, 269-286.

Dai, Y.-C., Korhonen, K. (2009) *Heterobasidion australe*, a new polypore derived from the *Heterobasidion insulare* complex. *Mycoscience* 50, 353–356.

Dai, Y.-C., Yu, C.-J., Wang, H.-C. (2007) Polypores from eastern Xizang (Tibet), western China. *Annales Botanici Fennici* 44, 135–145.

Deacon, J. (1997) *Modern Mycology*, Blackwell Science, Oxford, 303 pp.

de Jonge, R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M.H.A.J., Thomma, B.P.H.J. (2010) Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science* 329, 953–955.

de Wit, P.J.G.M., Mehrabi, R., van den Burg, H.A., Stergiopoulos, I. (2009) Fungal effector proteins: past, present and future. *Molecular Plant Pathology* 10, 735–747.

Drummond, A.J., Rambaut, A. (2007) "BEAST: Bayesian evolutionary analysis by sampling trees." *BMC Evolutionary Biology* 7, 214.

Eckert, A.J., Hall, B.D. (2006) Phylogeny, historical biogeography, and patterns of diversification for *Pinus* (Pinaceae): Phylogenetic tests of fossil-based hypotheses. *Molecular Phylogenetics and Evolution* 40, 166–182.

Egan, J.D., García-Pedrajas, M.D., Andrews, D.L., Gold, S.E. (2009) Calcineurin is an antagonist to PKA protein phosphorylation required for postmating filamentation and virulence, while PP2A is required for viability in *Ustilago maydis*. *Molecular Plant-Microbe interactions* 22, 1293–1301.

Erickson, D.L., Fenster, C.B., Stenøien, H.K., Price, D. (2004) Quantitative trait locus analyses and the study of evolutionary process. *Molecular Ecology* 13, 2505–2522.

Falconer, D.S., Mackay, T.F.C. (1995) *Introduction to Quantitative Genetics*, 4th edn. Addison-Wesley Longman, Harlow, UK.

Flor, H. (1971) Current status of the gene for gene concept. *Annual Review of Phytopathology* 9, 275–296.

Garbelotto, M., Ratcliff, A., Bruns, T.D., Cobb, F.W., Otrosina, W.J. (1996) Use of taxon-specific competitive-priming PCR to study host specificity, hybridization, and intergroup gene flow in intersterility groups of *Heterobasidion annosum*. *Genetics* 86, 543–551.

Garbelotto, M., Otrosina, W.J., Cobb, F.W., Bruns, T.D. (1998) The European S and F intersterility groups of *Heterobasidion annosum* may represent sympatric protospecies. *Canadian Journal of Botany* 76, 397–409.

Gonthier, P., Warner, R., Nicolotti, G., Mazzaglia, A., Garbelotto, M. (2004) Pathogen introduction as a collateral effect of military activity. *Mycological Research* 108, 468–470.

Hall, D., Tegström, C., Ingvarsson, P.K. (2010) Using association mapping to dissect the genetic basis of complex traits in plants. *Briefings in Functional Genomics* 9, 157–165.

Harel, A., Bercovich, S., Yarden, O. (2006) Calcineurin is required for sclerotial development and pathogenicity of *Sclerotinia sclerotiorum* in an oxalic-acid independent manner. *Molecular Plant-Microbe Interactions* 19, 682–693.

Harrington, T.C., Stenlid, J., Korhonen, K. (1998) Evolution in the genus *Heterobasidion*. In: *Root and Butt Rots of Forest Trees. Proceedings of the 9th International Conference on Root and Butt Rots* (Delatour, C., Guillaumin, J.J., Lung-Escarmant, B., Marcais, B., eds), INRS, France, pp. 63–74.

Hawthorne, B., Rees-George, J., Bowen, J., Ball, R. (1997) A single locus with a large effect on virulence in *Nectria haematococca* MPI. *Fungal Genetics Newsletter* 44, 24–26.

Heslin, M.C., Stuart, M.R. (1983) Fomannoxin production by different spruce isolates of *Fomes annosus*. *European Journal of Forest Pathology* 13, 403–409.

Heslin, M.C., Stuart, M.R., Murchú, P.O., Donnely, D.M.X. (1983) Fomannoxin, a phytotoxic metabolite of *Fomes annosus*: *in vitro* production, host toxicity and isolation from naturally infected Sitka spruce heart wood. *European Journal of Forest Pathology* 13, 11–23.

Hibbet, D.S. (2006) A phylogenetic overview of the Agaricomycotina. *Mycologia* 98, 917–925.

Hibbet, D.S., Donoghue, M.J. (1995) Progress towards a phylogenetic classification of the polyporaceae through parsimony analysis of mitochondrial ribosomal DNA sequences. *Canadian Journal of Botany* 73 (Suppl. 1), S853–S861.

Holdenrieder, O. (1982) Kristallbildung bei *Heterobasidion annosum* (Fr.) Bref. (*Fomes annosus* P. Karst.) und anderen holzbewohnenden Pilzen. *European Journal of Forest Pathology* 12, 41–58.

Hüttermann, A., Herche, C., Haars, A. (1980) Polymerisation of water-insoluble lignins by *Fomes annosus*. *Holzforschung* 34, 64–66.

JGI *Heterobasidion annosum* v.2.0 Genome Browser, http://genome.jgi-psf.org/Hetan2/Hetan2.home.html

Johannesson, H., Stenlid, J. (2003) Molecular markers reveal genetic isolation and phylogeography of the S and F intersterility groups of the wood-decay fungus *Heterobasidion annosum*. *Molecular Phylogenetics and Evolution* 29, 94–101.

Johansson, M. (1988) Pectic enzyme activity of spruce (S) and pine (P) strains of *Heterobasidion annosum*. *Physiological and Molecular Plant Pathology* 33, 333–349.

Jones, J.D.G., Dangl, J.L. (2006) The plant immune system. *Nature* 444, 323–329.

Karababa, M., Valentino, E., Pardini, G., Coste, A.T., Bille, J., Sanglard, D. (2006) *CRZ1*, a target of the calcineurin pathway in *Candida albicans*. *Molecular Microbiology* 59, 1429–1451.

Karlsson, J.-O., Stenlid, J. (1991) Pectic isozyme profiles of the intersterility groups in *Heterobasidion annosum*. *Mycological Research* 95, 531–536.

Karlsson, M., Olson, Å., Stenlid, J. (2003) Expressed sequences from the basidiomycetous tree pathogen *Heterobasidion annosum* during early infection of Scots pine. *Fungal Genetics and Biology* 39, 51–59.

Karlsson, M., Stenlid, J., Olson, Å. (2005) Identification of a superoxide dismutase gene from the conifer pathogen *Heterobasidion annosum*. *Physiological and Molecular Plant Pathology* 66, 99–107.

Karlsson, M., Hietala, A.M., Kvaalen, H., Solheim, H., Olson, Å., Stenlid, J., Fossdal C.G. (2007) Quantification of host and pathogen DNA and RNA transcripts in the interaction of Norway spruce with *Heterobasidion parviporum*. *Physiological and Molecular Plant Pathology* 70, 99–109.

Karlsson, M., Elfstrand, M., Stenlid, J., Olson, Å. (2008) A fungal cytochrome P450 is expressed during the interaction between the fungal pathogen *Heterobasidion annosum sensu lato* and conifer trees. *DNA Sequence*, 19, 115–120.

Kasuga, T., Woods, C., Woodward, S., Mitchelson, K. (1993) *Heterobasidion annosum* 5.8s ribosomal DNA and internal transcribed spacer sequence: rapid identification of European intersterility groups by ribosomal DNA restriction polymorphism. *Current Genetics* 24, 433–436.

Kasuga, T., White, T.J., Taylor, J.W. (2002) Estimation of nucleotide substitution rates in eurotiomycete fungi. *Molecular Biology and Evolution* 19, 2318–2324.

Kim, J.-E., Myong, K., Shim, W.-B., Yun, S.-H., Lee, Y.-W. (2007) Functional characterization of acetylglutamate synthase and phosphoribosylamine-glycine ligase genes in *Gibberella zeae*. *Current Genetics* 51, 99–108.

Knoche, H., Duvick, J. (1987) The role of fungal toxins in plant disease. In: *Fungal Infection of Plants* (Pegg, G., Ayres, P., eds), Cambridge University Press, Cambridge, pp. 158–191.

Koch, M., Vorwerk, S., Masur, C., Sharifi-Sirchi, G., Olivieri, N. Schlaich, N.L. (2006) A role for a flavin-containing mono-oxygenase in resistance against microbial pathogens in *Arabidopsis*. *The Plant Journal* 47, 629–639.

Korhonen, K. (1978) Intersterility groups of *Heterobasidion annosum*. *Communicationes Instituti Forestalis Fenniae* 94, 25 pp.

Korhonen, K., Stenlid, J. (1998) Biology of *Heterobasidion annosum*. In: *Heterobasidion annosum, Biology, Ecology, Impact and Control* (Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A., eds), CAB International, Wallingford, Oxon, UK, pp. 43–70.

Korhonen, K., Capretti, P., Karjalainen, R., Stenlid, J. (1998) Distribution of *Heterobasidion annosum* intersterility groups in Europe. In: *Heterobasidion annosum, Biology, Ecology, Impact and Control* (Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A., eds), CAB International, Wallingford, Oxon, UK, pp. 93–104.

Kück, U., Hoff, B. (2010) New tools for the genetic manipulation of filamentous fungi. *Applied Microbiology and Biotechnology* 86, 51–62.

Lander, E.S., Botstein, D. (1988) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* 121, 185–199.

Lind, M., Olson, Å., Stenlid, J. (2005) An AFLP-markers based genetic linkage map of *Heterobasidion annosum* locating intersterility genes. *Fungal Genetics and Biology* 42, 519–527.

Lind, M., Stenlid, J., Olson, Å. (2007) Genetics and QTL mapping of somatic incompatibility and intraspecific interactions in the basidiomycete *Heterobasidion annosum s.l. Fungal Genetics and Biology* 44, 1242–1251.

Linzer, R.E., Otrosina, W.J., Gonthier, P., Bruhn, J., Laflamme, G., Bussieres, G., Garbelotto, M. (2008) Inferences on the phylogeography of the fungal pathogen *Heterobasidion annosum*, including evidence of the interspecific horizontal genetic transfer and of human-mediated, long-range dispersal. *Molecular Phylogenetics and Evolution* 46, 844–862.

Maijala, P., Raudaskoski, M., Viikari, L. (1995) Hemicellulolytic enzymes in P- and S-strains of *Heterobasidion annosum*. *Microbiology*, 141, 743–750.

Maijala, P., Harrington, T.C., Raudaskoski, M. (2003) A peroxidase gene family and gene trees in *Heterobasidion* and related genera. *Mycologia*, 95, 209–221.

Matzinger, P. (2007) Friendly and dangerous signals: is the tissue in control? *Nature Immunology* 8, 11–13.

McDonald, B.A., Linde, C. (2002) Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology* 40, 349–379.

Meek, I.B., Peplow, A.W., Ake, C., Jr., Phillips, T.D., Beremand, M.N. (2003) *Tri1* encodes the cytochrome P450 monooxygenase for C-8 hydroxylation during trichothecene biosynthesis in *Fusarium sporotrichioides* and resides upstream of another new *Tri* gene. *Applied and Environmental Microbiology* 69, 1607–1613.

Michielse, C.B., Hooykaas, P.J.J., van den Hondel, C.A.M.J.J., Ram, A.F.J. (2005) *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Current Genetics*, 48, 1–17.

Millar, C.I. (1993) Impact of the Eocene on the evolution of *Pinus* L. *Annals of the Missouri Botanical Garden*, 80, 471–498.

Miller, S.L., Larsson, E., Larsson, K-H., Verbeken, A., Nuytinck, J. (2006) Perspectives in the new Russulales. *Mycologia*, 98, 960–970.

Niemelä, T., Korhonen, K. (1998) Taxonomy of the Genus *Heterobasidion*. In: *Heterobasidion annosum, Biology, Ecology, Impact and Control* (Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A., eds), CAB International, Wallingford, Oxon, UK, pp. 1–25.

Nord, F.F., Hata, K. (1969) Fungal degradation of pine bark lignin. In: *Current Aspects of Biochemical Energetics* (Kaplan, N.O., Kennedy, E.P., eds), Academic Press, New York, pp. 315–329.

Oliva, J., Gonthier, P., Stenlid, J. (2010) Gene flow and inter-sterility between allopatric and sympatric populations of *Heterobasidion abietinum* and *H. parviporum* in Europe. *Forest Pathology* doi: 10.1111/j.1439-0329.2010.00682.x.

Olson, Å., Stenlid, J. (2001) Mitochondrial control of fungal hybrid virulence. *Nature* 411, 438.

Olson, Å., Lind, M., Stenlid, J. (2005) In vitro test of virulence in the progeny of a *Heterobasidion* interspecific cross. *Forest Pathology* 35, 321–331.

Ota, Y., Buchanan, P.K., Hattori, T. (2006) Phylogenetic relationships of Japanese species of *Heterobasidion-H. annosum sensu lato* and an undetermined *Heterobasidion* sp. *Mycologia*, 98, 717–725.

Otrosina, W.J., Garbelotto, M. (2010) *Heterobasidion occidentale* sp. nov. and *Heterobasidion irregulare* nom. nov.: A disposition of North American *Heterobasidion* biological species. *Fungal Biology* 114, 16–25.

Otrosina, W.J., Chase, T.E., Cobb, F.W., Korhonen, K. (1993) Population structure of *Heterobasidion annosum* from North America and Europe. *Canadian Journal of Botany* 71, 1064–1071.

Rao, P.V., Krishna, C.M., Zigler, J.S., Jr. (1992) Identification and characterization of the enzymatic activity of ζ-crystallin from guinea pig lens. A novel NADPH:quinone oxidoreductase. *The Journal of Biological Chemistry* 267, 96–102.

Ruiz-Díez, B. (2002) Strategies for the transformation of filamentous fungi. *Journal of Applied Microbiology* 92, 189–195.

Samils, N., Elfstrand, M., Lindner Czederpiltz, D.L., Fahleson, J., Olson, Å., Dixelius, C., Stenlid, J. (2006) Development of a rapid and simple *Agrobacterium tumefaciens* mediated transformation system for the fungal pathogen *Heterobasidion annosum*. *FEMS Microbiology Letters* 255, 82–88.

Sanmartín, I., Enghoff, H., Ronquist, F. (2001) Patterns of animal dispersal, vicariance and diversification in the Holarctic. *Biological Journal of the Linnean Society* 73, 345–390.

Schumacher, J., de Larrinoa, I.F., Tudzynski, B. (2008) Calcineurin-responsive zinc finger transcription factor CRZ1 of *Botrytis cinerea* is required for growth, development, and full virulence on bean plants. *Eukaryotic Cell* 7, 584–601.

Shuman, S., Glickman, M.S. (2007) Bacterial DNA repair by non-homologous end joining. *Nature Reviews Microbiology* 5, 852–861.

Siewers, V., Viaud, M., Jimenez-Teja, D., Collado, I.G., Schulze Gronover, C., Pradier, J.-M., Tudzynski, B., Tudzynski, P. (2005) Functional analysis of the cytochrome P450 monooxygenase gene *bcbot1* of *Botrytis cinerea* indicates that botrydial is a strain-specific virulence factor. *Molecular Plant-Microbe Interactions* 18, 602–612.

Sonnenbichler, J., Lamm, V., Gieren, A., Holdenrieder, O., Lotter, H. (1983) A cyclobenzopyranone produced by the fungus *Heterobasidion annosum* in dual cultures. *Phytochemistry* 22, 1489–1491.

Sonnenbichler, J., Buestle, I.M., Peipp, H., Holdenrieder, O. (1989) Secondary fungal metabolites and their biological activities, I. Isolation of antibiotic compounds from cultures of *Heterobasidion annosum* synthesized in the presence of antagonistic fungi or host plant cells. *Biological Chemistry Hoppe-Seyler* 370, 1295–1303.

Sonnenbichler, J., Dietrich, J., Peipp, H. (1994) Toxins of *Heterobasidion annosum*, *Gloeophyllum abietinum* and *Armillaria ostoyae* – induction of their synthesis. In: *Proceedings of the Eighth IUFRO Conference on Root and Butt Rots. Sweden/Finland, August 1993* (Johansson, M., Stenlid, J., eds), Swedish University of Agricultural Sciences, Uppsala, Sweden, pp. 152–166.

Stalpers, J.A. (1996) The aphyllophoraceous fungi 2. Keys to the species of the *Hericales*. *Studies in Mycology* 40, 1–185.

Stenlid, J. (1985) Population structure of *Heterobasidion annosum* as determined by somatic incompatibility, sexual incompatibility, and isoenzyme patterns. *Canadian Journal of Botany* 63, 2268–2273.

Stenlid, J., Karlsson, J.-O. (1991) Partial intersterility in *Heterobasidion annosum*. *Mycological Research* 95 1153–1159.

Stephens, M., Balding, D.J. (2009) Bayesian statistical methods for genetic association studies. *Nature Genetics* 10, 681–690.

Stergiopoulos, I., Zwiers, L.-H., De Waard, M.A. (2002) Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and major facilitator superfamily. *European Journal of Plant Pathology* 108, 719–734.

Struhl, K. (1983) The new yeast genetics. *Nature* 305, 391–396.

Sugiura, R., Sio, S.O., Shuntoh, H., Kuno, T. (2001) Molecular genetic analysis of the calcineurin signaling pathways. *Cellular and Molecular Life Sciences* 58, 278–288.

Swedjemark, G., Johannesson, H., Stenlid, J. (1999) Intraspecific variation in *Heterobasidion annosum* for growth in sapwood of *Picea abies* and *Pinus sylvestris*. *European Journal of Forest Pathology* 29, 249–258.

Tajima, F. (1993) Simple methods for testing molecular clock hypothesis. *Genetics* 135, 599–607.

Taylor, J.W., Berbee, M.L. (2006) Dating divergences in the fungal tree of life: review and new analyses. *Mycologia* 98, 838–849.

Tokuda, S., Ota, Y., Hattori, T. (2007) Root and butt rot of Todo fir (*Abies sachalinensis*) caused by *Heterobasidion annosum s.l.* in Hokkaido, Japan. *Forest Pathology* 37, 155–166.

Tokuda, S., Hattori, T., Dai, Y.-C., Ota, Y., Buchanan, P.K. (2009) Three species of *Heterobasidion* (Basidiomycota, Hericiales), *H. parviporum*, *H. orientale* sp. nov. and *H. ecrustosum* sp. nov. from East Asia. *Mycoscience* 50, 190–202.

van Ooijen, J.W., Boer, M.P., Jansen, R.C., Maliepaard, C. (2002) *MapQTL® 4.0 Software for the Calculation of QTL Positions on Genetic Maps*, Biometris, Wageningen, The Netherlands.

Vacher, C., Piou, D., Desprez-Loustau, M.-L. (2008) Architecture of an antagonistic tree/fungus network: the asymmetric influence of past evolutionary history. *PLoS ONE* 3, e1740.

Volger, von C., Hesse, C., Vogt, A. (1982) Über das Vorkommen von Calciumoxalat-Kristallen in *Heterobasidion annosum* (Fr.) Bref. *European Journal of Forest Pathology* 12, 59–70.

Wang, X.-Q., Tank, D.C., Sang, T. (2000) Phylogeny and divergence times in Pinaceae: evidence from three genomes. *Molecular Biology and Evolution* 17, 773–781.

Wolpert, T., Dunkle, L., Ciuffetti, L.M. (2002) Host-selective toxins and avirulence determinants: What's in a name? *Annual Review of Phytopathology* 40, 251–285.

Woodward, S., Stenlid, J., Karjalainen, R., Hütterman, A., eds (1998) *Heterobasidion annosum, Biology, Ecology, Impact and Control*, CAB International, Wallingford, Oxon, UK, 589 pp.

Zhang, H., Zhao, Q., Liu, K., Zhang, Z., Wang, Y., Zheng, X. (2009) *MgCRZ1*, a transcription factor of *Magnaporthe grisea*, controls growth, development and is involved in full virulence. *FEMS Microbiological Letters* 293, 160–169.

Acknowledgements

The projects in this thesis were financially supported by the Swedish Research Council of Environmental, Agricultural and Spatial planning (FORMAS), the Swedish Foundation for Strategic research (SSF), Stiftelsen August T Larssons fond, Wallenbergska stiftelsen and SLU.

I thank my main supervisor, Professor Jan Stenlid, for giving me the opportunity to work as a PhD student. I have always been impressed by your intelligence, how you can memorize everything so well and lead the research. I also appreciate that I got the chance to work with the *H. irregulare* genome project.

I am also grateful to my assisting supervisor, Associate Professor Åke Olson, for all his time spent on the projects that I have been involved in, especially for reading and revising manuscripts, and for all the everyday advice about all parts of my research education.

I thank Mårten Lind for teaching me how to set up the virulence experiments, for being a nice and helpful colleague and for help with this thesis.

Kajsa Himmelstrand is greatly acknowledged for all her hard work with the association mapping.

I thank the former head of the department, Professor Roger Finlay, Karin Backström and Erica Häggström for all their administrative work.

A warm thank you goes to my room mates Fredrik Heyman and Johanna Boberg. Fredrik taught me how to organize references and Johanna taught

me a lot about Illustrator, Excel and statistics. Thank you for being such nice colleagues and friends!

I want to express my gratitude to Heriberto Vélëz for being a nice colleague and for all the time spent on supervision during the transformation experiments.

Thank you Karl Lundén for reading and commenting on this thesis, for the dinner parties and for being a supportive friend.

I thank Maria Jonsson and Katarina Ihrmark for all their lab assistance. Maria Jonsson was especially helpful with planting during the virulence experiments and lent an extra hand with the PCR and DNA extractions. I thank Katarina Ihrmark for guiding me in the lab as a total new comer. You have also been very nice and generous with advice in the laboratory. In addition, I thank Rena Gadjieva for helping out with DNA extractions in study III. Thanks also to Joakim Stenlid who worked hard in the laboratory during the hot summer.

I am grateful to Associate Professor Folke Sitbon and Lisa Arnqvist for everything that I was taught during work on my Master's thesis. I also thank all the people that I got to know at the Genetic Center: Jeppe and Dimitra, Magnus and Emma, Mattias and Lilijana, Per, Mats, Emma and Erik, Mattias and Cissi, Joel, Jon, Maria and Elke.

I am grateful to the group in Göttingen: Professor Ursula Kües, Dr Andrzej Majcherczyk, Dr Patrik Hoegger, Dr Mojtaba Zomorrodi, Dr Katerina Svoboda, Wassana Chaisaena, Banyat Cherdchim, Dorothea Fragner, Ihtzaz Malik, Annette Naumann, Monica Navarro-Gonzáles, Sudhakar Peddireddi, Martin Rühl and Karin Lange. Thank you Ursel for being so hospitable and for everything you taught me, especially annotation.

I am grateful to Cajsa Lithell for contributing to the positive atmosphere and for being a nice friend and colleague.

Petra Fransson is greatly acknowledged for organizing the research school activities, for reviewing a manuscript and for all the fun we had together.

Many thanks to Leslie Paul and Patrik Söderström for helping out with computer issues.

I thank Malin Elfstrand for her support during the transformation experiments and the last part of my education. Thanks for being such a positive colleague!

I also acknowledge the researchers Nils Högberg and Magnus Karlsson for their helpful advice during the work on study I.

I thank Eva Blixt for assisting me in the lab during my pregnancy.

Thanks to all the PhD students and colleagues at our department for the positive and open atmosphere.

The linguistic review was kindly provided by Caroline Woods.

A warm thanks to all the 35 friends I got to know from the 'Seventh Heaven'; it was a very rewarding experience to share a kitchen and a living room with eleven people at a time. Thanks Kristina and Anton for all the crazy nights and all the fun.

Jag vill tacka mormor för allt stöd, speciellt det finansiella så att jag kunde ta körkort och köpa möbler till lägenheten.

Jag vill också tacka farmor för att du är en så positiv förebild.

Many hugs go to my mom, brother and sister. I love you guys.

Till min kära familj Per, Elliot och Iris; tack för allt.