# Genetics of Virulence and Intraspecific Interactions in *Heterobasidion annosum s.l.*

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# Abstract

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**Abstract**

The present thesis summarizes and concludes the work of four separate studies on *Heterobasidion annosum* (Fr.) Bref. *s.l.*. The basic aim of these studies was to provide a tool for investigation and cloning of genetic regions in *H. annosum s.l.* and apply this tool to different regions of interest. The progeny isolates of a cross between North American intersterility groups S and P were used in an inoculation experiment on two-week-old *Pinus sylvestris* and demonstrated the nuclear contribution to be important for virulence. From the same population, a genetic linkage map was constructed based on 358 AFLP markers, consisting of 19 larger and 20 smaller linkage groups (LG). Based on further virulence tests on both one-year-old *Pinus sylvestris* and two-year-old *Picea abies*, several regions controlling virulence were identified and mapped to these LG. In particular, LG 15 was of interest, containing QTLs for pathogenicity to both pine and spruce. The tests also indicated that hybridization negatively affects virulence. An investigation of somatic incompatibility was conducted, using two heterokaryotic populations made from the progeny, backcrossed with a parental strain or crossed with an unrelated strain. The number of incompatibility loci was estimated to four and it was shown that the allelic differences alone are not enough to determine the degree of the incompatibility reaction. Furthermore, several strong QTLs for different intraspecific interactions that may be important to the incompatibility phenomenon were located.

Future work includes map based cloning and investigation of the QTLs identified in this thesis; a task that will benefit substantially from the upcoming sequencing of the *H. annosum s.l.* genome.

*Keywords:* Genetic linkage map, QTL, quantitative trait loci, intersterility, incompatibility, pathogenicity, forest pathogen

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# Contents

# Appendix

## Papers I-IV

This thesis is based on the following studies, which will be referred to by their Roman numerals.

**I** Olson, Å., Lind, M. and Stenlid, J. 2005. Genetic analysis of virulence in *Heterobasidion annosum s.l.*, casual agent of conifer root rot. Forest pathology 35, 231-331.

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

**III** Lind, M., Dalman, K., Stenlid, J., Karlsson, B. and Olson, Å. Identification of quantitative trait loci affecting virulence in *Heterobasidion annosum s.l.*.

**IV** Lind, M., Stenlid, J. and Olson, Å. Genetics and QTL mapping of somatic incompatibility and intraspecific interactions in the basidiomycete *Heterobasidion annosum s.l.*.

# Abbreviations

The following is a list of the less common abbreviations used in this thesis.

**AFLP** Amplified fragment length polymorphisms. A DNA fingerprinting method.

**cM** Centimorgan. A unit of distance in linkage maps. A distance of one cM equals one recombination in one hundred meioses.

**IS** Intersterility. A system controlling mating compatibility over species boundaries.

**LG** Linkage group. Group of linked markers in a linkage map not linked to any other linkage groups or markers.

**LOD** Logarithm of odds. A logarithmic value of the likelihood of markers or traits to be physically linked.

**QTL** Quantitative trait loci. Regions on a linkage map involved in controlling a given trait, defined by phenotypic observations.

**SI** Somatic incompatibility. A fungal self/non-self recognition system.

# Preface

I always wanted to be a writer.

Not necessarily an author, for an author by definition needs to publish and while I certainly don't mind that, the driving force behind my urge is the writing itself. The British author and satirist Terry Pratchett once said "writing is the most fun anyone can have by themselves" and although I do second that there is more to the writing than mere fun. Writing soothes me. It clears my mind. It helps me to achive structure and comprehension. While not really having problems expressing myself orally, it is by the written word I can give true flight to the thoughts in my head.

So I always wanted to write, and as a distant dream, I wanted to make a living out of it. Deep inside I have always had a feeling that someday, somehow I would indeed write at a professional level, but apart from that the vision of my crystal ball was obscured by the shrouds and mists of a lack of confidence and devotion and the ever-present urge to postpone for tomorrow what I could have done today. Especially the last issue has been a burden on my shoulders. To me, deadlines appear to be targets to hit rather than disasters to avoid. I need the carrot to be productive, and as so often in life, a coincidence shifted the table and a juicy carrot appeared before my eyes. Well, not so much a carrot as a flailing whip, but that's even better.

This whipping carrot appeared in the shape of my wife getting the opportunity to pursue *her* lifelong dream in Vietnam. Circumstances forced her to leave several months before the writing and defence of this thesis, which put me in the position of meeting a deadline or keeping my family divided, and my son apart from his mother. I can not imagine a stronger force of productivity.

With the trials of the past months behind me I look upon the fruit of my endeavours, a rather thin but dearly loved book about a fungus. Blended with emotions of pride and relief is the feeling that there is more to come. No matter whether the future holds scientific adventures, more books about fungi or other literate conquests in store, I know now what I have in me. That is my own prime accomplishment with this thesis.

For I have written a book.

Fly, my thoughts.

# Introduction

In every kind of cultivation of plants, a main part of losses is due to the toll taken by pathogens. Be it agricultural or silvicultural production, the cultivator will always have to deal with the adaptivity of nature's opportunists, always ready to make use of whatever new ecological niches the hand of man digs open in its exploits. The story of *Heterobasidion annosum* is the story of such an opportunist. It is a fungal pathogen whose life cycle has been favoured immensely by the development of modern forestry. The fungus has probably gone from being of small pathogenic importance in unmanaged boreal forest in terms of occurrence and rate of dispersal, to the main forest pathogen in sustained yield forestry of conifer trees all over the northern hemisphere, nowadays causing losses of €790 million yearly in Europe (Woodward *et al.*, 1998). In managed Swedish forests without actions for control, *H. annosum* has been estimated to infect approximately 15% of all spruce. This drastic development is due to the abundance of infection sites from newly cut stumps, a venue of infection not often available in unmanaged forests. The fungus has been studied under different names, e.g. *Polyporus annosus* (Fries, 1821), *Trametes radiciperda* (Hartig, 1874), *Fomes annosus* (Karsten, 1879) and *Fomitopsis annosa* (Bondartsev & Singer, 1941) for over 200 years but even though its consequences are so well known and so very costly in all forest management, little action is taken by forest owners to limit the losses (Thor, 2005). Apart from stump treating agents and conscious forest management, the arsenal against the ravaging fungus has been almost empty. This is partly related to the scarce knowledge of the pathogenesis at the molecular level. However, many current projects are about to change this.

## Fungal pathogenicity to plants

Before delving any deeper into the nature of pathogens, it is important to define the terms virulence and pathogenicity. Throughout this thesis, pathogenicity is defined as the binary character of being able to cause disease in a host. Virulence, on the other hand, is a relative trait describing the severity of the disease caused. Thus, by this definition, an organism is either pathogenic or non-pathogenic, and may be more or less virulent depending on genetic variation, host and environmental conditions. In order to understand a specific fungal pathogen, it is important to also understand the general basis of pathogenicity. Although there are certain aspects of pathogenicity specific to different pathogens that determine host specificity, fitness and virulence, there are also many features common among many pathogenic species.

A crude distinction between pathogenic species can be made by the division into biotrophic and necrotrophic organisms. Biotrophic fungi require a living host to obtain nutrients, parasitizing within the organism on its own biosynthetic pathways. The biotrophic fungus often penetrates the cell wall, or gains access through natural openings or wounds, and forms a feeding structure called haustorium between the cell wall and membrane (Deacon, 1997). When the host dies, the fungus is unable to fulfil its lifecycle unless it can switch to a

necrotrophic or saprophytic growth, and must seek a new host elsewhere. As the fungus is dependent on the host keeping part of its metabolism intact, it is crucial to avoid being recognized as an invading pathogen, because this would lead to a hypersensitive response (HR) (Gilchrist, 1998; Greenberg & Yao, 2004; Heath, 2000; Morel & Dangl, 1997) and apoptosis in the infected plant tissue, making further spread impossible. Generally, the host plant has HR-triggering receptors for certain general or pathogen-specific membrane proteins, exuded metabolites or residual products of degradation of plant material. In turn, the pathogen might try to disguise those proteins, or block the host's receptors to avoid detection. This hide-and-seek strategy is often referred to as the gene-for-gene system (Dangl & Jones, 2001; Flor, 1971) where resistance genes of the host are recognizing avirulence genes of the pathogen. Because this calls for highly specialized pathogens, biotrophic fungi are usually more host specific than necrotrophs.

While biotrophs seek to deftly sneak past the host's defences and act unnoticed within its very cells, there is nothing subtle about the approach of the necrotrophic fungi. The pathogen often enters the host by the formation of a structure called appressoria (Deacon, 1997), forming on the surface of host cells and forcing its way through the cell wall by a built-up pressure inside the appressoria, aided by the exudation of degrading enzymes. The fungus then grow filamentously through plant tissues and kills off the plant cells by secretion of toxins (Knoche & Duvick, 1987; Wolpert, Dunkle & Ciuffetti, 2002) and degrading enzymes, feeding by absorbing nutrients thereby made available. The fungal presence induces the plant HR, producing toxins and enzymes to kill and degrade the invading fungi, and trying to block the path of the pathogen by lignification and papillae formation. The pathogen deals with this by exuding toxin-degrading compounds. Against necrotrophs, the HR is generally not an efficient defence because they often can grow saprotrophically through the lignified cells (Asiegbu, Daniel & Johansson, 1994; Lindberg & Johansson, 1991). Apart from the biotroph, the necrotroph does not need the host to stay alive to live inside it. Indeed, the pathogen might benefit from killing the host and not having to cope with the defences; on the other hand, once the host is dead it will be the target of saprotrophs, not able to live inside a living host but possibly well suited to compete with the pathogens over dead substrate.

## *Heterobasidion annosum*

### *Species complex*

The basidiomycete *Heterobasidion annosum* (Fr.) Bref *sensu lato* (*s.l.*) is a facultative necrotrophic fungus, living on a wide range of coniferous and broadleaf trees all over the northern hemisphere (Korhonen & Stenlid, 1998). It is a species complex made out of several intersterility (IS) groups belonging to different phylogenetic species (Chase & Ullrich, 1983; Garbelotto *et al.*, 1993; Johannesson & Stenlid, 2003; Otrosina *et al.*, 1993; Worrall, Parmeter Jr & Cobb, 1983). Three IS groups have been reported from Europe; *H. annosum sensu stricto* (*s.s.*), *H. parviporum* and *H. abietinum*, with a host preference of pine, spruce and fir respectively and previously referred to as belonging to the P, S and F

intersterility groups. *H. annosum s.s.* has been reported from all over Europe and as far north as southern Norway and Finland. *H. parviporum* occurs all over Fennoscandia and central Europe, but has not been reported from Spain, Britain, western France and Ukraine. *H. abietinum* is present in central and southern Europe, from southern Poland and Germany to Mediterranean Italy and Greece (Thor, 2005). The mentioned preferences aside, the host range of these species do partly overlap. The main host of *H. annosum s.s.* is *Pinus* species but has been reported on *Picea*, *Larix*, *Betula*, *Alnus* and *Juniperus* (Korhonen & Stenlid, 1998). *H. parviporum* has a more narrow host range, mainly attacking *Picea abies* but occasionally also infecting *P. sylvestris* and *Abies sibirica* (Korhonen *et al.*, 1997; Stenlid & Swedjemark, 1988). *H. abietinum* primarily lives on *Abies* species (Capretti *et al.*, 1990). In North America, two IS groups has been identified but so far not given Latin names. They are called the S and P group after their main host preference spruce and pine, although S also attacks *Abies*, *Pseudotsuga*, *Tsuga* and *Sequoiadendron* and P has been found on *Juniperus* and *Calocedrus* (Garbelotto *et al.*, 1996).

### *Life cycle and pathogenesis*

Fruiting bodies of *H. annosum s.l.* are formed on stumps, roots and logs of dead or diseased trees (Redfern & Stenlid, 1998). The sporocarps release great amounts of basidiospores, dispersed mainly by wind. Their spatial distrubition does not seem to go farther than 100-1000 meter from the fruiting body because at this distance the number of spores of *H. annosum s.l.* equals background levels (Möykkynen, von Weissenberg & Pappinen, 1997). Infection by *H. annosum s.l.* mainly takes place by basidiospores through fresh wounds and newly cut stumps within a few days of felling (Isomäki & Kallio, 1974; Redfern & Stenlid, 1998; Risbeth, 1949; Swedjemark & Stenlid, 1993). Upon spore contact with host tissue, the infection begins with adhesion to the surface within 1-2 hours (Asiegbu, 2000; Snape, Preston & Woodward, 1993), probably mediated by a lipid spore component (Asiegbu, 2000). Once established in a tree or a stump, *H. annosum s.l.* grows down in the root system and infects adjacent living trees through root-root contact because the ability for the fungus to spread in soil is limited (Redfern and Stenlid 1998). In the infection of the new root, the fungus forms virulence structures such as appressoria and infection pegs although their impact on infection remains to be fully investigated (Asiegbu, Daniel & Johansson, 1993). Being a necrotrophic organism, *H. annosum s.l.* grows filamentously inside the host, exuding toxins and enzymes to kill and degrade the plant tissues (Asiegbu *et al.*, 1998). At least 12 such toxins has been identified in *H. annosum s.l.*, including fomannosin and fomannoxin (Asiegbu*, et al.*, 1998). Both of these are known to cause browning and death in conifers (Bassett *et al.*, 1967; Heslin *et al.*, 1983). The host defends itself by significant lignification, formation of papillae, necrosis and exudation of toxifying agents (Asiegbu, Daniel & Johansson, 1993; Karjalainen, Ernst & Woodward, 1998) although these responses seldom stop the infection. In pine, the infection spreads in the phloem and cambial zone, which leads to losses in forms of growth reduction due to root death and mortality. However, during infection in most other hosts, mortality is less of a problem than wood decay (Stenlid & Redfern, 1998). Losses from wood decay are primarily due to ruining of timber

value. In a spruce trunk, the decay column can rise as high as 12 m (Stenlid & Wästerlund, 1986) and grow at a rate of 10-30 cm yearly, but the variation is high and the rate has even been reported to be as high as 2 m in one year (Bendz-Hellgren *et al.*, 1999; Thor, 2005; Vasiliauskas, 2001).

### *Intraspecific interactions*

Various intraspecific interactions define species borders and control mating and individualism in *H. annosum s.l.*. Mating is controlled by a unifactorial, bipolar mating system, where each spore resulting from a sporocarp carries one mating type, from either of the parents (Chase & Ullrich, 1983; Chase & Ullrich, 1990; Korhonen, 1978). Each mating type is determined by allelic differences in a mating type locus. There are probably over 100 different mating type alleles in the *H. annosum s.l.* species complex (Chase & Ullrich, 1983; Stenlid, 1985). Therefore, any two spores picked at random are in most cases compatible. Compatible mating is defined as anastomosis and the formation of a heterokaryotic mycelium carrying nuclei from both of the parental strains (Korhonen & Stenlid, 1998). The resulting mycelium, also referred to as secondary mycelium, is the normal vegetative state of *H. annosum s.l.* and enables the fungus to attack living trees and form fruiting bodies (Korhonen & Piri, 1994; Platt, Cowling & Hodges, 1965). However, Garbelotto (1997) showed that homokaryotic isolates can be as virulent as heterokaryons and be stable for at least 12 months. In *H. annosum s.l.*, secondary mycelium is readily identified by the presence of clamp connections at septa although they may be few in numbers and hard to find under the microscope.

The mating type system is not the sole control of mating. While mating type can be described as a way to prevent self-mating, intersterility genes control mating over species boundaries. These have only been described in detail for the North American S and P isolates (Chase & Ullrich, 1990). Each individual has either a + or a - allele for each of five intersterility loci, named S, P, $V_1$, $V_2$ and $V_3$. To mate, two individuals must share a + allele for at least one of the five loci. S + and P + alleles are carried by individuals from the S and P groups, respectively, while the other three loci regulate interfertility between intersterility groups. Thus, a common $V_3$ + allele in an S and P isolate would allow compatible mating among them. The same system has not been identified between other IS groups and interfertility among those is not necessarily controlled in the same way. Compatibility between these described groups has been reported to 18% (Garbelotto*, et al.*, 1996; Harrington, Worrall & Rizzo, 1989). In Europe, compatibility between *H. annosum s.s* and *H. parviporum* is lower than between *H. abietinum* and *H. parviporum*, 10% and 25-75% respectively (Korhonen *et al.*, 1992; Korhonen*, et al.*, 1997; Stenlid & Karlsson, 1991). Compatibility between North American P and *H. annosum s.s* seems to be high, like between both North American S and *H. parviporum* and *H. abietinum* (Capretti, et al., 1990; Stenlid & Karlsson, 1991). However, although all groups have the ability to mate with the others in the laboratory, such hybrids seem to be rare in nature and have only been reported once (Garbelotto*, et al.*, 1996), suggesting some kind of negative effect of hybridization. This thesis shows that this negative effect strongly affects the virulence **(Paper III)**.

Somatic incompatibility is an intraspecific system by which fungi can distinguish self mycelia from non-self, thus being able to protect own mycelium and territory from other individuals. The system also provides a defence against mycoviruses (usually dsRNA) and lethal plasmids (Hartl, Dempster & Brown, 1975; Milgroom, 1999; Worrall, 1997). However, in *H. annosum s.l.*, this particular function seems to be of limited importance. Ihrmark and colleagues (2002) showed that dsRNA are transferred between incompatible isolates and even between isolates of different IS groups. The incompatibility in *H. annosum s.l.* is expressed as a demarcation line between the isolates, made out of a clearing zone and varying degrees of concentrated hyphae on agar plates (Korhonen, 1978; Malik & Vilgalys, 1999; Stenlid, 1985) and dark zone lines on woody substrates (Johannesson & Stenlid, 2004; Swedjemark & Stenlid, 2001). The demarcation has very few aerial hyphae although hyphae within agar seem to intermingle freely. Incompatibility also leads to changed metabolic activity (Hansen, Stenlid & Johansson, 1993a), which was attempted to map in **Paper IV**. Hansen and Stenlid (1993b) showed that incompatibility is controlled by three or four loci in *H. annosum s.l.*. In **Paper IV**, this observation was sharpened and a four loci system suggested.

# Aims of this study

The basic aim of the work presented in this thesis was to provide a tool for investigation and cloning of genetic regions in *H. annosum s.l.* and apply this tool to different regions of interest. More specifically, we wanted to

- Construct a genetic linkage map for future identification and cloning of genomic regions of interest
- Determine the nuclear influence on virulence
- Elucidate the effect of hybridization on virulence
- Map QTLs for general and host specific virulence against both spruce and pine using different virulence measures
- Map intersterility genes, incompatibility genes and other factors involved in intraspecific interactions
- Determine the number of somatic incompatibility loci and challenge the assumption that only the SI allelic differences in a pairing determine the level of incompatibility.

# Materials and Methods

## Biological material

### *Fungal material*

The main fungal isolates used in the works described in this thesis (**Paper I-IV**) were progeny isolates of a cross between the North American P-type isolate TC-32-1 and S-type isolate TC-122-12 (Chase, 1985). One hundred and two single spore progeny isolates were collected from a sporulating fruiting body of the S/P dikaryotic hybrid (AO 8) and named AO 8-1 to AO 8-105 (Olson & Stenlid, 2001; Stenlid & Rayner, 1991). These particular parental strains were used because of their previously characterized intersterility genotypes, which enabled a compatible mating over the reproduction barrier of the S and P intersterility groups and the segregation of IS genes (Chase & Ullrich, 1990). Such a mating was preferred over mating within the group because the greater differences made for more and easier spotted polymorphisms; an advantage in the subsequent linkage mapping. It also allowed for mapping of several traits of interest that differ distinctly between the two parents, such as host specificity and IS genes. In **Paper IV** an unrelated North American P-type isolate, TC-39-7, was used to backcross the progeny isolates (Chase, 1985). These isolates were all kindly provided by Dr Thomas Chase.

### *Plant material*

In **Paper I**, two-week-old seedlings of *P. sylvestris* and *P. abies* were used for the inoculations, grown from seeds on water agar in petri dishes in a growth chamber. In **Paper III**, one-year-old *P. sylvestris* and two-year-old *P. abies* seedlings were used, replanted in pots of fertilized peat and grown in green house or outdoors for four weeks. All plant material was acquired from Swedish nurseries.

## Linkage mapping

### *Map construction*

A genetic linkage map is based on the segregation of parental polymorphisms in the progeny. Such differences can be identified by different modes of DNA fingerprinting. In the map constructed in **Paper II**, we used the amplified fragment length polymorphism (AFLP) technique (Vos *et al.*, 1995) with several different primer combinations to collect an adequate number of markers. AFLP is a powerful method for this because it amplifies DNA fragments with high specificity and it proved to be well suited for *Heterobasidion annosum s.l.* The markers found in the parental strains were then searched for in the progeny isolates, using the Genescan 3.1.2 and Genotyper 2.5 softwares (PE Applied Biosystems). Based on the observed segregation, the markers were organized into linkage groups using the JoinMap 3.0 software (Van Ooijen & Voorrips, 2001). JoinMap organizes the markers together by a statistical logarithmic measure called logarithm of odds (LOD), here used to describe the likelihood of co-segregating

markers to be physically linked (Lander & Botstein, 1988). The LOD score is the log value of the ratio between the likelihood of an observed value and the probability of the null hypothesis. Because the null hypothesis generally is that there is no linkage, it is usually 0.5. Since the LOD score is logarithmic, a LOD value of e.g. 3 means that the likelihood of the observed value is 1000 higher than the probability of 0.5; i.e a strong indication of linkage. The physical distance is measured in centimorgans (cM), where a distance of one cM equals one recombination per one hundred progeny isolates.

### *QTL analysis*

Quantitative trait loci (QTL) analysis links phenotype (the expression of traits) to genotype (the organization of markers). Any quantitatively distinguishable trait can be linked to the genomic regions regulating them in this fashion. For each trait measured in this thesis (**Paper III** and **IV**), quantitative data were collected for each of the progeny isolate and analysed together with the linkage map data using the MapQTL 4.0 software (Van Ooijen *et al.*, 2002). The software assigned regions to each trait based on the level of co-segregation between observed trait expression and the markers of the region. The likelihood of a QTL to be valid is described in LOD, with a significance threshold calculated for each individual trait and LG.

## Infection systems

The infection system in **Paper I** was applied on two-week-old seedlings, growing bare-root in homogenized mycelia. The virulence was measured as frequency of killed seedlings per day. This is a useful regime to measure fine root infection and provides information of the fungus ability to penetrate living cells. In **Paper III**, one-year-old pine seedlings and two-year-old spruce seedlings were inoculated by fungus-infected wooden plugs attached to a cambial wound. Virulence was measured as rate of spread through the sapwood and as length of lesions in the inner bark, a visual determinant for the hypersensitive response (HR) of the host. This method highlights the fungus' ability to spread through the phloem inside a living host and to some extent the level of alert it causes in the host.

## Interaction systems

### *Intersterility*

The parental strains used in all papers in this study are able to compatibly mate over the intersterility boundaries due to a common $V_3$ + allele (Chase & Ullrich, 1990). The state of the $V_1$ allele is unknown for TC-32-1, but the parental strains are heterozygous for IS loci P, S and $V_2$, causing these alleles to segregate in the progeny. Thus, they can be mapped in the same fashion as a marker, coding each isolate as carrying or not carrying the + allele for each of the three IS loci. This was tested in **Paper II** by crossing each progeny isolate with three tester strains, TC-111-4, Sä 16-4 and Sä 159-5, carrying the + allele for the $V_2$, P, and S loci,

respectively and the - allele for all other IS loci. Compatible matings between progeny isolates and tester strains indicated present + allele in the progeny.

*Somatic incompatibility*

Somatic incompatibility and other intraspecific interactions were measured by observing the reactions in all possible pairing combinations between dikaryons of the progeny isolates. Those were obtained by either crossing the progeny homokaryons with TC-39-7 or backcrossing them with parental strain TC-122-12. The number of somatic incompatibility loci was then estimated by observing the frequency of compatible reactions between the isolates. These crosses and backcrosses were also used to determine whether all nuclei in a mycelial encounter affect incompatibility. The other intraspecific interactions were scored quantitatively for each isolate as the cumulative value of the reactions towards all other isolates and mapped as QTLs.

# Results and Discussion

## Nuclear influence on virulence

Olson and Stenlid (2001) showed that the mitochondrion plays a vital role in the virulence of *Heterobasidion annosum s.l.* on *P. sylvestris*. The aim was therefore to determine the role of the nucleus in the virulence (**Paper I**). This was done by the use of an experimental population of parental strains TC-122-12 and TC-32-1 (Chase, 1985) and the progeny resulting from the cross between them. All progeny isolates carried the S-mitochondrion; hence, any potential variation in virulence among them cannot be explained by mitochondrial factors alone. Such variation can however still be due to mitochondrial-nuclear interactions. Two-week-old seedlings of *P. sylvestris* were grown in homogenized mycelia of our experimental population and virulence was measured as disease incidence rate for each isolate. The results show a continuous variation of virulence, indicating a multigenic control (Fig 1). Apparently, both the nucleus and the mitochondrion play vital parts in the virulence. This nuclear involvement was also confirmed by the appearance of a QTL for this virulence on LG 11 of our linkage map (**Paper II**, QTL data not shown). The QTL peaked at LOD 3.09 with LOD 2.3 being significant at the 1% level. The results also show that some isolates are more virulent than both of the parental strains. However, only one such natural hybrid has been found (Garbelotto*, et al.*, 1996) and that hybrid was less virulent than isolates from the S and P group respectively. This correlates with our findings (**Paper III**). The lack of these natural hybrids could be the result of negative interactions leading to isolates with low virulence and thus low fitness. The data show that these negative interactions do not affect the aspect of virulence measured in this method, invasion of living root cells. On the contrary, there seem to be a wider virulence potential in this regard in S/P hybrids than in natural isolates.

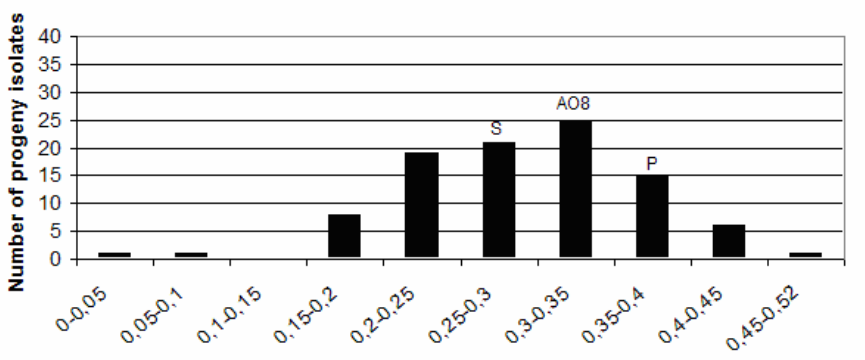


*Fig 1.* Frequencies of distinct virulence classes in progeny isolates of *Heterobasidion annosum s.l.*. Virulence of parental strains P (TC-32-1), S (TC-122-12) and heterokaryotic hybrid AO 8 are indicated. X-axis values describe relative virulence. Reproduced from **Paper I**.

We also investigated the correlation between virulence on spruce and virulence on pine by selecting three high-virulent and three low-virulent isolates to infect spruce seedlings. Although the observed variation in virulence was much higher in pine, 80% of the variation in pine was explained by the variation in spruce, showing at a strong correlation in virulence using this infection system. The same six isolates were also used to investigate the correlation of virulence to wood decay and growth rate on agar. Although neither of these correlated to virulence, the speed of growth trait has been studied in greater detail later (Olson, 2006) and shown to be tightly linked to, or even controlled by, the same loci as the intersterility genes S and P.

## Linkage mapping

A genetic linkage map is a powerful tool to assign traits to the genomic regions controlling them. Based on the segregation of 358 AFLP markers, polymorphic for the parental strains TC-32-1 and TC-122-12, a genetic linkage map of 19 larger (Fig 2) and 20 smaller linkage groups was constructed (**Paper II**). The map is clearly not completely saturated. We assume *H. annosum s.l.* to have 9-11 chromosomes (Anderson, Kasuga & Mitchelson, 1993) and thus a map covering the entire genome would have a number of LG corresponding to this. It is safe to assume that the map would be more saturated if more markers were collected and that this would cause some of the smaller groups to merge into larger ones. However, there might also be biological explanations for the uncharted areas of the map. Because the markers are positioned based on recombinations, areas where no recombination occurs would be impossible to map. This could be the case if the parental strains are too genetically distant, causing reduced homologies in end chromosomal regions and preventing recombinations in those parts. Recombinational hot-spots could also cause chromosomal sections to be impossible to map because all recombinations always occur in the same spot. Another striking feature of the map is the relatively high frequency of distortedly

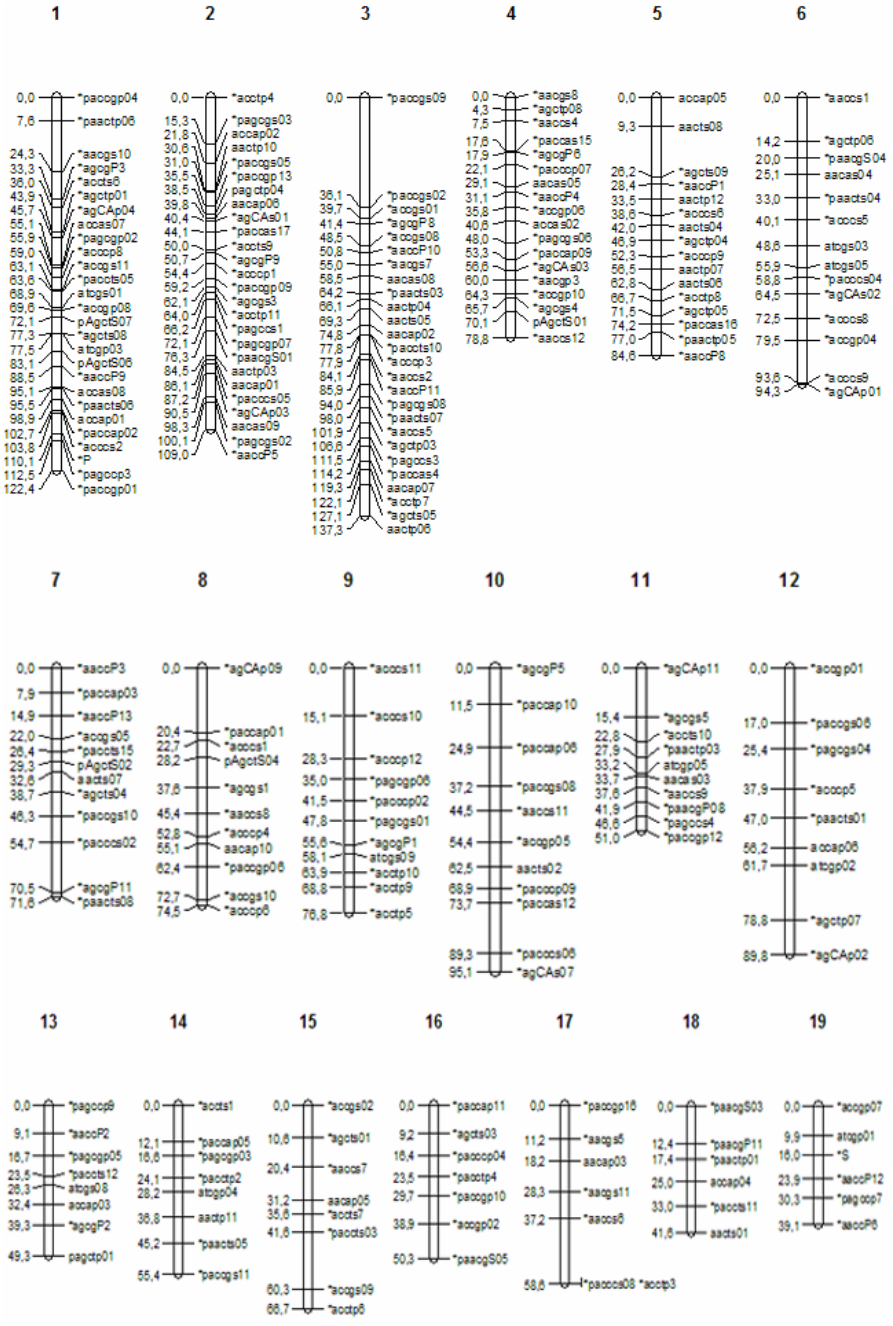


*Fig 2.* The 19 largest groups of a genetic linkage map of *Heterobasidion annosum s.l.* based on 358 AFLP markers. Intersterility genes P and S have been mapped in groups 1 and 19, respectively. Reproduced from **Paper II**.

segregated markers compared to other studies (Kerrigan *et al.*, 1993; Larraya *et al.*, 2000; Pongam, Osborn & Williams, 1998; Xu & Leslie, 1996). These can potentially be caused by screening errors and thus need to be investigated before being incorporated in the map. Because the distorted markers did not cluster in the map but were evenly spaced among and within linkage groups, and because removing them did not significantly alter the map in a different way than removing any other marker did, they were included. The distortion is thus probably the result of biological causes. There could for example be some kind of bias involved in the selection of spores which would leave out certain parts of the genome from the mapping (Kerrigan*, et al.*, 1993; Larraya*, et al.*, 2000). Another possible explanation for a non-Mendelian segregation is gene conversion caused by the assumed differences between the parental strains (Davis & Smith, 2001). Even though the map is partially fragmented and some markers are distorted, we consider the map to be reliable, which is also supported by the high density measured in physical size per genetic distance, 11.1 kb/cM. This is significantly denser than many other reported maps (Larraya*, et al.*, 2000; Muraguchi *et al.*, 2003; Sicard *et al.*, 2003; Xu & Leslie, 1996) and well above the suggested minimum density for map based cloning of 70 kb/cM (Sicard*, et al.*, 2003). The reliability has also been proven by the many successful applications of the linkage map so far (Olson, 2006).

## Pathogenicity mapping

During infection of a host, *H. annosum s.l.* makes use of a wide range of gene products previously reported to be involved in different aspects of virulence (Karlsson, 2005). These include several toxins and degrading agents for invasive growth and detoxifying factors for tolerance of the host's defence. (Bradshaw *et al.*, 2002; Gardiner *et al.*, 2004; Karlsson, Olson & Stenlid, 2003; Wasmann & Van Etten, 1996). To pin-point the genomic regions most critical for controlling *H. annosum s.l.* virulence on *P. sylvestris* and *P. abies*, infection systems on both species were set up. The progeny isolates and parental strains were used for the infections. Virulence was measured as length of lesion and fungal growth in sapwood (FGS) around the infection site. Least square mean values for both of these virulence measures on both hosts in every progeny isolate were used for QTL mapping.

The continuous distribution of virulence in the progeny isolates demonstrated in all experiments supports the previous findings (**Paper I**) that a nuclear involvement affects virulence, possibly through mitochondrial interactions (Olson & Stenlid, 2001). Lesion lengths and FGS within both host studied in these experiments correlates (**Paper III**), which is in concordance with previous observations (Swedjemark, Johannesson & Stenlid, 1999; Swedjemark & Stenlid, 1996; Swedjemark, Stenlid & Karlsson, 1998) but in contrast to other studies (Swedjemark, Johannesson & Stenlid, 1999; Swedjemark & Karlsson, 2004). The mean virulence of hybrid progeny isolates compared to the mean virulence of parental strains suggested a strong negative effect on virulence in the hybrid heterokaryon and its offspring. Interestingly, this was reversed in winter-hardened plants, where the hybrids seemed more virulent than the parents (Fig 3). Possibly,

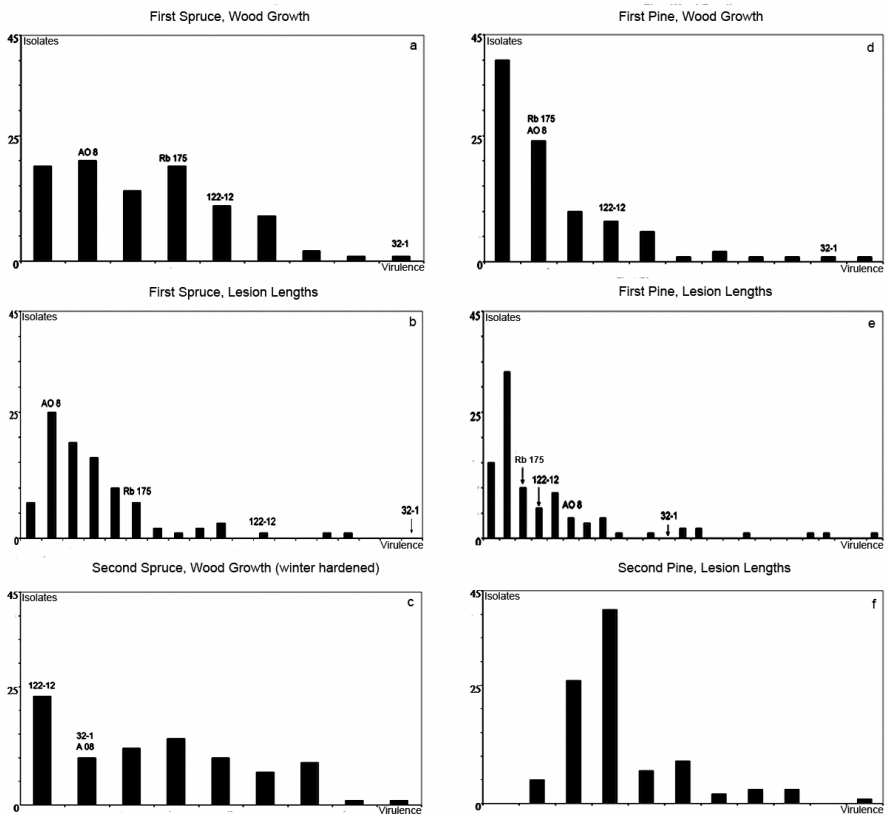


*Fig 3a-f.* Frequencies of virulence in a progeny population of *Heterobasidion annosum s.l.* Tests were performed on *P. sylvestris* (a-c) and *P. abies* (d-f) and measured in wood (a, c, and d) and the inner bark (b, e and f). Each isolate was tested in at least 5 replications. Virulence of parental strains (32-1, 122-12), heterokaryotic hybrid (AO 8) and European S-type control strain (Rb 175) are indicated when measured. Reproduced from **Paper III**.

a mutual host/pathogen recognition is needed for the host specificity to be expressed. Virulence on spruce seems to be controlled to a large extent by regions on LG 15, where we found QTL for both lesion lengths and FGS (Fig 4). We also found a QTL for lesion lengths on pine on this group. By the appearance of the QTLs, two separate regions of the LG are involved and both of these are controlling sapwood growth in spruce; while one of them affects lesion lengths in spruce, the other affects lesion lengths in pine. Apparently, there are factors of both host specificity and general virulence here. Lesion lengths in pine also identified a second QTL on LG 20, while sapwood growth in pine had a QTL on LG 36, a third involved group. According to these observations, virulence on spruce is controlled by primarily two regions, while three are involved in virulence on pine. One of these regions seems general and redundant for aspects of both pine and spruce virulence. It is possible, however, that two or all three of these groups actually belong to the same chromosome, because they are all small and might well be linked by yet undiscovered markers.

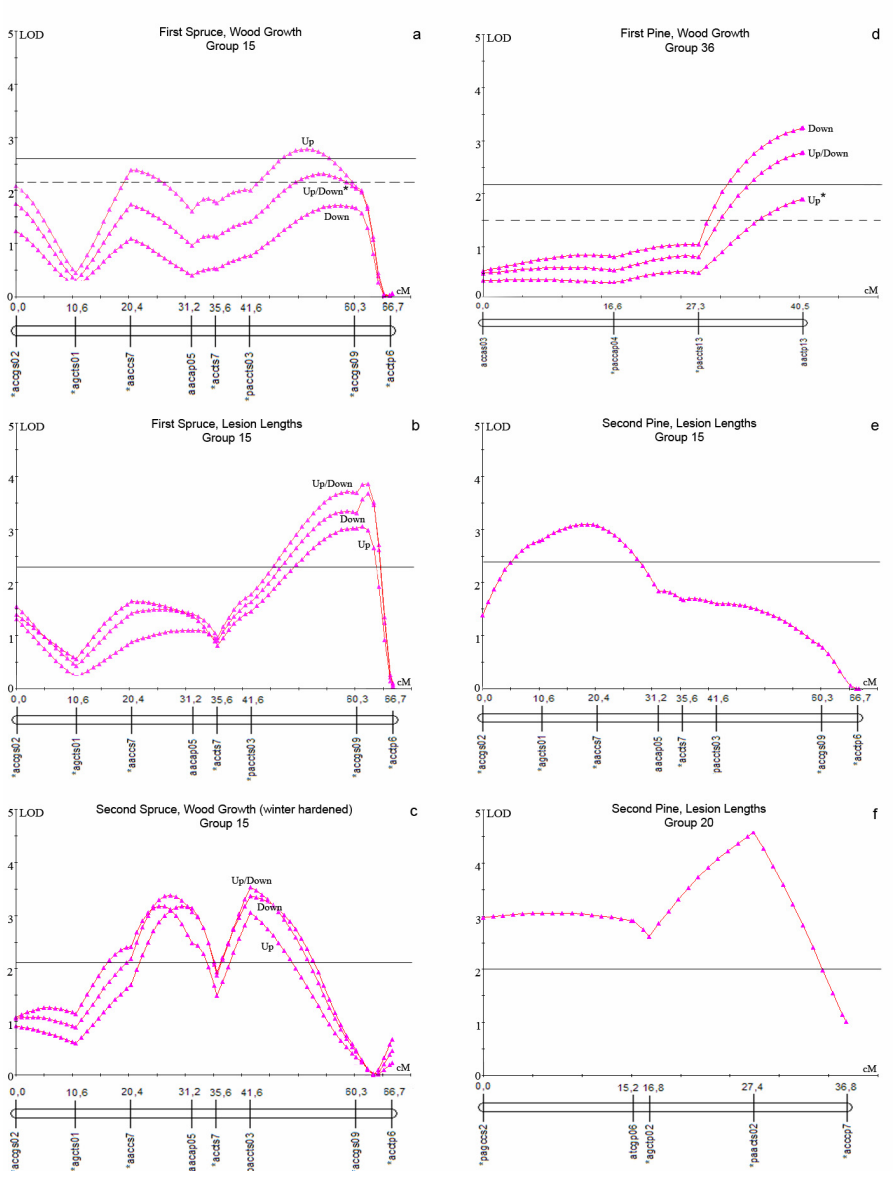


*Fig 4a-f.* QTLs for virulence to *P. abies* and *P. sylvestris* in *H. annosum s.l.* In figure a-d, data for virulence upstem, downstem and up/down combined are presented. The horizontal line denotes the highest LOD value for the 1% level of significance for the measurements included in each figure. The dashed line denotes the LOD value for the 1% level of significance for the measurement marked with an asterisk only. Reproduced from **Paper III**.

These findings support previous theories that the rareness of S/P hybrids in nature may come from reduced fitness due to low virulence (Garbelotto*, et al.*, 1996). However, our observations (**Paper I**) indicate that this low virulence is not ubiquitous in every stage of the pathogenic process, not affecting the fungus potential for root infection of seedlings but severely limiting the hybrids' potential for spread inside the stem. Moreover, our data show that *H. annosum s.l.* has at least partially diverging loci for lesion lengths and sapwood growth as well as for pine and spruce specificity. Lesion length is an indicator of dead plant material (Swedjemark, 1995) that one might speculate to be the result of certain host-specific toxins. Lesions also indicate a plant cellular reaction to the fungal presence. Thus, they might be the result of the fungus growing invasively through plant tissues and not entering the phloem. Fungal sapwood growth however might be seen as the fungus ability to overcome host defence and quickly spread in the phloem. These regions might be more important for detoxification of host toxins.

The only other tree pathogen analyzed in this way, *Venturia inaequalis*, seems to be controlled by only one major locus (Sierotzki & Gessler, 1998) while both single- and multiloci systems have been reported from different agricultural pathogens such as *Gibberella zeae*, *Phytophthora infestans* and *Cochliobolus sativus* (Cumagun *et al.*, 2004; Jiang *et al.*, 2006; Zhong & Steffenson, 2002).

## Interaction mapping

### *Intersterility*

The intersterility system controls mating between IS groups and over species boundaries. According to the system described by Chase (1990), fungal isolates need to share + alleles for at least one IS loci in order to mate. The intersterility genes controlling interspecific compatibility are still relatively unstudied. To identify and clone them could provide crucial novel information of reproductive barriers and speciation. In our S/P cross, three intersterility + alleles segregate among the progeny, being heterozygous in the parents (Chase & Ullrich, 1990). The progeny isolates were screened for IS genotype by crossing with tester strains carrying the IS + alleles. It is expected that each allele segregates 1:1 and thus appears in approximately 50% of the progeny isolates. However, looking for clamps in *H. annosum s.l.* isolates is a tedious process because very few, and often indistinct, clamps are formed. Therefore crossing must be attempted several times to collect a sufficient part of the positives in the population. We managed to do this for the S-allele, which was found in 50.5% or the progeny. The P-allele was found in 38.6% and $V_2$ in 29.2% of the progeny isolates. It is possible that a significant part of the P + allele positives was found, but we almost certainly lack many $V_2$ + positives. Treating the data as genetic markers, both the P and S intersterility loci was located in the map, on LG 1 and 19, respectively. The data for the $V_2$ locus, however, was probably too fragmented to link to any other markers. Further attempts to collect $V_2$ positive isolates are likely to locate the locus on the map, although it is also possible that $V_2$ is located on an uncharted part. If that is the case, the locus can only be located through the adding of more markers to the map.

*Somatic incompatibility*

Somatic incompatibility is the way of fungal individuals to separate self from non-self when challenged with other mycelia. If the encountering mycelia do not share incompatibility genes, a clearing zone will be formed between them to prevent being overrun or having to fight for their territories (Malik & Vilgalys, 1999). This system is poorly studied in basidiomycetes. In *H. annosum s.l*, three or four loci have been suggested to be responsible for the reaction (Hansen, Stenlid & Johansson, 1993b). Many questions about fungal individualism could be answered if the incompatibility genes were identified, or more light shed on the molecular biology behind the interaction. As we observed in laboratory pairings between different isolates (**Paper IV**), the morphology of the interactions take on other appearances than just a clearing zone. Dark pigmentations, increased production of aerial hyphae and wall-like mycelial interaction zone structures are other features that might be observed. Locating QTLs for these interactions paves the way for future identification and cloning, possibly also adding pieces to the incompatibility puzzle.

Our data show that four loci control incompatibility in *H. annosum s.l.* (**Paper IV**). The results also indicate that in pairings between two heterokaryotic mycelia, all nuclei affect incompatibility, and separate reactions containing the exact same allelic differences in compatibility thus can still give quite different levels of incompatible reactions. In interactions between homokaryotic mycelia, an incompatibility reaction can often be seen prior to the recognition of the mating types which eventually overrides the incompatibility and induces the mating. This indicates that incompatibility is a continuous, constitutively expressed character in any mycelia and one that can be shut down by recognition by the SI loci. No conclusions about the nature of this recognition can be drawn from these data, but if both nuclei affect the reaction, it is possible that a formation of dimers of the gene product from the respective nuclei is involved, and the recognition could be due to a tetramer formation between dimers from the interacting mycelia.

Furthermore, several interesting QTLs were located for our studied interactions (Fig 5). We found one QTL at least partially regulating the visible strength of the incompatibility reaction. We also found that in general interactions between mycelia, regardless of self or non-self mycelia, a very strong QTL controls both pigmentation and production of aerial hyphae. Interestingly, pigmentation increase when encountering exclusively non-self mycelia was controlled by a very strong, very thin QTL, located on a different LG than the QTL for pigmentation/aerial hyphae, and apparently not in any way affecting the production of aerial hyphae. Moreover, we found the hyphal barrier structures did not depend on the mycelium constructing them, but rather to be a reaction to something present in the other mycelium.

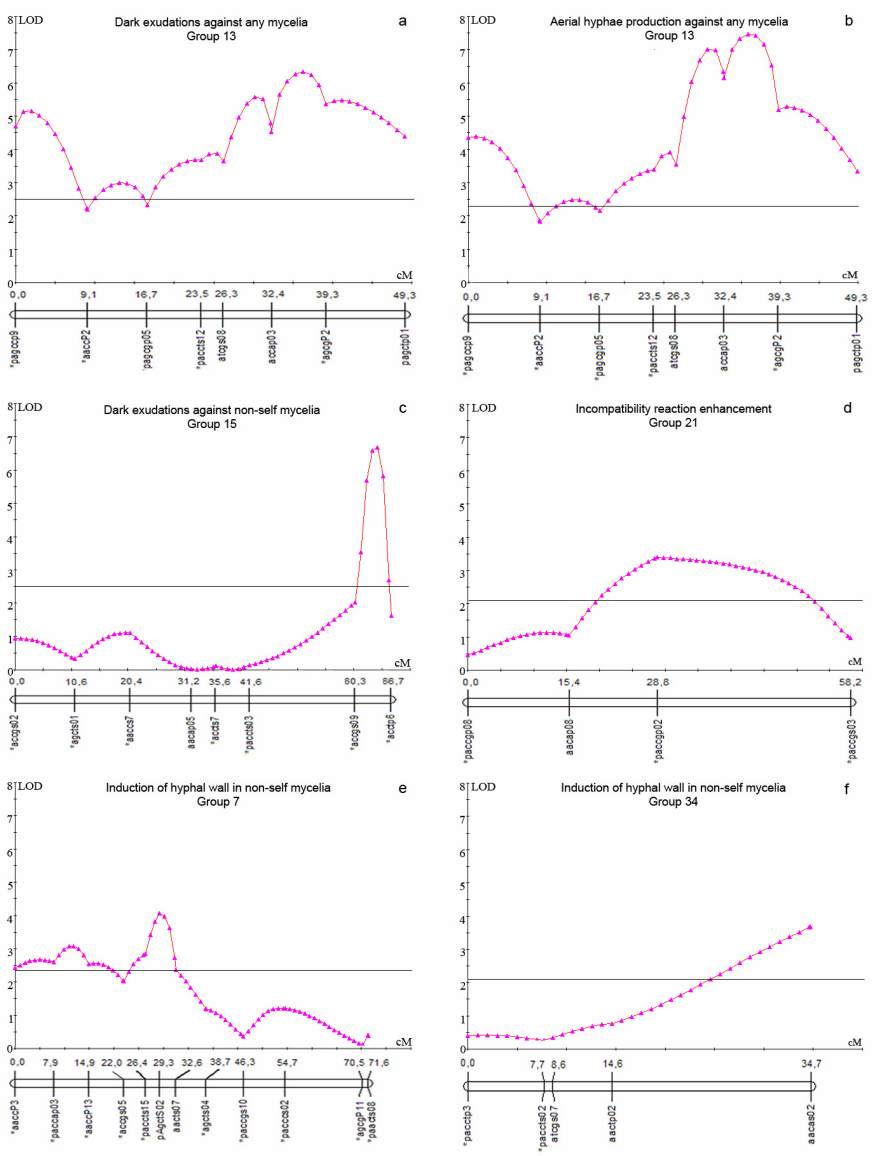


*Fig 5a-f.* QTLs for various intraspecific interactions in H. annosum s.l. **a** and **b**, data for exudation of dark metabolites and production of aerial hyphae under interaction with any mycelia. **c**, data for increased exudation of metabolites under interaction with non-self mycelia as opposed to self mycelia. **d**, data for stronger reactions to incompatibility and **e-f** data for the ability to induce hyphal barriers in other mycelia. The line denotes the LOD value for the 1% level of significance. Reproduced from **Paper IV**.

# Conclusions

The aim of this project was to construct a genetic linkage map and to use this to locate genetic regions controlling different traits. The finished map has already proved its reliability on several occasions by the mapping of pathogenicity factors, intersterility genes, factors controlling speed of growth and various elements affecting intraspecific interactions.

Furthermore, the importance of nuclear involvement in the virulence of *H. annosum s.l.* during infection of seedling roots was shown. This was previously thought to be mainly controlled by mitochondrial factors (Olson & Stenlid, 2001) and suggests a mitochondrial-nuclear interaction to be fundamental for at least this step of infection. The nuclear involvement was also demonstrated during infections following inoculation through cambial wounds.

Identified QTLs concluded that fungal sapwood growth and the induced formation of lesions on *P. sylvestris* originate from at least three different regions of the genome. During infection of *P. abies*, the same virulence traits originated from only one LG, which appears to be a real hot spot for pathogenesis. LG 15 is of interest for general as well as spruce specific virulence and guided by the profile of the respective QTLs on this group, there could be two separate regions of importance. None of the QTLs correlated with the previous found QTLs for speed of growth on agar (Olson, 2006), suggesting that these QTLs really are involved in pathogenesis and do not control the ability to quickly cover dead substrate. Judging from observed QTLs, virulence on *P. abies* is controlled by fewer regions than virulence on *P. sylvestris*. Perhaps the pathogenesis on pine is more complicated than on spruce. This could be due to the fact that the fungal growth inside pine is concentrated to the phloem and cambial zone, where a more active defence might be encountered. In spruce on the other hand, the fungal impact is most significant in heartwood decay (Stenlid & Redfern, 1998), which is a biologically less active area that might require a less specific virulence.

No host specificity was expressed by the pathogen on dormant *P. abies* material not responding to fungal infection. This suggests that to express host specificity, a mutual recognition between the host and the pathogen is needed. This thesis has also shed light on the previously suggested negative effects of hybridization on virulence (Garbelotto*, et al.*, 1996). The negative effect is clear in the studies in **Paper III** but non-existant in **Paper I**, indicating that the negative effect is limited to a part of the pathogenic process and not affecting root infection. Thus, the formation of infection structures and the penetration of the root surface cells do not appear to suffer from the hybridization, while the ability to grow through the sapwood and inner bark of a more mature host might well do.

Furthermore, intersterility genes S and P were successfully mapped to different LG in the linkage map (**Paper II**). This is exciting not only because these genes are largely unknown, but also because of the later findings that speed of radial growth correlates to the same locations (Olson, 2006). In fact, the $V_2$ intersterility

loci, which could not be located in the map, could also be linked to speed of growth using a Kruskal-Wallis test (unpublished data). Moreover, QTLs for several interactions between *H. annosum s.l.* isolates have been located (**Paper IV**). Even though only 63 of the 102 isolates from the map could be used the QTLs still exceed LOD 6 in several cases. Both the induction of the hyphal wall and the increased exudation of dark metabolites were traits explicitly expressed under interaction with non-self mycelia, indicating that these are some kind of antagonistic reactions. They can thus be related to the somatic incompatibility concept, which has previously primarily been described in *H. annosum s.l.* as visible by a clearing zone. However, between wood growing individuals SI is identified by dark lines of metabolic exudations. Even if the increased pigmentation *per se* does not infer any actual incompatibility in terms of limiting mycelial growth and migration of nuclei, it is a consequence of interactions between incompatible mycelia. Another interesting find was that pigmentation and aerial hyphae production was completely linked when involved in reaction to any mycelia, self or non-self, but when involved in interactions with non-self mycelia exclusively, the pigmentation was controlled by a different LG and apparently not linked to any increased aerial hyphae production. Thus, it is likely that the aerial hyphae growth and dark exudations during any mycelial interaction are the results of the same process, probably a general state of metabolic alert induced by the recognized presence of a mycelium. The dark exudation reaction during non-self encounters however appears to be the result of a different process, as a direct or indirect effect of antagonism.

The results from **Paper IV** provide evidence of a four-loci control of somatic incompatibility, which strengthens and sharpens the previous assumption of three or four loci (Hansen, Stenlid & Johansson, 1993a). Finally, the observation was made that more than the allelic differences in a pairing determines the level of an incompatibility reaction. In other descriptions of SI, differences at SI loci have been mentioned as the determinant of the reaction, but these new data suggest that some kind of nuclear interactions control the process. This is the first report of this kind of findings in any basidiomycete. Apparently, both nuclei in each interacting individual will contribute to the effect even if an identical nucleus is present in the corresponding mycelium. This nuclear interaction might consist of dimer formations between the gene products of both nuclei, possibly forming tetramers with the dimers from the corresponding mycelium as a mode of SI recognition.

# Future prospects

The discoveries presented in this thesis ask many new questions to be answered. The finding that hybridization does not negatively affect virulence under root infections (**Paper I**) highlights the differences in virulence expressed under root contact and during infection through cambial wounds. This calls for further elucidation of the proposed mitochondrial-nuclear interaction impact on virulence, so far only demonstrated during root infections. Cambial wound infections using

both S- and P-type mitochondria-carrying isolates would increase the understanding of this interaction.

The theory of *H. annosum s.l.* losing host specificity on winter hardened spruce (**Paper III)** is a step into an unstudied area of most tree pathogens. Very few reports exist on comparisons between virulence caused on winter hardened and fully active hosts. To further investigate this, P and S isolates could be used to infect trees over a time gradient with increasing states of winter hardening under the hypothesis that the host specificity would gradually disappear.

The somatic incompatibility study (**Paper IV**) presented new theories regarding the SI system in *H. annosum s.l.*. The idea of compatibility depending on interactions between the gene products from corresponding loci on all nuclei in a pairing calls for further investigation. This might be difficult without any information of the SI loci. Attempts were made to locate SI loci by manually screening groups of between themselves compatible isolates for homologies in marker genotypes (data not shown), to find markers linked to SI loci. The primary results were inconclusive but still brought hopes of success should more effort be put into this.

*Heterobasidion annosum s.l.* is the most devastating forest pathogen in the northern hemisphere and reducing related financial losses is the focus of many research projects. Until recently, none of these projects had equipped the scientific community with many tools in molecular biological research. This has recently changed drastically. Karlsson (2005) provided extensive collections of ESTs from different infection systems, while in this project, a genetic linkage map has been constructed (**Paper II**), a powerful tool for cloning and investigation of the genetic regions behind any measurable trait. An efficient transformation system for *H. annosum s.l.* has also been developed (Samils *et al.*, 2006).

When this work is being published, a sequencing project of the genome of *H. annosum s.l.* will be under way. At the same time, a genomic library of 40kb inserts is being constructed. This opens up all sorts of possibilities for future research. Using map based cloning, all identified QTLs from **Paper III** and **IV**, as well as the intersterility genes and their linkage to the QTLs discovered by Olson (2006) can easily be identified and analyzed. This will give us abundant information about the exact nature of the virulence and population biology of *Heterobasidion,* which is a prerequisite for reducing its damage. In the future, this could lead to more resistant hosts, new biocontrol agents and prevention of adaptation to existing biocontrol agents.

However, for some QTLs it might be that the location suggested is too vague and the physical distance between the markers involved too large. In that case the map itself needs to be strengthened by the addition of more progeny isolates, to sharpen the location of the current markers, or more markers, to map a larger area of the genome. Ideally, to create the best tool for future studies, this should be done until the number of LG corresponds to the number of chromosomes. If such a map could be obtained, we would have a molecular tool that together with the

genome sequence and the library could be used to identify the exact genomic regions involved in any phenotypically measurable trait. If the goal is to unravel the mysteries of an organism's molecular nature, it is hard to imagine a more powerful weapon.

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