Identification of quantitative trait loci affecting virulence in the basidiomycete *Heterobasidion annosum s. l.*

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Abstract
Quantitative trait loci (QTL) for virulence on one-year-old *Pinus sylvestris* and two-year-old *Picea abies* seedlings were identified and positioned on a genetic linkage map of *Heterobasidion annosum sensu lato (s.l.),* a major root rot pathogen of conifers. The virulence among 102 progeny isolates was analysed using two measurements of virulence; lesion length around and fungal growth in sapwood from a cambial infection site. In general, we found negative virulence effects of hybridization although this was contradicted on winter-hardened spruce. On *P. abies*, both measurements identified several partially overlapping QTLs on linkage group (LG) 15 of significant logarithm of odds (LOD) values ranging from 2.77 to 3.85. On *P. sylvestris*, the lesion length measurement also identified a QTL (LOD 3.09) on LG 15. Moreover, QTLs on two separate smaller LG, with peak LOD values of 3.24 and 4.58 were identified for fungal sapwood growth and lesion lengths respectively. The QTL probably represent loci important for specific as well as general aspects of virulence on *P. sylvestris* and *P. abies*. This is the first report of virulence located in a genetic linkage map of the *Heterobasidion spp.* genome. The mapping of virulence QTLs forms the basis for map based cloning and identification of the corresponding virulence genes, which may give answers to the so far largely unknown mechanisms of the infection process of *H. annosum s.l.*.

Keywords
Forest pathogen, QTL, JoinMap, genetic linkage map, pathogenicity, virulence, host specificity, root rot, conifer, hybridization

Introduction

Virulence is a complex feature in any organism and the genetics controlling it may differ significantly from one pathogen to another. Avirulence genes (Dangl & Jones, 2001; Flor, 1971), toxins (Knoche & Duvick, 1987; Wolpert, Dunkle & Ciuffetti, 2002), host defence detoxifying agents and different arrays of degrading enzymes (Asiegbu *et al.*, 1998) are examples of genetic factors for host specific and general virulence that might be involved. Avoiding host recognition is the central theme for virulent races in gene for gene systems that are common in rust fungi and other biotrophic pathogens. Although many necrotrophic pathogens are well documented (Elad *et al.*, 2004; Fox, 2000; Woodward *et al.*, 1998), a limited amount of genetic studies has been reported. Virulence of apple scab causing pathogen *Venturia inaequalis* has been shown to be controlled by a single major locus (Sierotzki & Gessler, 1998). This locus that has its gene-for-gene counterpart in the apple host (Vinatzer *et al.*, 2001). Analysing only the pathogenic isolates in a progeny population of *Gibberella zeae* suggested that only few loci for pathogenicity and aggressiveness differ in the progeny (Cumagun *et al.*, 2004). In the barley pathogen *Cochliobolus sativus,* studies indicate a single locus controlling virulence on a particular cultivar (Zhong & Steffenson, 2002), while in *Phytophthora infestans*, six loci were identified as possible virulence factors, as they changed their copy numbers in microarrays on isolates of differing virulence (Jiang *et al.*, 2006).

Host specificity in fungal pathogen-host interactions is a concept of great importance for the virulence factors of a specific organism. Physical attachment to the host, mediated by specific binding of homologous fungal material to the cell surface (Albersheim & Anderson-Prouty, 1975; Jones, 1994; Manocha, 1984), and the subsequent host-parasite recognition are early stage examples of such specificity. During actual infection, host resistance and pathogen avirulence genes control the outbreak of a hypersensitive response (HR) in the host (Flor, 1971; Gilchrist, 1998; Greenberg & Yao, 2004; Heath, 2000; Morel & Dangl, 1997) where rapid adaptation to avoid recognition by plant resistance genes is a crucial feature for any successful pathogen. While the HR generally is an efficient mode of resistance against biotrophs (Atkinson, 1993; Siegee, 1993), necrotrophic fungi seem able to cope with the HR induced by their own presence (Asiegbu, Daniel & Johansson, 1994; Lindberg & Johansson, 1991) or even benefit from it (Lyon, Goodman & Williamson, 2004). *Botrytis cinerea* has also been reported to utilise host defence fluids aimed at insects as substrates for germination (Holz, Coertze & Williamson, 2004). Different pathogens also induce different HR, probably due to different sets of avirulence/resistance genes. *Heterobasidion annosum* and *Fusarium* spp. induce deep HR necroses but no lignin formation, while *Phlebiopsis gigantea* and *Resinicium bicolor* induce much more superficial, but highly lignified, necroses (Johansson, Lundgren & Asiegbu, 1994).

Heterobasidion annosum (Fr.) Bref. *sensu lato (s.l.)*, the casual agent of annosum root rot, is one of the most devastating pathogens on conifers in boreal and temperate forests (Asiegbu, Adomas & Stenlid, 2005). The economic losses

from devaluation of timber and growth reductions caused by rot in the trunk equals at least 790 million € a year in Europe (Woodward*, et al.*, 1998). *H. annosum s.l.* is a species complex consisting of several closely related species and intersterility (IS) groups. Two IS groups of *H. annosum s.l.*, P and S*,* have been found in North America, and preferentially infect pine and spruce respectively (Harrington, Worrall & Rizzo, 1989). In Europe three IS groups with partly overlapping distributions and host specificity have been identified (Capretti *et al.*, 1990; Korhonen, 1978) and are today referred to by their Latin names *H. annosum sensu stricto (s.s.)*, *H. parviporum* and *H. abietinum*, and preferentially infect pine, spruce and fir species, respectively (Niemelä & Korhonen, 1998). Individuals from the distinct groups are intersterile, i.e. generally do not mate outside the group. However, the reproductive barrier is not complete, and in laboratory mating is possible between some isolates from distinct IS groups (Stenlid & Karlsson, 1991). Although compatible mating over intersterility borders is possible (Korhonen & Stenlid, 1998; Olson, Lind & Stenlid, 2005) only one natural S/P hybrid isolate has been found (Garbelotto *et al.*, 1996), suggesting a negative effect on hybridization. The hybrid was also found to be less virulent than pure S or P isolates (Garbelotto*, et al.*, 1996). However, in a previous study on *in vitro* infections of *P. sylvestris* roots we could not prove any such effect to be originating from reduced virulence (Olson, Lind & Stenlid, 2005).

The molecular biology behind virulence of *H. annosum s.l.* is largely unknown. It has been shown that virulence of homokaryotic and heterokaryotic isolates do not differ significantly and that homokaryons can stay stable during the initial 12 months after infecting trees (Garbelotto *et al.*, 1997). Nuclear genetic factors of importance for virulence segregate in interspecific hybrid progeny isolates between the North American S and P groups (Olson, Lind & Stenlid, 2005). Several degrading enzymes and toxins have been suggested as such virulence factors in *Heterobasidion spp.* (Asiegbu*, et al.*, 1998). *H. annosum s.s.* isolates have a higher laccase production than *H. parviporum* isolates. Laccase is known to be involved in lignin degradation (Johansson, Denekamp & Asiegbu, 1999) and hypothesised to also be involved in pathogenicity. Pectinases also differ between IS groups (Karlsson & Stenlid, 1991) and may play a crucial role in the infection process (Johansson, 1988). Spore surface receptors for carbohydrates such as mannose and fructose have been suggested to be of relevance for the spore development during the infection process (Asiegbu, 2000). Among other putative virulence genes, a cytochrome P450 gene as well as a manganese-dependent superoxid dismutase with increased expression during infection have been found (Karlsson, Olson & Stenlid, 2003; Karlsson, Stenlid & Olson, 2005). Furthermore, it has been shown that the mitochondrion also plays a vital role for the virulence of interspecific hybrid isolates between the S and the P groups (Olson, Lind & Stenlid, 2005; Olson & Stenlid, 2001). This is also supported by the discovery that genes encoding proteins involved in mitochondrial functions were upregulated during interaction with roots (Karlsson, Olson & Stenlid, 2003).

Although QTL analyses have been applied to many plant species, such reports in fungi are rare. In *H. annosum s.l,* successful attempts have been made to map both intersterility genes S and P, as well as link radial growth rate to these loci (Lind,

Olson & Stenlid, 2005; Olson, 2006). In *Pleurotus ostreatus*, QTLs controlling growth rate and some important industrial production traits have been found (Larraya *et al.*, 2003; Larraya *et al.*, 2002). One QTL controlling resistance to *Pseudomonas tolaasii* has been described in *Agaricus bisporus* (Moquet *et al.*, 1999). Cumagun and colleagues (2004) identified a QTL associated with pathogenicity and aggressiveness on wheat in *Gibberella zeae*. In *Nectria haematococca* MPI, several QTLs for pathogenicity on *Cucurbita maxim* were discovered (Hawthorne *et al.*, 1997), while Welz and Leonard (1994) identified QTLs for lesion size and pseudothecia formation in *Cochliobolus carbonum.*

The two major *H. annosum s.l.* hosts, *P. sylvestris* and *P. abies*, have different main antagonists, *H. annosum s.s.* and *H. parviporum* (Harrington, Worrall & Rizzo, 1989). This indicates that quite different loci might be critical for successful infection of the respective host. The aim of this study was to identify and locate QTLs responsible for virulence in *H. annosum s.l.* using infection systems on *P. abies* and *P. sylvestris,* which could provide information of general as well as host specific virulence factors for future identification and map based cloning. We also wanted to test the hypothesis that hybridization has a negative effect on virulence.

Material and methods

Experimental population and genetic linkage map

The experimental population used in this study consisted of 102 homokaryotic progeny isolates derived from a compatible mating between North American S and P isolates as previously described (Olson, Lind & Stenlid, 2005). The genetic linkage map constructed from the same progeny is based on 358 AFLP markers, organized in 19 larger and 20 smaller linkage groups (LG) (Lind, Olson & Stenlid, 2005).

Virulence analysis

In this study, four separate infection experiments have been carried out; two on two-year-old *P. abies* seedlings and two on one-year-old *P. sylvestris* seedlings. The experiments will henceforth be named **Spruce 1**, **Spruce 2**, **Pine 1** and **Pine 2**. The *P. abies* seedlings were planted in two-litre pots of fertilized peat. The *P. sylvestris* seedlings were planted in one-litre pots. All plants were grown for one month in the green house at 20-25 °C. The fungal isolates were grown in darkness at 21 °C for one week, in Petri dishes containing 10 % nutrient strength Hagem media (Stenlid, 1985). Four weeks prior to infection, 5x5x5-mm autoclaved wood blocks of *P. sylvestris* were placed on the mycelia allowing thorough colonisation. The inoculation of seedlings was carried out by cutting a small (5x10mm) window in the cambium of the plant, halfway between two nodes. One colonised wooden block was then placed in the wound and wrapped in place with parafilm. Each of the 102 progeny strains was used to infect 5 plants (10 for **Pine 2**). The parental

TC-122-12 (S-type), TC-32-1 (P-type) and AO 8 (heterokaryotic hybrid) strains were included apart from in **Pine 2**. A European S-type isolate Rb 175 was also used in **Spruce 1** and **Pine 1**. The infections were carried out in blocks with one set of replicates done every day for five days, apart from **Pine 2** which was unblocked and done on 20 isolates and 10 plants per isolate per day for five days. For **Spruce 1** and **Pine 1**, the seedlings were kept outdoors during the infection process; while **Spruce 2** and **Pine 2** were conducted in the green house (Table 1). After four weeks of infection, the tests were ended and the plants harvested. For all plants, the lesion length around the wound was measured upstem and downstem. The plants were then cut in five-mm-pieces and placed on wet paper in Petri dishes for one week. The pieces were investigated for presence of *H. annosum s.l.* conidiophores, and the length of fungal growth thus acquired was noted. Plants with no visible lesion, no length of growth and no fungal material in the wound were regarded as unsuccessfully infected due to bad inoculum and discarded. An exception to the procedure mentioned above was **Spruce 2** that was carried out in October on seedlings that were partially winter-hardened and therefore did not produce any active responses in terms of lesion lengths. Similarly, **Pine 2** got aborted prematurely due to a high temperature spell and did therefore not yield any notable conidiophores for growth length determination.

Statistical and QTL analysis

The variance components were estimated using the following mixed linear model:

$$\boldsymbol{y}_{ijk} = \mu + \boldsymbol{b}_i + \boldsymbol{g}_{ij} + \boldsymbol{e}_{ijk}$$

where $\boldsymbol{y}_{ijk}$ is the observation of each trait of the ijk^{th} inoculation, μ is the overall mean, $\boldsymbol{b}$ is the fixed block effect, $\boldsymbol{g}$ is the random isolate effect, $\boldsymbol{e}$ is the random residual. The random effects were assumed to be normally distributed with expectation zero and to be independent of each other. The model was the same for the unblocked **Pine 2** experiment, without the fixed block effect.

Genotypic $(\hat{\sigma}^2_{\mathrm{G}})$, environmental $(\hat{\sigma}^2_{\mathrm{E}})$ and phenotypic $(\hat{\sigma}^2_{\mathrm{P}})$ variance components were estimated as $\hat{\sigma}^2_{\mathrm{G}} = \hat{\sigma}^2_{\mathrm{g}}$, $\hat{\sigma}^2_{\mathrm{E}} = \hat{\sigma}^2_{\mathrm{e}}$ and $\hat{\sigma}^2_{\mathrm{P}} = \hat{\sigma}^2_{\mathrm{G}} + \hat{\sigma}^2_{\mathrm{E}}$ where $\hat{\sigma}^2_{\mathrm{g}}$ and $\hat{\sigma}^2_{\mathrm{e}}$ are the estimated clonal and residual variances, respectively. The estimates of individual observation broad-sense heritability $(\hat{H}^2)$ were obtained by $\hat{H}^2 = \hat{\sigma}^2_{\mathrm{G}} / \hat{\sigma}^2_{\mathrm{P}}$ (Falconer, 1989).

QTL for virulence estimates were analysed using interval mapping (Lander & Botstein, 1988) for every 1 cM of the genetic linkage map of *H. annosum s.l.* to identify associations between AFLP markers and virulence, significant at the 1% level as calculated by the permutation test from the MapQTL 4.0 software (Van Ooijen *et al.*, 2002). All calculations were made using MapQTL 4.0. QTL analysis was performed with least square mean values of lesion lengths and fungal spread in the stem; upstem, downstem and up- and downstem combined.

Results

All 102 isolates from the segregating population of the *H. annosum s.l.* cross were used in all four experiments to inoculate the plants through stem wounds. The parental strains TC-122-12 and TC-32-1 and the AO8-hybrid were included in **Spruce 1** and **2** and in **Pine 1**. In **Spruce 1** and **Pine 1**, another S-type control strain, Rb 175, was also included (Table 1, Fig 1a-f). Negative controls in form of seedlings inoculated with wooden plugs without fungal material was performed in every experiment. These never produced any lesions apart from in the area of the actual wound and less than one mm into the inner bark. No fungal reisolations could be made from any of the negative controls. For most hosts, virulence measures and growing conditions, the TC-32-1 parental strain and the median of both the parental strains were significantly more virulent than the mean of the progeny isolates. Exceptions to this were **Spruce 2**, conducted on dormant host material, where the parental median was significantly *lower* than the progeny mean, and the lesion lengths of **Pine 1** where no significant differences could be found at all (Table 1). The mean lesion lengths caused by the fungal infections were continuously distributed (Fig. 1) and isolates differed significantly.

Table 1. *Heterobasidion annosum s.l.* virulence analysis on *Picea abies* and *Pinus sylvestris*

Experiment	Isolates	Inf. freq. (%)[1]	Progeny[2]	TC-32-1[2]	TC-122-12[2]	AO 8[2]	Rb175[2]
Spruce 1							
Outdoors							
Lesion	95	64	10.8 (+/-1.3) [AB]	54.3 (+/-42.2)	33.0 (+/-10.0)	3.3 (+/- 0.5) [AB]	16.3 (+/-1.8) [B]
Sapwood	96	66	27.7 (+/-2.4) [AB]	88.8 (+/-19.1)	47.5 (+/-27.5)	15 (+/-4.6) [AB]	31.3 (+/-9.0) [B]
Spruce 2							
Green house							
Sapwood	87	86	10.9 (+/-0.9) [A]	6.0 (+/-2.4)	0[3]	4.0 (+/-2.9)	ND
Pine 1							
Outdoors							
Lesion	94	68	11.5 (+/-1.7)	34.3 (+/-17.8)	11.4 (+/-4.5)	15.0 (+/-5.1)	8.8 (+/-4.2)
Sapwood	96	68	8.7 (+/-1.5) [AB]	48.8 (+/-23.6)	16.3 (+/-8.0)	5.0 (+/-5.0) [B]	9.0 (+/-6.0) [B]
Pine 2							
Green house							
Lesion	97	86	1.9 (+/-0.1)	ND	ND	ND	ND

Footnotes:
[1] Frequency of successfully infected seedlings. Unsuccessful infections have been omitted in subsequent calculations.
[2] Mean virulence, measured as lesion or growth length in mm, for all progeny isolates or specific isolate. SE values indicated in parenthesis.
[3] Zero means isolate colonised the wound but spread less than 5 mm in the stem.
[A] Mean value significantly differing from the median between the parental isolates TC-32-1 and TC-122-12 in Bonferroni t-test at the 0.05 level of significance.
[B] Mean value significantly differing from the mean of TC-32-1 in Bonferroni t-test at the 0.05 level of significance.
ND = not determined.

The broad sense heritability estimate ($\hat{H}^2$) varied from 0.12 (SE=0.06) to 0.39 (SE=0.07), with genotypic variance components $(\hat{\sigma}^2_G)$ from 0.4 (SE=0.1) to 106.5 (SE=27.7) and a phenotypic variance component $(\hat{\sigma}^2_P)$ from 2.1 to 275.3 for lesion induced (Table 2), while the broad sense heritability estimate ($\hat{H}^2$) for sapwood growth varied between 0.23 (SE=0.16) to 0.57 (SE=0.05), with genotypic variance components $(\hat{\sigma}^2_G)$ from 4.4 (SE=0.8) to 25 (SE=5.1) and a phenotypic variance component $(\hat{\sigma}^2_P)$ from 27.7 to 54 (Table 2).

Table 2. *Heritability estimates and variance components of Heterobasidion annosum s.l. virulence on Picea abies and Pinus sylvestris*

Infection	H^2 [A]	σ^2_G [B]	σ^2_P [C]
Spruce 1			
Lesion	0.12 (+/- 0.06)	19.5 (+/- 10.5)	168.7
Sapwood	0.46 (+/- 0.06)	250.5 (+/- 50.9)	540.2
Spruce 2			
Sapwood	0.57 (+/- 0.05)	43.8 (+/- 8.0)	77.4
Pine 1			
Lesion	0.39 (+/- 0.07)	106.5 (+/- 27.7)	275.3
Sapwood	0.23 (+/- 0.06)	52.6 (+/- 16.2)	227.0
Pine 2			
Lesion	0.21 (+/- 0.06)	0.4 (+/- 0.1)	2.1

Footnotes:
[A] Broad sense heritability estimate.
[B] Genotypic variance component.
[C] Phenotypic variance component.
SE values indicated in parenthesis.

First spruce infection

For the fungal spread upwards in the plant, a QTL effect was found on LG 15 (Fig. 2a). Although the QTL profile over the group looked similar for up- and downstem growth and these correlated (R^2=0.836), no significance could be noted for downward spread. The LOD peak of 2.77 lied 10 cM from marker *paccts03* and 9 cM from marker *accgs09* and explained 20.4% of the variation. For the lesion lengths, another LG 15 QTL could be located (Fig. 2b). This QTL was significant for upstem as well as and downstem growth, and most significant for up/down combined. Marker *accgs09* was the only marker within the area of significance. For the up/down combined trait, it was located 2 cM from the QTL peak of LOD 3.85. The QTL explained 34.1% of the variation.

Second spruce infection

For the wood growth, another LG 15 QTL could be located (Fig. 2c). This QTL was significant both for upstem and downstem growth, and even more so for both combined. The QTL had two nominal peaks. For the combined trait, the highest

peak had a LOD of 3.53 and was located at marker *paccts03.* This QTL explained 19.4% of the variation. The second peak of LOD 3.38, 7 cM from *aaccs7* and 3.8 cM from *aacap05*, explained 21.2% of the variation. No lesion lengths could be measured since this experiment was carried out on partially winter-hardened plants.

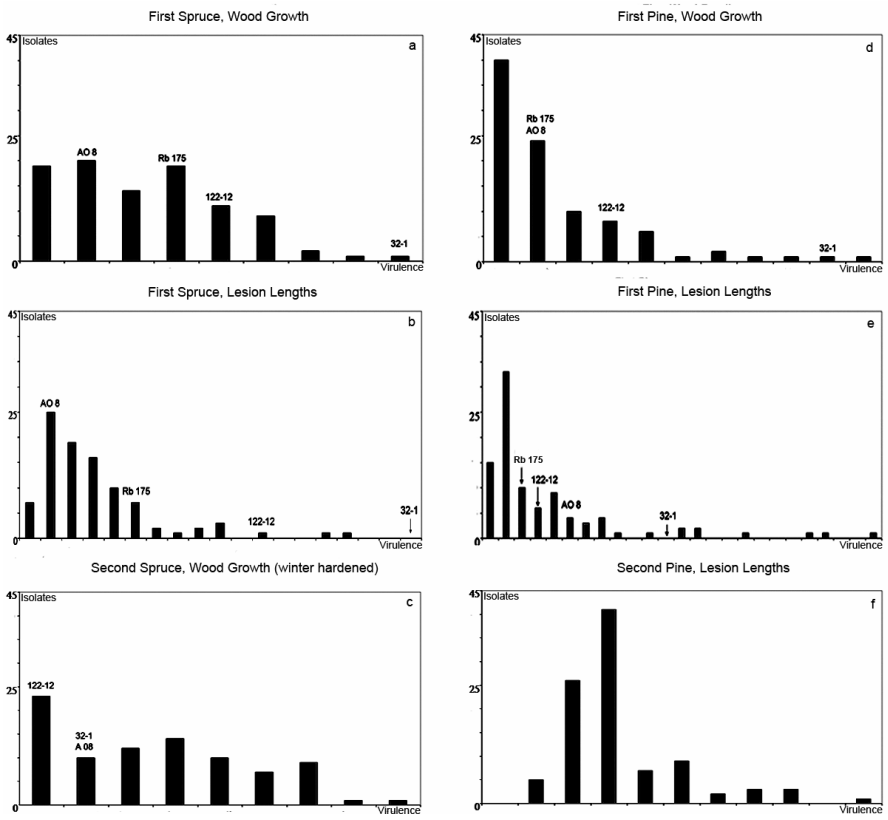

Fig. 1a-f. Frequencies of virulence in a progeny population in *Heterobasidion annosum s.l.*. Experiments were performed on pine (a-c) and spruce (d-f) and measured in wood (a, c, and d) and the inner bark (b, e and f). Each isolate was tested in at least 5 replications. Virulence of parental strains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

First pine infection

The linkage mapping of *H. annosum s.l.* published earlier generated 20 LGs containing fewer than 6 markers (Lind, Olson & Stenlid, 2005). One of them, here named LG 36 and containing 4 markers over 40.5 cM, contained a QTL for fungal downstem growth in wood (Fig. 2d), as well as for up- and downstem growth combined. No significance could be found for upstem growth, although the group-wide QTL profile looked similar for up- and downward growth and the traits correlated well (R^2=0.663). Only marker *aactp13* at the end of the group had

significant LOD values. The peak of LOD 3.24 for the downstem growth lied at this marker and explained 16.6% of the variation. No significant QTL could be found for the lesion lengths in this experiment.

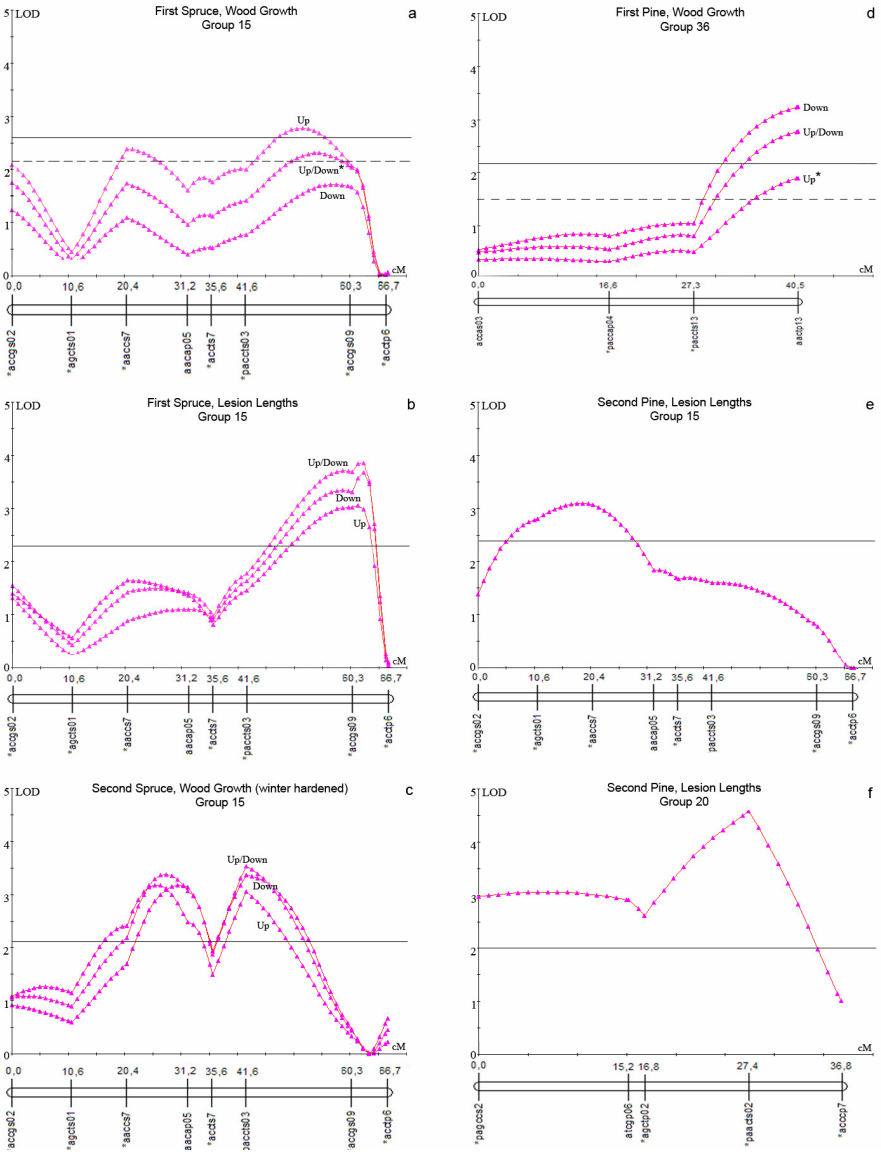

Fig. 2a-f. QTLs for virulence to spruce and pine in *Heterobasidion annosum s.l.* In figure a-d, data for virulence upstem, downstem and up/down combined are being presented. The complete line denotes the highest LOD value for the 1% level of significance for the measurements included in each figure. The spaced line denotes the LOD value for the 1% level of significance for the measurement marked with an asterisk only.

Second pine infection

Due to a high temperature spell after two weeks of infection, this experiment was terminated prematurely. It was however still harvested after four weeks. No living fungi could be reisolated from the plants. For virulence estimated from mean lesion lengths, a QTL was identified on LG 15 with a LOD peak of 3.09 0.8 cM from marker *aaccs7* (Fig. 2e). This QTL explained 14.9% of the variation of lesion lengths found in the pine tree stems. One of the smaller LGs mentioned above, here referred to as LG 20 and containing 5 markers and spanning 36.8 cM, also had a strong QTL. Its LOD peaked at 4.58 at marker *paacts02*, but apart from marker *acccp7*, every other marker in the group had LOD values above the level of significance, from 2.61 to 2.97 (Fig. 2f). The variation of virulence estimated from lesion lengths explained by this QTL was 19.8%.

Discussion

In this study, we assigned specific chromosomal regions in the fungus *H. annosum s.l.* to virulence on *P. abies* and *P. sylvestris* using single spore isolates, originating from a cross between North American S and P type parental strains. We found that LG 15 in a previously constructed linkage map (Lind, Olson & Stenlid, 2005) is of high interest for these traits. We also found two more LGs of interest for virulence and demonstrated a distinct negative effect on virulence of S/P hybridization.

Two experiments were made on two-year-old *P. abies* seedlings and two on one-year-old *P. sylvestris* seedlings. The seedlings were inoculated through a cambial wound. After four weeks of infection, lesion lengths and fungal spread in the stem were measured and used as estimations of virulence. Our data (Table 1, Fig. 1a-f) show that the TC-32-1 parental strain and the median of both parental strains were significantly more virulent than the mean of the progeny isolates in most experiments and significantly more virulent than the heterokaryotic AO 8 hybrid in **Spruce 1** (Table 1). These observations are in concordance with observations made earlier (Garbelotto*, et al.*, 1996) and support the conclusion that the hybrid isolates suffer negative effects on virulence. We could not find such an effect in a previous study (Olson, Lind & Stenlid, 2005), indicating that the negative hybridization effect is not expressed under the conditions used in that study; infection of young seedling roots. The negative effect does not occur in the spruce experiment on winter-hardened seedlings (Table 1, Fig. 1c) where the median of the parental isolates was significantly lower than the progeny isolates. This might have to do with the fact that the winter-hardened seedlings do not express their defence mechanisms and thus likely also lack recognition responses. It is possible that in order to express its host specificity, the fungus needs to recognize or counteract certain defence reactions within the host.

Olson and Stenlid (2001) showed a strong mitochondrial influence on virulence in *H. annosum*. In a previous study, we showed that the nuclear involvement also

plays a major part, possibly in some mitochondrial interaction (Olson, Lind & Stenlid, 2005). The continuous distribution of virulence in the progeny in all experiments in this study further strengthens this observation.

Data on growth in stem from both spruce experiments identified QTLs on LG 15. These QTLs had significant peaks of LOD 2.77 for **Spruce 1** (Fig. 2a) and LOD 3.53 and 3.38 for **Spruce 2** (Fig. 2c). Lesion length data from **Spruce 1** also locates a QTL on LG 15 with a LOD of 3.85. Lesion lengths were not measured in **Spruce 2**. The position of this QTL is similar to the QTLs for the spread in stem. It seems possible that there are two regions of LG 15 with factors of relevance to the virulence on spruce, of which both are of great importance for growth in wood while one is much more important than the other for the lesion lengths in the inner bark.

In **Pine 1**, one QTL was found for fungal growth in wood, of LOD 3.24 on LG 36. Fungal growth in wood was not measured in **Pine 2**. In the first experiment, no significant QTLs for lesion lengths could be found; while in **Pine 2** two QTLs were located for this trait. One of them, of LOD 3.09, emerged on LG 15, while the other was mapped to LG 20 at LOD 4.58. This further supports the idea of LG 15 being of great interest for virulence in *H. annosum s.l.*. The profile of this QTL also adds to the impression of two separate regions being relevant on this group. Interestingly, it is not the same region that seemed to be of importance to lesion length in spruce as that of lesion lengths in pine. LG 20 and LG 36 seem to harbour pine-specific factors of *H. annosum s.l.* virulence, while LG 15 apparently contains elements that are both spruce specific and more general.

The results from the separate spruce experiments correlated well, resulting in QTLs on the same LG. The lesion lengths could not be repeated due to the winter-hardened state of the saplings, but the correlation between lesion length and sapwood growth in the first assay makes it plausible that the lesion length factors still are closely linked to the sapwood growth. This is also in concordance with previous reports where lesion length and fungal growth in the sapwood were correlated (Swedjemark, Johannesson & Stenlid, 1999; Swedjemark & Stenlid, 1996; Swedjemark, Stenlid & Karlsson, 1998). However, the same author has not been able to find such a correlation in other experiments (Swedjemark, Johannesson & Stenlid, 1999; Swedjemark & Karlsson, 2004). It has also been shown that the susceptibility of spruce to *H. annosum s.l.* sapwood growth during different vegetative stages correlates and that difference between clones explained a larger part (24%) of the difference in susceptibility than the vegetative state (4%) (Swedjemark & Stenlid, 1996). This supports our comparison between sapwood growth in winter-hardened and non-hardened spruce saplings.

In pine, we could not repeat the sapwood growth analysis in **Pine 2** as the experiment got terminated prematurely. For lesion lengths however, **Pine 2** managed to locate QTLs not found during **Pine 1**. A reason for this might be the higher number of replicates in the second assay. Furthermore, the variation in lesion lengths was much larger in the first experiment. This can partly be explained by **Pine 2** getting aborted after two weeks, but might also have to do

with the fact that the plants were kept outdoors, where one-year-old seedlings are much more exposed to abiotic differences than in a green house. As this would have affected the general status of the seedlings, it is likely that it also would have affected their susceptibility to the infections.

Olson (2006) located QTLs for rate of growth on agar on LG 1 and 19 on the linkage map used in this study (Lind, Olson & Stenlid, 2005). Our QTLs do not correlate to those identified by Olson, indicating that there is more to the genetic factors behind them than the ability to quickly cover dead substrate. This strengthens the theory that our QTLs are involved in the pathogenic process.

The individual heritability of virulence in our study varied from 0.12 to 0.57 (Table 2) which is comparable to the individual heritability of susceptibility to bacteria or toxin in *A. bisporus* which Moquet estimated to 0.38 respective 0.45 (1999). The variance in virulence in these experiments not explained by the QTLs found might be associated with additional QTLs for virulence, but since part of the *H. annosum s.l.* genome is not fully covered in the genetic linkage map, the map would have to be extended in order to identify these QTLs. There are other possible reasons why not all QTLs for virulence would be identified. Some QTLs could be masked by environmental variation or not expressed under these experimental conditions. The parents might also not display any polymorphism for some QTLs for virulence measured in our analysis and thus remain undetected. Performing alternative infections, such as using more mature trees or clonal host material, or different measures of virulence such as rate of caused death in the host, might result in identification of additional QTLs, highlighting other virulence aspects than the analyses used in this study.

In *H. annosum s.l.* we found that the virulence measured as either fungal growth in wood or lesion length observed in inner bark segregates as continuous characters. In our interspecies cross, virulence towards spruce seem to be controlled by either few or closely situated regions, while there are several regions affecting virulence towards pine. Not many other tree pathogens has been studied in this way, but the virulence of *Venturia inaequalis*, causing apple scab, has been shown to be controlled by a single major locus (Sierotzki & Gessler, 1998). However, in agricultural systems both multi and single locus control of virulence have been identified. In a progeny population of *G. zeae* pathogenicity towards wheat segregates 61:38 for pathogenic vs. non-pathogenic and maps to a location that is adjacent to loci affecting colony pigmentation, perithecium production and trichothecene toxin production (Cumagun*, et al.*, 2004). Analysing only the pathogenic isolates, a QTL for aggressiveness was detected suggesting that only few loci for pathogenicity and aggressiveness differ in their cross (Cumagun*, et al.*, 2004). In the barley pathogen *C. sativus* virulence is segregating 1:1 for low respectively high virulence, indicating that a single locus controls virulence on a particular cultivar in this cross (Zhong & Steffenson, 2002). Also, in *P. infestans*, six possible virulence factor loci were observed (Jiang*, et al.*, 2006).

Although our data indicate that virulence factors are originating from several parts of the *H. annosum s.l.* genome, there is still the possibility that two or all

three of the groups belong to the same chromosome. All three LGs are relatively small and there is a clear discrepancy in the number of LGs to the expected number of chromosomes in *H. annosum s.l.* (Anderson, Kasuga & Mitchelson, 1993). The aspects of virulence represented by the QTLs could thus be physically closer than indicated by their genetic localization. Even when located on different chromosomes there might be a physiological linkage between the gene products and they could be involved in the same biochemical process.

In this study we have shown a negative effect on virulence of hybridization between North American S and P isolates. On winter-hardened spruce, this effect could not be detected, suggesting that a mutual host-pathogen recognition is required for host specificity to be expressed. Moreover, we have identified several QTLs in *H. annosum* s.l. for virulence on pine and spruce. QTLs controlling both lesion lengths and fungal sapwood growth in spruce were found on LG 15 in our previously reported linkage map (Lind, Olson & Stenlid, 2005). A QTL for lesion lengths in pine was also found here, while yet another pine lesion length QTL and one QTL for fungal growth in pine sapwood was found on two different, smaller LG. This QTL mapping of the virulence trait form the basis for map based cloning and identification of virulence genes, providing basic information on the mechanistic interactions between conifers and the annosum rot root pathogen.

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References

Albersheim, P. & Anderson-Prouty, A. 1975. Carbohydrates, proteins, cell surfaces and the biochemistry of pathogenesis. *Annual Review of Plant Physiology 26*, 31-52.

Anderson, M., Kasuga, T. & Mitchelson, K. 1993. A partial physical karyotype of *Heterobasidion annosum*. In *Eighth International Conference on Root and Butt Rots*, Edited by M. Johansson & J. Stenlid. Wik, Sweden & Haikko, Finland

Asiegbu, F.O. 2000. Adhesion and development of the root rot fungus (Heterobasidion annosum) on conifer tissues: effects of spora and host surface constituents. *FEMS Microbiology 33*, 101-110.

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.

Asiegbu, F.O., Daniel, G. & Johansson, M. 1994. Defence related reactions of seedling roots of Norway spruce to infection by *Heterobasidion annosum* (Fr.) Bref. *Physiological and Molecular Plant Pathology 45*, 1-19.

Asiegbu, F.O., Johansson, M., Woodward, S. & Hüttermann, A. 1998. Biochemistry in the Host-Parasite Interaction. In *Heterobasidion annosum. Biology, Ecology, Impact and Control*. Edited by S. Woodward, J. Stenlid, R. Karjalainen & A. Hüttermann. CAB International. Wallingford, UK. 167-193. pp.

Atkinson, M. 1993. Molecular mechanisms of pathogen recognition by plants. *Advances in Plant Pathology 10*, 35-64.

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

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.

Elad, Y., Williamson, B., Tudzynski, P. & Delen, N. 2004. *Botrytis:* Biology, Pathology and Control. In. Kluwer Academic Publishers. Dordrecht.

Falconer, D. 1989. *Introduction to quantitative genetics*. 3rd 3rd. Longman Group Limited. London pp.

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

Fox, R. 2000. *Armillaria* Root Rot: Biology and Control of Honey Fungus. In. Intercept Press. Andover, UK. 240. pp.

Garbelotto, M., Ratcliff, A., Bruns, T.D., Cobb, F.W. & Otrosina, W.J. 1996. Use of Taxon-Specific Competetive-Priming PCR to Study Host Specificity, Hybridization, and Intergroup Gene Flow in Intersterility Groups of Heterobasidion annosum. *Phytopathology 86*, 543-551.

Garbelotto, M.M., Lee, H.K., Slaughter, G., Popenuck, T., Cobb, F.W. & Bruns, T.D. 1997. Heterokaryosis is not required for virulence of *Heterobasidion annosum*. *Mycologia 89*, 92-102.

Gilchrist, D. 1998. Programmed cell death in plant disease: the purpose and promise of cellular suicide. *Annual Review of Phytopathology 36*, 393-414.

Greenberg, J. & Yao, N. 2004. The role and regulation of programmed cell death in plant-pathogen interactions. *Cellular Microbiology 6*, 201-211.

Harrington, T., Worrall, J.J. & Rizzo, D. 1989. Compatibility among host-specialized isolates of *Heterobasidion annosum* from western North America. *Phytopathology 79*, 290-296.

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.

Heath, M. 2000. Hypersensitive response-related death. *Plant Molecular Biology 44*, 321-334.

Holz, G., Coertze, S. & Williamson, B. 2004. The ecology of *Botrytis* on plant surfaces. In *Botrytis: Biology, Pathology and Control*. Edited by Y. Elad, B. Williamson, P. Tudzynski & N. Delen. Kluwer Academic Publishers. Dordrecht. 9-27. pp.

Jiang, R., Weide, R., de Vondervoort, P. & Govers, F. 2006. Amplification generates modular diversity at an avirulence locus in the pathogen Phytophthora. *Genome Research 16*, 827-840.

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

Johansson, M., Denekamp, M. & Asiegbu, F.O. 1999. Production and isozyme pattern of extracellular laccase in the S and P intersterility groups of the root pathogen *Heterobasidion annosum*. *Mycological Research 103*, 365-371.

Johansson, M., Lundgren, L. & Asiegbu, F.O. 1994. Initial interactions in living bark and sapwood of conifers infected by root rot fungi. In *Proceedings from the SNS Meetings in Forest Pathology.* Edited by D. Aamlid. SNS. Biri, Norway. 12-16. pp.

Jones, E. 1994. Fungal adhesion. *Mycological Research 98*, 961-981.

Karlsson, J. & 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.
Knoche, H. & Duvick, J. 1987. The role of fungal toxins in plant disease. In *Fungal Infection of Plants*. Edited by G. Pegg & P. Ayres. Cambridge University Press. Cambridge. 158-191. pp.
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*. Edited by S. Woodward, J. Stenlid, R. Karjalainen & A. Hüttermann. CAB International. Wallingford, UK. 43-70. pp.
Lander, E.S. & Botstein, D. 1988. Mapping Mendelian Factors Underlying Quantitative Traits Using RFLP Linkage Maps. *Genetics 121*, 185-199.
Larraya, L.M., Alfonso, M., Pisabarro, A.G. & Ramirez, L. 2003. Mapping of Genomic Regions (Quantitative Trait Loci) Controlling Production and Quality in Industrial Cultures of the Edible Basidiomycete Pleurotus ostreatus. *Applied and Environmental Microbiology 69*, 3617-3625.
Larraya, L.M., Idareta, E., Arana, D., Ritter, E., Pisabarro, A.G. & Ramirez, L. 2002. Quantitative Trait Loci Controlling Vegetative Growth Rate in the Edible Basidiomycete Pleurotus ostreatus. *Applied and Environmental Microbiology 68*, 1109-1114.
Lind, M., Olson, Å. & Stenlid, J. 2005. An AFLP-marker Based Genetic Linkage Map of *Heterobasidion annosum* Locating Intersterility Genes. *Fungal Genetics and Biology 42*, 519-527.
Lindberg, M. & Johansson, M. 1991. Growth of *Heterobasidion annosum* through bark of *Picea abies. European Journal of Forest Pathology 21*, 377-388.
Lyon, G.D., Goodman, B.A. & Williamson, B. 2004. *Botrytis cinerea* perturbs redox strategies as an attack strategy in plants. In *Botrytis: Biology, Pathology and Control*. Edited by Y. Elad, B. Williamson, P. Tudzynski & N. Delen. Kluwer Academic Publishers. Dordrecht. 119-141. pp.
Manocha, M. 1984. Cell surface characteristics of *Mortierella* and their interaction with a mycoparasite. *Canadian Journal of Microbiology 30*, 290-298.
Moquet, F., Desmerger, C., Mamoun, M., Ramos-Guedes-Lafargue, M. & Olivier, J.-M. 1999. A Quantitative Trait Locus of Agaricus bisporus Resistance to Pseudomonas tolaasii Is Closely Linked to Natural Cap Color. *Fungal Genetics and Biology 28*, 34-42.
Morel, J.-B. & Dangl, J. 1997. The hypersensitive response and the induction of cell death in plants. *Cell Death and Differentiation 4*, 671-683.
Niemelä, T. & Korhonen, K. 1998. Taxonomy of the Genus *Heterobasidion*. In *Heterobasidion annosum. Biology, Ecology, Impact and Control*. Edited by S. Woodward, J. Stenlid, R. Karjalainen & A. Hüttermann. CAB International. Wallingford, UK. 27-33. pp.
Olson, Å. 2006. Genetic linkage between growth rate and the intersterility genes S and P in the basidiomycete *Heterobasidion annosum s.lat. Mycological Research 110*, 979-984.
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.
Olson, Å. & Stenlid, J. 2001. Mitochondrial control of fungal hybrid virulence. *Nature 411*, 438.
Siegee, D. 1993. Bacterial Plant Pathology; Cell and Molecular Aspects. In. Cambridge University Press. Cambridge, UK. 126-171. pp.
Sierotzki, H. & Gessler, C. 1998. Genetic analysis of a cross of two *Venturia inaequalis* strains that differ in virulence. *Journal of Phytopathology 146*, 515-519.

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. 1991. Partial intersterility in *Heterobasidion annosum. Mycological Research 95*, 1153-1159.

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

Swedjemark, G. & Karlsson, B. 2004. Genotypic variation in susceptbility following artificial Heterobasidion annosum inoculation of Picea abies clones in a 17-year-old field test. *Scandanavian Journal of Forest Research 19*, 103-111.

Swedjemark, G. & Stenlid, J. 1996. Variation in spread in *Heterobasidion annosum* in clones of *Picea abies* grown at different vegetation phases under greenhouse conditions. *Scandanavian Journal of Forest Research 11*, 137-144.

Swedjemark, G., Stenlid, J. & Karlsson, B. 1998. Genetic variation among clones of *Picea abies* in resistance to growth of *Heterobasidion annosum*. *Silvae Genetica 46*, 369-374.

Van Ooijen, J., Boer, M.P., Jansen, R.C. & Maliepaard, C. 2002. *MapQTL 4.0, Software for the calculation of QTL positions on genetic maps.* Plant Research International, Wageningen, the Neterlands.

Welz, H. & Leonard, K. 1994. Genetic analysis of two race 0 X race 2 crosses in *Cochliobolus carbonum*. *Phytopathology 84*, 83-91.

Vinatzer, B., Patocchi, A., Gianfranceschi, L., Tartarini, S., Zhang, H., Gessler, C. & Sansavini, S. 2001. Apple contains receptor-like genes homologous to the *Cladosporium fulvum* resistance gene family of tomato with a cluster of genes cosegregating with Vf apple scab resistance. *Molecular Plant-Microbe Interactions 14*, 508-515.

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. & Hûttermann, A. 1998. *Heterobasidion annosum*. Biology, Ecology, Impact and Control. In. CAB International. Cambridge, UK.

Zhong, S. & Steffenson, B.J. 2002. Identification and characterization of DNA markers associated with a locus conferring virulence on barley in the plant pathogenic fungus *Cochliobolus sativus*. *Theoretical and Applied Genetics 104*, 1049-1054.

Genetics and QTL mapping of somatic incompatibility and intraspecific interactions in the basidiomycete *Heterobasidion annosum s.l.*

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Abstract
In this study, we show that somatic incompatibility (SI) in the basidiomycete *Heterobasidion annosum sensu lato* is controlled by four loci. This was done by observing the frequency of compatible pairings in two matrices of pairings using every possible combination. The first included 63 heterokaryons with one unique sibling nucleus and one unrelated nucleus, the second 39 heterokaryons with the sibling nuclei backcrossed with one of the parental strains. We concluded that SI allelic differences in a pairing alone are not enough to determine the degree of somatic incompatibility. In the first matrix, we also observed other interactions such as hyphal walls in interaction zones, increased exudation of dark colored metabolites and increased production of aerial hyphae. These data were used to map QTLs for the respective traits to a genetic linkage map of the *H. annosum* genome. Many of these QTLs were strong and will be identified in upcoming studies using map based cloning. The strengthened data for the number of loci and the identified QTLs, of which several represent reactions exclusive for encounters with non-self mycelia, shed much new light on the SI concept in basidiomycetes.

Keywords
Basidiomycete, genetic linkage mapping, quantitative trait loci, fungal interactions, recognition

Introduction

Somatic incompatibility (SI) is a system by which filamentous fungi can distinguish self from non-self by delimiting its own mycelia from that of other individuals of the same species. The SI system maintains the separate identity of individuals by restricting nuclear and cytoplasmic exchange between different genotypes. In this way the system provides a protection against the transmission of infectious elements such as mycoviruses (Milgroom, 1999) and lethal plasmids, and the mycelial individual can avoid becoming subjugated by aggressive nuclear genotypes (Hartl, Dempster & Brown, 1975; Worrall, 1997). It is the SI system that allows vegetative mycelia to maintain their individuality in every sense; genetically, physiologically and ecologically. The macroscopic expression of SI can vary greatly among species, from a band of hyphal knots along the edges of the interaction (Barrett & Uscuplic, 1971) to very strong pigmentation and deep, well-defined clearing zones (Hansen, Stenlid & Johansson, 1993b) with no or very little aerial hyphae. When observing SI between individuals in wood however, the most apparent indicator of mycelial interactions is dark zonal lines, albeit those can also appear in interspecific interactions. No matter the physical appearance, the basic principle of isolation of interacting mycelia remains the same (Malik & Vilgalys, 1999).

The somatic incompatibility reaction is not a strict binary character. The intensity and morphology of mycelial interactions usually vary among pairings and also depend on media and growth conditions (Worrall, 1997). There is a general trend that distantly related secondary mycelia express somatic incompatibility more frequent (Worrall, 1997). In this paper, we define secondary mycelia as the dikaryotic product of a compatible mating between two homokaryotic (primary) mycelia. The intensity of the SI reaction has been reported to decrease with relatedness (Kay & Vilgalys, 1992; Stenlid, 1985) although a quantitative estimate of this association in *Coprinus cinereus* could not prove a significant difference between mycelial pairs sharing a common nucleus as compared with no nuclei in common (May, 1988). Incompatibility reactions can also be observed between homokaryotic isolates prior to mating. When mating type compatibility has been recognized, the mating system is believed to override the SI (Rayner *et al.*, 1984; Rayner & Todd, 1979).

Somatic incompatibility is associated with genetic dissimilarity in the SI loci. Generally, individuals are considered to be compatible if they share alleles for all SI loci and incompatible if heterozygous for at least one locus (Caten, 1972; Malik & Vilgalys, 1999; Perkins & Turner, 1988). The intensity of the reaction generally increases with the number of differences (Caten, 1972). Under this assumption the SI reaction has been used to delimit fungal individuals (genets) and to study the population biology of basidiomycetes (Johannesson & Stenlid, 2004; Swedjemark & Stenlid, 2001). In ascomycetes the somatic incompatibility (generally known as vegetative or heterokaryon incompatibility) has been shown to be controlled by several multiallelic and biallelic unlinked loci, referred to as *het* (Saupe, Clavé & Béguret, 2000). Interactions at these loci are generally allelic but non-allelic

interaction has been described (Labarère, Béguret & Bernet, 1974). In allelic systems, heterokaryon incompatibility is triggered by allelic differences at a single *het* loci, while in non-allelic systems, the incompatibility is controlled by interactions between specific alleles in two different loci (Béguret, Turcq & Clavé, 1994; Glass & Kuldau, 1992; Leslie, 1993). The number of identified *het* loci varies between species; from 11 and 9 in *Neurospora crassa* (Glass & Kuldau, 1992) and *Podospora anserina* (Béguret, Turcq & Clavé, 1994) to 8 and 7 in *Aspergillus nidulans* (Anwar, Croft & Dales, 1993; Dales & Croft, 1983) and *Cryphonectria parasitica* (Cortesi & Milgroom, 1998; Huber, 1996). Microscopic and molecular heterokaryon incompatibility features have been identified in only a few ascomycetes (Garnjobst & Wilson, 1956; Mylyk, 1976; Newhouse & MacDonald, 1991). The features are generally consistent with those observed during programmed cell death in many organisms, such as cytoplasmic vacuolization, organelle degradation and DNA fragmentation (Jacobson, Beurkens & Klomparens, 1998; Konopleva *et al.*, 1999; Marek *et al.*, 1998). Among basidiomycetes the genetic basis of somatic incompatibility has been estimated to consist of 3 or 4 genes in *H. annosum* (Hansen, Stenlid & Johansson, 1993b), 3 or more in *Pleurotus ostreatus* and *Collybia fusipes* (Marcais, Caël & Delatour, 2000), two biallelic loci in *Serpula lacrymans* (Kauserud *et al.*, 2006) and a major single locus in *Phellinus* spp (Rizzo, Rentmeester & Burdsall, 1995). Compared to ascomycetes, basidiomycetous somatic incompatibility is very scanty studied. No SI genes are cloned or identified from basidiomycetes, and there have been no reports so far of homologies to any vegetative incompatibility genes from ascomycetes.

Heterobasidion annosum (Fr.) Bref *sensu lato* (*s.l.*) is an economically very important basidiomyceteous pathogen, mostly attacking conifers in the Northern Hemisphere (Asiegbu, Adomas & Stenlid, 2005). Three intersterility (IS) groups of *H. annosum s.l.* have been found in Europe, P, S, and F, preferentially infecting pine, spruce and fir respectively, and two IS groups are reported from North America, P and S (Korhonen & Stenlid, 1998). Mating between isolates from distinct IS groups is possible (Chase & Ullrich, 1990; Garbelotto *et al.*, 1996; Korhonen & Stenlid, 1998). The fungus has a unifactorial, multiallelic mating system (Holt, 1983; Korhonen, 1978; Stenlid & Rayner, 1991). The primary mycelium stems from a single basidiospore and lacks clamp connections while the secondary mycelium has clamps at irregularly spaced septa. Morphologically, the *H. annosum s.l.* SI typically consists of a distinct clearing zone between interacting isolates, with no or sparse aerial hyphae (Hansen, Stenlid & Johansson, 1993b; Korhonen, 1978; Stenlid, 1985). In *H. annosum s.l.*, the SI function of preventing transmission of mycoviruses seems to be of limited importance. Ihrmark and colleagues (2002) showed that dsRNA is transferred between incompatible isolates, both homo- and heterokaryotic, and even between isolates of different IS groups. In the field, SI might be an important factor defining mycelial domains and borders for substrate occupancy in *H. annosum s.l.*. In a study of a population of *H. annosum* individuals studied using SI and microsatellite markers, Johannesson and Stenlid (2004) found that nuclei in the interaction zone might remate with other partner nuclei and escape into new

substrate in wood. This was favoured by combinations of nuclei with low degree of somatic incompatibility with each other.

The encounter between individual *H. annosum s.l.* vegetative mycelia triggers hyphal reactions traditionally not considered being direct parts of the *H. annosum s.l.* SI system, such as increased production of aerial hyphae, increased exudation of metabolites and formation of thick mycelia at the fungal-fungal interaction zones. Although not involved in the formation of the typical SI clearing zone usually described as the prime indicator of an SI effect in *H. annosum s.l.* (Hansen, Stenlid & Johansson, 1993a), these reactions are still clearly elicited by the presence of another mycelium and therefore relevant in characterizing the interaction.

The assumption that allelic differences at SI loci alone determine the degree of somatic incompatibility (Caten, 1972; Malik & Vilgalys, 1999; Perkins & Turner, 1988) infers that the effect from identical nuclei, or the genetic background, can be ignored. In this report, we thoroughly challenged that assumption in an ambition to widen the understanding of somatic incompatibility in *H. annosum s.l.*. Moreover, we examined the effect of relatedness on SI. We also wanted to further estimate the number of loci responsible for intraspecific fungal-fungal interactions in *Heterobasidion annosum s.l.* as well as locate them on a previously constructed genetic linkage map (Lind, Olson & Stenlid, 2005). This would enable the subsequent identification and map based cloning of the SI loci.

Material and methods

Experimental populations and genetic linkage map

The genetic linkage map used in this study was made out of 102 homokaryotic progeny isolates, named AO8-1 to AO8-105 (some original isolates were missing), collected from a fruiting of a compatible mating between North American S and P isolates of *H. annosum s.l.* (Olson, Lind & Stenlid, 2005). The genetic linkage map is based on 358 AFLP markers, clustered in 19 larger and 20 smaller linkage groups (LG) (Lind, Olson & Stenlid, 2005). Out of the 102 homokaryotic progeny isolates, 63 were successfully mated with a homokaryotic North American P-isolate, TC-39-7, using a method previously described (Stenlid, 1985). The mating resulted in 63 heterokaryotic isolates (hereafter referred to as the sib-unrelated data set), all carrying one full-sib nucleus, one unrelated (TC-39-7) nucleus and the mitochondrion from the TC-39-7 isolate. The 102 homokaryons were also backcrossed with the homokaryotic parental strain TC-122-12 (S-type). Thirty-nine homokaryons were found compatible with the parental isolate, forming heterokaryotic isolates carrying one full-sib nucleus, one parental (TC-122-12) nucleus (hereafter referred to as sib-parental data set) and the mitochondrion from the TC-122-12 isolate.

Somatic incompatibility analysis

On Petri dishes containing 18 ml Hagem media (Stenlid, 1985) with 10 g/l malt extract instead of 5 g/l, two 5 mm plugs of actively growing heterokaryotic isolates were placed 2 cm apart. The plates were incubated at 25 °C in darkness for 5 weeks and then visually analysed. For each plate the morphology of the interaction was studied. The general somatic incompatibility of the interaction was estimated on a scale of 1-4, where 1 means no incompatibility and 4 means very strong incompatibility. These estimations were made by first-impression assessments of antagonism between the interacting mycelia. Also, the width and strength of the clearing zone between the two isolates was estimated in the same fashion, where 1 meant no visible clearing of aerial hyphae and 4 a very distinct clearing zone. Each isolate on each plate was also analysed for its general tendency to form aerial hyphae, to produce brown mycelia and exudates and to form denser mycelia in the interaction with the other isolate. The same scale was used for these categories, with 1's meaning no brown color, very little aerial hyphae, and no denser mycelia in the interaction zone, respectively. For the 63 sib-unrelated heterokaryotic isolates 2016 plates were set up in an incompatibility matrix with all possible pair wise interactions, including self pairings. In total, 8 different values were estimated for each plate (degree of general somatic incompatibility, distinction of clearing zone, density of hyphae in interaction zone for both isolates, amount of aerial hyphae for both isolates and amount of brown hyphae and agar staining from exudates for both isolates), all together 16128 observations. For the sib-parental heterokaryotic isolates a similar matrix, including self-pairings, was set up resulting in 780 plates. In this matrix only the general somatic incompatibility trait was estimated according to the same scale as above (1-4). In both matrices, each pairing appeared only once, i.e. if the Y x Z pairing existed, no Z x Y pairing was made. However, this caused the self-pairings to be over represented in the data. Therefore, half the effect of self pairings was subtracted from the analysis in the calculations.

Mapping of QTLs

For each heterokaryotic isolate, corresponding to the homokaryotic progeny isolate of the mapping population, and each trait a mean value was calculated by summing up the scored values toward all the other individuals in the matrix and dividing it by the total number of interactions analysed for the particular isolate. Values for all traits were calculated both as absolute values and as values of increase compared to self-pairings. An inductive effect of the aerial hyphal growth, dark metabolite exudation and hyphal wall building traits was also calculated for each individual by summing up the scores observed for the counterpart individual in each pairing. To locate the QTLs of importance, interval mapping (Lander & Botstein, 1988) included in the MapQTL 4.0 software was performed (Van Ooijen *et al.*, 2002) based on the calculated mean values from the different traits measured. To determine the significance threshold for logarithm of odds (LOD) for each putative QTL, the permutation test included in the MapQTL software was run and the 1% level of significance was used to determine a QTL as valid.

Results

Somatic incompatibility pairings

Somatic incompatibility pairings were conducted in two experiments utilising synthesized heterokaryons, 1) pairings among heterokaryons composed of one sib-related and one common unrelated nucleus (sib-unrelated) and 2) pairings among heterokaryons composed of one sib-related and one common parental nucleus (sib-parental).

The sib-unrelated data set consisted of 63 isolates which were paired in all combinations. In the estimation of general compatibility for the complete data set, the number of highly compatible (score 1) pairings in the assay was, counting self pairings as half, 112.5 out of 1947.5 informative pairings. Numbers of observations of scores 2, 3, and 4 were 562, 608 and 665 respectively. The number of pairings with the score 1 for individual isolates varied between 0 and 15 for the sib-unrelated data-set. For the data set of the 39 sib-parental isolates the number of general compatibility scores 1, 2, 3, and 4 were 68.5, 295, 176, and 117, respectively, for the complete data set of 656.5 informative pairings. For individual isolates in the sib-related data set, the number of pairings with the score 1 varied between 0 and 15.

The clearing zone between isolates was estimated in the sib-unrelated data set where a score of 1 meant no visible clearing and 4 a very distinct clearing zone. In the matrix the number of 1, 2, 3, and 4 were 924.5, 620, 277 and 126 respectively, while the number of pairings with the score 4 for individual isolates varied between 0 and 14.

Number of SI loci

To estimate the number of genes involved in SI, the frequency of compatible reactions (1's) in the general somatic incompatibility trait assay was calculated. The entire data set as well as each individual isolate were analysed for deviation from a segregation pattern indicating two, three or four loci control of the SI reaction, i.e. 25%, 12.5%, or 6.25% of the pairings being compatible. Counting every self-pairing as half, the percentage of compatible 1's for the entire sib-unrelated data set was 5.8% which did not differ significantly from the four loci model. Percentage of score 2, 3 and 4 was 28.9%, 31.2% and 34.1%, respectively. Screening each of the 63 isolates individually for segregation of 1's in the somatic incompatibility trait assay, 49 (77.8%) did not differ significantly from the 1/16 ratio. Seven (11.1%) differed at the 0.05 level of significance and 7 differed at the 0.01 level of significance. For the sib-parental data set, the frequency of 1's (10.4%) did not match the 1/16 ratio, but did not differ significantly from a 1/8 ratio of segregation. Frequencies of scores 2-4 were 44.9%, 26.8% and 17.8%, respectively. Screening the 39 individual isolates, 32 (80.0%) did not differ

significantly from the 1/8 ratio while 7 (17.5%) differed at the 0.05 level of significance and 1 (2.5%) differed at the 0.01 level of significance.

Clearing zone was used for the sib-unrelated data set as an estimate of SI. The proportion of highly incompatible pairings with the scoring 4, was 6.5% which was not differing significantly from a 1/16 ratio. The frequencies of scores 1, 2 and 3 was 47.5%, 31.8% and 14.2%, respectively. For individual isolates screening for 4's in the clearing zone assay, 42 (66.7%) did not differ significantly from the 1/16 ratio, while 11 (17.5%) differed at the 0.05 level of significance and 10 (15.9%) differed at the 0.01 level of significance.

SI allelic difference influence of incompatibility reaction

Seventeen out of the 102 homokaryons mentioned above formed dikaryons both with the parental isolate TC-122-22 and with the unrelated isolate TC-39-7. To analyse if SI allelic differences alone determines the degree of somatic incompatibility, the 17 heterokaryons from the sib-unrelated set were paired with the corresponding heterokaryons from the sib-parental set. In the pairings the mycelia in each cross differed in one nucleus, originating from TC-39-7 and TC-122-12, and were similar for the nuclei from the progeny isolate. The pairings were set up twice and included self pairings. All pairings should in theory give the same somatic incompatibility reaction since the pair wise genetic difference is the same among the 17 replicates, albeit the genetic similarity is different. However, the degree of incompatibility still varied (Fig 1). Two pairings were fully compatible (1's) while 4 pairings had 2's, 4 had 3's and 7 crosses had the highest degree on incompatibility (4's). Self pairings of neither the sib-unrelated nor the sib-parental isolates gave anything other then fully compatible reactions (data not shown).

Mapping of intraspecific mycelial interactions and somatic incompatibility

In order to identify the genomic regions affecting intraspecific mycelial interactions and somatic incompatibility, the genetic linkage map (Lind, Olson & Stenlid, 2005) was divided into 1 cM sized segments, and for each segment, the presence of a QTL effect was tested (Fig 2a-f). All mapping calculations were made from average values calculated from the individual 63 sib-related isolates of the AO8-(n) x TC-39-7 data set paired in all possible combinations. For the general somatic incompatibility, one QTL was found on LG 21 (Fig 2d). The QTL had an 18cM long region above LOD 3 and its peak of LOD 3.39 at marker *paccgp02*. This QTL explained 26.6% of the variation of the SI trait.

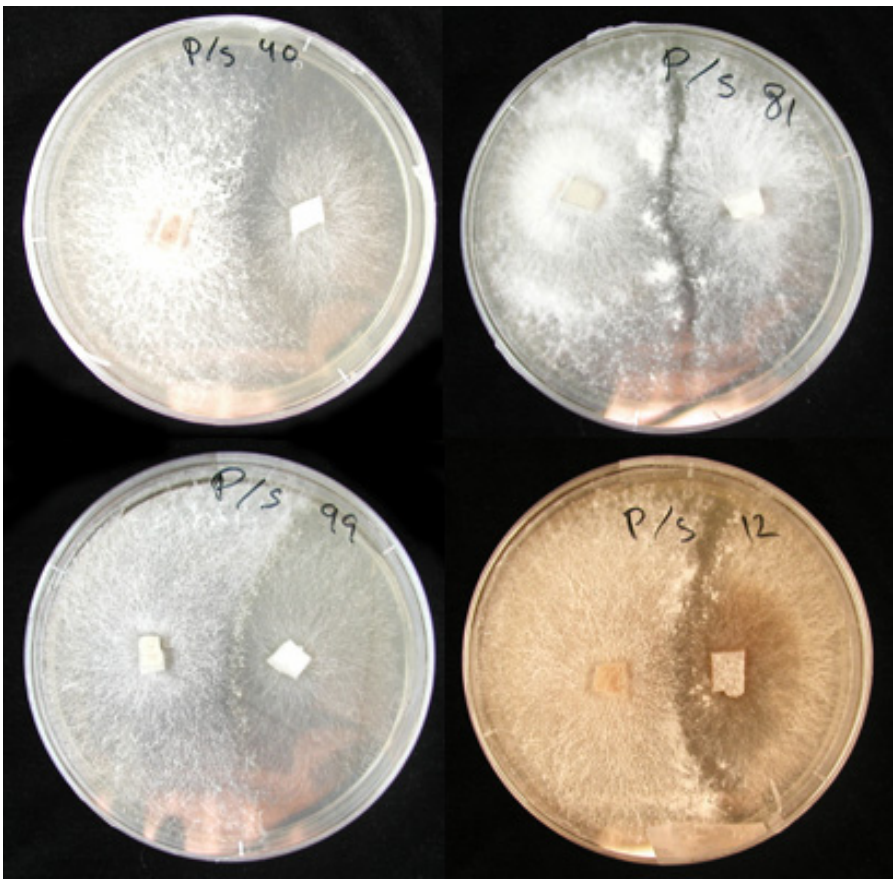

Fig 1. Pairings between *H annosum s.l.* of sib-unrelated and sib-parental heterokaryons. The mycelia in each pairing contain one common sib-nucleus (40, 99, 81 or 12) and either a TC-122-12 or a 39-7 nucleus. The pairings to the left display compatible or very weak incompatibility reactions while the pairings to the right are fully incompatible.

When encountering other mycelia, some fungal individuals tend to trigger the counterpart individual to form various degrees of thick hyphal structures along the interaction zone, ranging from a thin line of white dots to a centimetre-high wall (Fig 3). Two QTLs were found for the ability to induce the formation of these structures in other, non-self mycelia (Fig 2e-f). One of the QTLs was found on LG 7 with two peaks above the threshold value; one between markers *paccap03* and *aaccp13* with a peak of LOD 3.08, explaining 26% of the variation in wall-like hyphae formation induction, the other on marker *pagcts02* with a peak of LOD 4.06, explaining 27.3%. The other QTL was found on LG 34. The LOD score for the QTL increased from marker *aactp02* to marker *aacas02*, at LOD 3.68 at the latter marker, at the very end of the group. This QTL explained 30.5% of the variation in the trait.

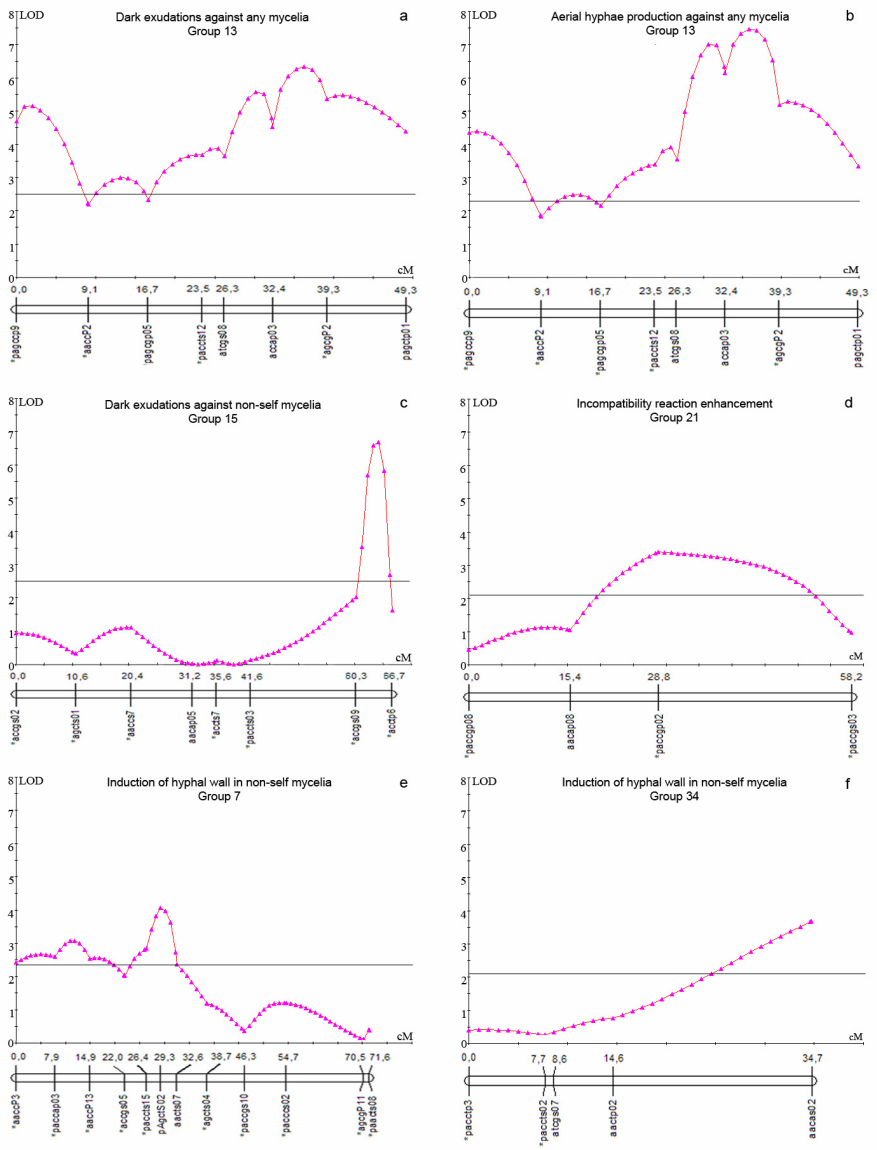

Fig 2a-f. QTLs for various intraspecific interactions in *H. annosum s.l.*. **a** and **b**: data for exudation of dark metabolites and production of aerial hyphae under interaction with any mycelia. **c**: data for increased exudation of metabolites under interaction with non-self mycelia as opposed to self mycelia. **d**: data for stronger reactions to incompatibility. **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.

Some fungal genotypes tend to change their morphology drastically when they meet other mycelia, whether its own mycelium or not, increasing the production of aerial hyphae and producing colored mycelia and exudates, ranging from yellow to dark brown. On LG 13, QTLs were found for both of these abilities when exposed to growing mycelia, self or non-self (Fig 2a-b). The profiles of the QTLs over the entire group were almost identical for both traits. There was a strong QTL close to marker *pagccp9*, with a peak of LOD 5.16 for the colorization trait, explaining 41.7% of the variation, and LOD 4.39 for the aerial hyphae trait, explaining 32.9%. Furthermore, the colorization and aerial hyphae traits both had a ~30 cM long region consistently above LOD 3, spanning from *paccts12* to *pagctp01*, with peaks of 6.33 and 7.47, respectively, located between markers *accap03* and *agcgp2*. These two peaks explained 42.5% and 48.6% of the variation in the respective trait. Some isolates exude dark metabolites and form stained hyphae only when encountering non-self mycelia. On LG 15, a very thin, sharp QTL was found for the ability to increase colorization when exposed to non-self mycelia, as compared to when exposed to self mycelia. The QTL has its peak between markers *accgs09* and *acctp6* at LOD 6.68 and explains 63.5% of the variation of this ability.

No QTL was found for the following traits: Ability to form a hyphal wall when challenged with non-self mycelia, and aerial hyphae production against non-self mycelia.

Fig. 3. Hyphal wall-like structures induced in interactions between *H. annosum s.l.* heterokaryons. To the left, isolate 41 is inducing a wall formation in isolate 73 and in the middle, a similar structure in isolate 40. On the right, isolate 69 cannot induce such a reaction in isolate 40.

Discussion

In our studies 63 heterokaryons carrying one common TC-39-7 nucleus and one AO8-(n) nucleus from a sporulating TC-122-12 x TC-32-1 hybrid was grown pair wise in all possible combinations. We used different screening methods to identify compatible and incompatible reactions. The general somatic incompatibility is a good way to screen for completely compatible reactions as compatible individuals do not react strongly to each other in terms of changed mycelial morphology. However, as we believe the clearing zone to be the prime indicator of *H. annosum s.l.* SI on agar plates, this was the most suitable method to screen for completely incompatible reactions. The frequencies of 5.8% compatible and 6.5% incompatible reactions strongly support the theory of a system of four loci, where complete homozygosis is needed for somatic compatibility and complete heterozygosis for somatic incompatibility. In our second set-up we paired 39 heterokaryons carrying one AO8-(n) nucleus from the hybrids and one parental TC-122-12 nucleus. We found 10.4% compatible reactions indicating three loci controlling SI. Thus, with a parental nucleus present in each pairing, on average 25% of the progeny SI loci are not informative due to monomorphy, i.e. both nuclei in both isolates being homozygous for a locus. This makes it look as if the SI reaction is controlled by 3 loci rather than 4 in the sib-parental pairing test. Although there is nothing in our results disputing the possibility of other loci for which the parental strains would be homozygous, these observations are well in line with Hansen and Stenlids (1993b) observations of 3 or 4 loci controlling SI in *H. annosum*. The observed 1/16 segregation ratio in the sib-unrelated system tells that the parental strains TC-122-12 and TC-32-1 are heterozygous for four loci and that the unrelated TC-39-7 nucleus also is different from both the parents for all four loci.

A common view has been that in pairings between secondary mycelia with a common nucleus the common nucleus would be neutral, i.e. not influencing the SI reaction. The outcome of the interaction would instead depend on the allelic differences in SI loci in the differing nuclei. The 17 heterokaryotic isolates with pair wise common sib-nuclei show that it is not the nuclear genetic differences between two isolates alone that determine the level of somatic incompatibility. In 17 pairings where this difference was the same in every pairing, we could still identify four levels of SI (Fig 1). This might be explained by a suppression of the TC-39-7 or TC-122-12 nuclei, preventing its SI alleles to be expressed, or a mycelium partially homokaryotic, largely dominated by the progeny nucleus, causing the TC-39-7/TC-122-12 nuclei to simply not be present in the interaction zone. Alternatively, this could indicate that all nuclei affect SI and that separate pairings containing the exact same allelic differences in compatibility thus can give different levels of incompatible reactions. In interactions between homokaryotic mycelia, a somatic incompatibility reaction can often be seen prior to the recognition of the mating types. When mating has occurred, the somatic incompatibility is suppressed. This indicates that somatic incompatibility is a constitutively expressed state in *H. annosum s.l.*, which can be turned off by mating type controlled signalling and possibly also by a somatic *compatibility*

recognition. Our data does not give any proof towards the nature of this recognition, but it is tempting to speculate that if both nuclei affect the reaction, a formation of dimers is involved, and the recognition could be due to a tetramer formation with corresponding dimers from the encountering mycelia.

In the sib-unrelated dataset, 1273 out of 1979 pairings (64%) received general incompatibility scores of 3 or 4, while in the sib-parental dataset, 293 out of 676 (43%) received those scores. This observation together with the higher frequency of fully compatible reactions in the sib-parental set indicates that relatedness decreases the total somatic incompatibility.

We discovered several QTLs for a number of different intraspecific interactions between the 63 dikaryotic isolates in the sib-unrelated pairing matrix (Fig 2a-f), where all isolates shared one unrelated nucleus (from TC-39-7) and were full siblings for the other nucleus. All the discovered QTLs have peaks above the level of significance but some of them were remarkably strong. The QTL for exudation of dark metabolites when encountering any mycelia peaks at LOD 6.33. This trait seems as good as completely linked to the ability to form aerial hyphae upon encountering any growing mycelia (Fig 2a-b). Apparently, these two traits are regulated by the same, or tightly linked, genetic factors. Interestingly, the QTL for increased exudation of such metabolites when encountering non-self mycelia is equally strong (LOD 6.68) but located on a different LG (Fig 2c). This QTL was also very thin which should make subsequent cloning and characterization less difficult. However, no QTL could be found for the ability to increase formation of aerial hyphae when encountering non-self mycelia. Thus, these traits do not seem linked when reacting to the presence of exclusively non-self mycelia, but are completely linked when reacting to any mycelia. The physiological background of the darkening of interaction zones associated with SI is poorly understood. In *Phellinus weirii*, interaction zone hyphae become melanized and the polyphenol oxidase activity is stimulated (Li, 1981) In *H. annosum s.l.*, the laccase and oxidative metabolic activity in the interaction is increased (Hansen, Stenlid & Johansson, 1993a).

Slightly weaker, but still ten times above the level of significance, is the stronger of the QTLs for induction of wall-like structures in encountering mycelia (LOD 4.06) (Fig 2e-f, Fig 3). These structures can easily be taken as somatic incompatibility reactions and to the eye give the impression of interactions between highly incompatible individuals. Apparently from our QTL analysis, however, the formation of these hyphal walls is not so much a consequence of the genetics of the reacting mycelia as it is a response reaction to something in the encountered mycelia. The induced hyphal walls and the increased pigmentations might not be generally considered SI reactions *per se*, but yet they are clearly expressed exclusively in interactions between distinct mycelia of separate individuals. However, their antagonistic appearance aside, we know nothing of their possible role in delimiting mycelia by preventing migration of nuclei or mycoviruses. This remains to be studied.

We tried to map the general somatic incompatibility trait by adding up all incompatibility reactions for each isolate and found a QTL for this (LOD 3.39) (Fig 2d). This is an interesting observation as statistically, all isolates should have approximately the same cumulative incompatibility if the strength of the incompatibility reaction was only due to the distribution of SI alleles. This does not seem to be the case; apparently, some isolates react to somatic incompatibility in a more flamboyant way than others. It seems possible that the QTL found on LG 21 is involved in this reaction enhancement.

In this study, we determined the number of SI loci in *H. annosum s.l.* to four. Furthermore, we investigated the effect of the identical genetic background in pairings between isolates being heterokaryotic for one nucleus and homokaryotic for the other. Our results show that the identical nuclei do affect the reaction. We have further showed that in pairings between related isolates of *H. annosum s.l.*, the reactions are generally more compatible than between more unrelated isolates. Additionally, we managed to locate several QTLs of great interest for their involvement in different intraspecific interactions. Future investigation and cloning of these QTLs will reveal their nature and how they are involved in the SI reactions.

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References

Anwar, M., Croft, J. & Dales, R. 1993. Analysis of heterokaryon incompatibility between heterokaryon-combatibility (h-c) groups R and Gl provides evidence that at least 8 *het* loci control somatic incompatibility in *Aspergillus nidulans*. *Journal of Genetic Microbiology 139*, 1599-1603.

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.

Barrett, D. & Uscuplic, M. 1971. 1971. *New Phytologist 70*, 581-598.

Béguret, J., Turcq, B. & Clavé, C. 1994. Vegetative incompatibility in filamentous fungi - *het* genes begin to talk. *Trends in Genetics 10*, 441-446.

Caten, C. 1972. Vegetative incompatibility and cytoplasmic infection in fungi. *Journal of General Microbiology 72*, 221-229.

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

Cortesi, P. & Milgroom, M. 1998. Genetics of vegetative incompatibility in *Cryphonectria parasitica*. *Applied and Environmental Microbiology 64*, 2988-2994.

Dales, R. & Croft, J. 1983. A chromosome assay method for the detection of heterokaryon incompatibility (*het*) genes operating between members of different heterokaryon compatibility (h-c) groups in *Aspergillus nidulans*. *Journal of Genetic Microbiology 129*, 3643-3649.

Garbelotto, M., Ratcliff, A., Bruns, T.D., Cobb, F.W. & Otrosina, W.J. 1996. Use of Taxon-Specific Competetive-Priming PCR to Study Host Specificity, Hybridization, and Intergroup Gene Flow in Intersterility Groups of Heterobasidion annosum. *Phytopathology 86*, 543-551.

Garnjobst, L. & Wilson, J. 1956. Heterocaryosis and protoplasmic incompatibility in *Neurospora crassa. Proceedings of National Academy of Science USA 42*, 613-618.

Glass, N. & Kuldau, G. 1992. Mating type and vegetative incompatibility in filamentous ascomycetes. *Annual Review of Phytopathology 30*, 201-224.

Hansen, E.M., Stenlid, J. & Johansson, M. 1993a. Genetic control of somatic incompatibility in the root-rotting basidiomycete *Heterobasidion annosum*. *Mycological Research 97*, 1229-1233.

Hansen, E.M., Stenlid, J. & Johansson, M. 1993b. Somatic incompatibility and nuclear reassortment in *Heterobasidion annosum*. *Mycological Research 97*, 1223-1228.

Hartl, D., Dempster, E. & Brown, S. 1975. Adaptive significance of vegetative incompatibility in *Neurospora crassa. Genetics 81*, 553-569.

Holt, C. 1983. The mating system of *Fomes annosus* (*Heterobasidion annosum*). *European Journal of Forest Pathology 13*, 174-181.

Huber, D. 1996. *Genetic analysis of vegetative incompatibility polymorphisms and horizontal transmission in the chestnut blight fungus, Cryphonectria parasitica,* Michigan State University.

Ihrmark, K., Johannesson, H., Stenström, E. & Stenlid, J. 2002. Transmission of double-stranded RNA in *Heterobasidion annosum*. *Fungal Genetics and Biology 36*, 147-154.

Jacobson, D., Beurkens, K. & Klomparens, K. 1998. Microscopic and ultrastructural examination of vegetative incompatibility in partial diploids heteroxygous at *het* loci in *Neurospora crassa. Fungal Genetics and Biology 23*, 45-56.

Johannesson, H. & Stenlid, J. 2004. Nuclear reassortment between vegetative mycelia in natural populations of the basidiomycete *Heterobasidion annosum*. *Fungal Genetics and Biology 41*, 563-570.

Kauserud, H., Saetre, G.-P., Schmidt, O., Decock, C. & Schumacher, T. 2006. Genetics of self/nonself recognition in *Serpula lacrymans*. *Fungal Genetics and Biology 43*, 503-510.

Kay, E. & Vilgalys, R. 1992. Spatial distribution and genetic relationships among individuals in a natural population of the oyster mushroom *Pleurotus ostreatus*. *Mycologia 84*, 173-182.

Konopleva, M., Zhao, S., Xie, Z., Segall, H. & Younes, A. 1999. Apoptosis. Molecules and mechanisms. *Advanced Experimental Medical Biology 457*, 217-236.

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*. Edited by S. Woodward, J. Stenlid, R. Karjalainen & A. Hüttermann. CAB International. Wallingford, UK. 43-70. pp.

Labarère, J., Béguret, J. & Bernet, J. 1974. Incompatibility in *Podospora anserina*: Comparative properties of the antagonistic cytoplamsic factors of a nonallelic system. *Journal of Bacteriology 120*, 854-860.

Lander, E.S. & Botstein, D. 1988. Mapping Mendelian Factors Underlying Quantitative Traits Using RFLP Linkage Maps. *Genetics 121*, 185-199.

Leslie, J. 1993. Fungal vegetative compatibility. *Annual Review of Phytopathology 31*, 127-50.

Li, C.Y. 1981. Phenoloxidase and peroxidase activities in zone lines of *Phellinus weirii. Mycologia 73*, 811-821.

Lind, M., Olson, Å. & Stenlid, J. 2005. An AFLP-marker Based Genetic Linkage Map of *Heterobasidion annosum* Locating Intersterility Genes. *Fungal Genetics and Biology 42*, 519-527.

Malik, M. & Vilgalys, R. 1999. Somatic Incompatibility in Fungi. In *Structure and Dynamics of Fungal Populations*. Edited by J.J. Worrall. Kluwer Academic Publishers. Dordrecht. 123-138. pp.

Marcais, B., Caël, O. & Delatour, C. 2000. Genetics of somatic incompatibility in *Collybia fusipes*. *Mycological Research 104*, 304-310.

Marek, S., Wu, J., Glass, N., Gilchrist, D. & Bostock, R. 1998. Programmed cell death in fungi: Heterokaryon incompatibility involves nuclear DNA degradation. *Phytopathology 88*, S58.

May, G. 1988. Somatic incompatibility and individualism in the coprophilous basidiomycete, *Coprinus cinereus. Transactions of the British Mycological Society 91*, 443-451.

Milgroom, M. 1999. Viruses in fungal populations. In *Structure and Dynamics of Fungal Populations*. Edited by J.J. Worrall. Kluwer Academic Publishers. Dordrecht. 283-305. pp.

Mylyk, O. 1976. Heteromorphism for heterokaryon incompatibility genes in natural populations of *Neurospora crassa. Genetics 83*, 275-284.

Newhouse, J. & MacDonald, W. 1991. The ultrastructure of hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. *Canadian Journal of Botany 69*, 602-614.

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.

Perkins, D. & Turner, B. 1988. *Neurospora* from natural populations: Toward the population biology of a haploid eukaryote. *Experimental Mycology 12*, 91-131.

Rayner, A., Coates, D., Ainsworth, A., Adams, T., Williams, E. & Todd, N. 1984. The biological consequences of the individualistic mycelium. In *The ecology and physiology of the fungal mycelium*. Edited by D. Jennings & A. Rayner. Cambridge University Press. Cambridge, United Kingdom. 509-540. pp.

Rayner, A. & Todd, N. 1979. Population and community structure and dynamics of fungi in decaying wood. In *Advances in Botanical Research*. Edited by H. Woolhouse. Academic Press. London. 333-420. pp.

Rizzo, D.M., Rentmeester, R.M. & Burdsall, H.H. 1995. Sexuality and somatic incompatibility in *Phellinus gilvus*. *Mycologia 87*, 805-820.

Saupe, S., Clavé, C. & Béguret, J. 2000. Vegetative incompatibility in filamentous fungi: *Podospora* and *Neurospora* provide some clues. *Current Opinion in Microbiology 3*, 607-612.

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. & Rayner, A. 1991. Patterns of nuclear migration and heterokaryosis in pairings between sibling homokaryons *Heterobasidion annosum*. *Mycological Research 95*, 1275-1283.

Swedjemark, G. & Stenlid, J. 2001. A highly diverse population of *Heterobasidion annosum* in a single stump of *Picea abies*. *Mycolgical Research 105*, 183-189.

Van Ooijen, J., Boer, M.P., Jansen, R.C. & Maliepaard, C. 2002. *MapQTL 4.0, Software for the calculation of QTL positions on genetic maps.* Plant Research International, Wageningen, the Neterlands.

Worrall, J.J. 1997. Somatic incompatibility in basidiomycetes. *Mycologia 89*, 24-36.