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Abstract

Heterobasidion annosum s.l. (*sensu lato*, in the broad sense) is a fungal complex consisting of five partly intersterile species and is regarded as the most devastating forestry pest in the northern hemisphere. The fungus is specialized on coniferous trees and utilizes lignin, cellulose and other structural compounds of the wood as its nutrient source. Infection by the fungus results in rotted wood, stunted growth and in death of the host. The fungus wood-decaying properties also results in timber unsuitable as construction material. *H. annosum s.l.* has a large economic impact on the forestry industry, causing annual losses estimated to exceed €1 billion in the EU alone. Scots pine, an economical important tree species, is infected by *H. annosum s.s.* (*sensu stricto*, in the narrow sense). There have been some studies of breeding Scots pine for enhanced resistance to *H. annosum s.s.*, but surprisingly little is known about this topic. Several quantative trait loci (QTLs) associated with resistance to *H. parviporum* – one of the species within the complex – in Norway spruce were recently identified. This thesis investigated if those loci also existed in the Scots pine genome. Five orthologues to the QTLs were successfully identified in genotypes present in the Swedish forest tree breeding programme. Four out of the five genes possessed SNPs, suggesting that there might be genetic potential for breeding future Scots pine trees with enhanced resistance to the pathogen. Field inoculation of *H. annosum s.s.* failed to detect any significant variation in resistance between the different Scots pine genets although there was a clear trend (p-value = 0.054). Future studies could potentially identify variation in resistance of the Scots pine genotypes and associate the phenotypic variation with variants of the QTL orthologues identified in this thesis.

Keywords: Heterobasidion annosum, H. annosum s.s., plant breeding, forest pathology, Scots pine, SNPs, Pinus sylvestris, QTL resistance.

Populärvetenskaplig sammanfattning

Svampar orsakar många allvarliga sjukdomar hos växter som varje år leder till stora förluster inom jord- och skogsbruket. En av de allvarligaste skadegörarna för skogsindustrin är rottickan, en svamp som orsakar sjukdomen rotröta. Svampen angriper främst barrträd där den bryter ner de ämnen som finns i veden (t.ex. cellulosa) för att sedan kunna utnyttja de frigjorda ämnena som sin näringskälla. Träd som drabbats av rotröta växer sämre, riskerar falla omkull vid blåst och kan även dö som följd av infektionen. Det rötskadade virket blir dessutom otjänligt som byggnadsmaterial. De skador som orsakas av rottickan ger stora ekonomiska konsekvenser för skogsbruket och beräknas endast inom EU uppgå till mer än en miljard euro årligen.

I Sverige är tall ett av det ekonomiskt mest betydelsefulla trädslaget och utgör nästan 40% av landets virkesförråd. För de industriellt sett viktigaste trädslagen i Sverige (tall samt gran) bedrivs det ett aktiv förädlingsarbete för att få fram plantor med så bra egenskaper som möjligt till nästa generations skog. Träd med högre tillväxt, bättre virkeskvalité och större motståndskraft mot sjukdomar är några av målen med förädlingsarbetet. Det har forskats en del om möjligheterna att korsa fram tallplantor som är mindre känsliga mot rotröta, men det är fortfarande många frågetecken som kvarstår inom detta ämne. I gran identifierades dock nyligen ett antal s.k. kvantitativa resistensgener som alla gav ett litet men betydelsefullt skydd mot rottickan. Trots att gran och tall skiljdes från varandra för 140 miljoner år sedan så har de fortfarande mycket gemensamt på genetisk nivå. Med detta i åtanke är det möjligt att tänka sig att de gener som gav skydd mot rottickan i gran möjligen kunde finnas även i tallens DNA. Dessa gener skulle då potentiellt kunna användas inom växtförädlingen av tall för att ta fram friskare plantor. Syftet med denna uppsats var både att undersöka om dessa gener överhuvudtaget fanns i tall och om så var fallet även se om det kunde finnas olika varianter av dem. Det analyserades även om det gick att se något samband mellan eventuell förekomst av dessa gener och skydd mot rottickan genom att ympa in svampen på tallar som ingår i det svenska förädlingsprogrammet.

Genom att använda molekylärbiologiska metoder lyckades fem gener som liknade de i gran identifieras även i tallens DNA, varav fyra av dessa förekom i olika varianter. Mer studier av dessa gener behövs men kanske kan de en dag vara en integrerad del i kampen mot rottickan. I försöket där svampen ympades in på tallplantorna gick det inte att se någon skillnad i motståndskraft hos träden, även om det fanns vissa trender. Detta kan dock bero på brister i försöksupplägget, t.ex. det kalla vårvädret som hindrat rottickan från att få sprida sig i sådan grad att någon märkbar skillnad gick att mäta. Förhoppningsvis kan framtida studier påvisa en skillnad i motståndskraft mot rottickan och eventuellt korrelera dessa skillnader med de gener och genvarianter som identifierats i denna uppsats!

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I would like to thank all the people (they are simply too numerous to mention) in the Department of Forest Mycology and Plant Pathology for the help I received during my laboratory work there. My friends and family who have supported me during the writing of this thesis should be credited as well. I also wish to show my appreciation to Curt Almqvist, senior researcher at Skogforsk, for his assistance with the field work during the beginning of this project. Finally, I want to thank my main supervisor Malin Elfstrand and my assistant supervisor Jan Stenlid for introducing me to this interesting topic and all the support they have given me during this project. Perhaps one day I will become as good researcher as they are!

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Abbreviations

ABI	Application binary interface (file format)
bHLH	Basic helix-loop-helix
BLAST	Basic local alignment search tool
BLASTX	BLAST, nucleotide 6-frame translation-protein
CH	Calponin homology
F-group	Fir group
FGS	Fungal growth in sapwood
IST1	Increased sodium tolerance 1 homolog
LAC	Laccase
LAR	Leucoanthocyanidin reductase
LL	Lesion length
MYB39	Myeloblastosis39 transcription factor
NAC	No apical meristem, *Arabidopsis* transcription activation factor, cup-shaped cotyledon
P-group	Pine group
P-loop	Phosphate-binding loop (Walker A motif)
PCR	Polymerase chain reaction
PLAC8	Placenta associated 8
QTL	Quantative trait loci
S-group	Spruce group
s.l.	*Sensu lato* (in the broad sense)
s.s.	*Sensu stricto* (in the narrow sense)
SB	Sodium borate
SNP	Single-nucleotide polymorphism
TAE	Tris base, acetic acid, EDTA
TF	Transcription factor

1 Introduction

1.1 Background

Heterobasidion annosum s.l. (*sensu lato*, in the broad sense) is a fungal complex consisting of five parasitic wood-decaying species and is regarded as the most devastating forest pest in the northern hemisphere (Asiegbu et al., 2005). Three of the species are found on the Eurasian continent, *H. annosum s.s.* (*sensu stricto,* in the narrow sense)*, H. parviporum* and *H. abietinum* (Gontier & Thor, 2013). The two other species, *H. irregulare* and *H. occidentale*, are both found on the North American continent (ibid), although *H. irregulare* was introduced to Italy during the second world war (Gonthier et al., 2004). More than 200 species of forest tree species are susceptible to infection by the fungus, most of them coniferous plants (Korhonen & Stenlid, 1998). The species within *H. annosum s.l.* are specialized on different hosts although there is some overlap within the host range (Mitchelson & Korhonen, 1998). Pines are mostly infected by *H. annosum s.s.* whereas spruces are targeted by *H. parviporum* (Korhonen et al., 1998[a]). *H. abietinum* are mostly observed on firs (Dalman et al., 2010). By utilizing cellulose, lignin and other structural compound of wood as its nutrient source, infection by the pathogen results in death and decay of the tree (Woodward et al., 1998). Because of this, the fungus has large economic impact on the forest industry. As of 1998, it was estimated that *H. annosum s.l.* caused annual losses of at least €790 million in the EU alone (equivalent to €1.14 billion today) (ibid). The severity of the disease is expected to rise in the future as long-term studies have found an increase in the frequency of the pathogen (Thor et al., 2005).

H. annosum s.l. infects its host either by air-borne basidiospores landing on freshly exposed wood such as a wound or a newly cut stump (primary infection) and by root-to-root transmission from already infected trees or stumps (secondary infection) (Korhonen & Stenlid, 1998). There exist efficient control methods to prevent the primary infection to occur, usually by applying chemical or biological agents to

the stump surface during the felling of the stand (Pratt et al., 1998). Although these measures are often effective, they also come with increased costs mostly due to prolonged maintenance work and costs for the stump treatment agent. Also, there is no widely adapted method for managing the secondary (root-to-root) infection pathway. As the fungus can survive on wood debris for many decades (Greig & Pratt, 1976) or perhaps even centuries (Korhonen et al., 1998[b]), alternative methods for managing this forest pest is still a topic of ongoing research.

Most countries that practice artificial forest regeneration in large scale have established ambitious breeding programmes for forest trees with increased field performance and enhanced wood quality (Ruotsalainen, 2014). There has also been some fruitful work of forestry breeding for enhanced plant health such as resistance to certain rust diseases of North American pines (Sniezko et al., 2014). Studies have found no negative impact on either plant growth or wood quality in Norway spruce associated with resistance to *H. annosum s.l.*, suggesting there is great potential for breeding conifers resistant to this devastating pathogen (Chen et al., 2018). Several quantative trait loci (QTLs) associated with resistance in the *H. parviporum*-Norway spruce pathosystem have already been identified thanks to research conducted in the last decade (e.g. Elfstrand et al., 2020). Because of the strong synteny between coniferous species (Pavy et al., 2012) it is reasonable to assume that some of the QTLs also exist as orthologues in other coniferous plants. Since phylogenetic data of *H. annosum s.l.* suggests that the divergence of species within the complex occurred by plate tectonics and not host specialization (Dalman et al., 2010), it is also possible that potential QTLs present in other conifers would function in a similar manner.

Scots pine (*Pinus sylvestris*) is an important species globally, both with respect to ecology and economy. In Sweden, its standing volume is just slightly less than that of Norway spruce, the country's most common tree (40.4% versus 39.3%; Nilsson et al., 2019). Many pine species, including Scots pine, are susceptible to infection by *H. annosum s.s.* (*sensu stricto,* in the narrow sense) that ultimately results in death of the tree (Bendz-Hellgren et al., 1998). The problem with *H. annosum s.s.* in pine plantations is of great magnitude but is by some reason often overlooked by the Swedish forest industry. Fortunately, there has been some studies identifying potential QTLs for *H. annosum s.s.* resistance in Scots pine (e.g. Mukrimin et al., 2019). Incorporating QTLs associated with resistance into breeding programmes for Scots pine could potentially be the future solution to this devastating pathogen. This thesis project aims to increase the knowledge of potential resistance QTLs against *H. annosum s.s.* in Scots pine by conducting a first pilot study within the Swedish forestry breeding programme.

1.2 Objectives

The intention of this study is to provide insight into the *H. annosum s.s.* resistance levels in the Scots pine as well as to investigate the presence of potential QTLs associated with resistance. By doing the first pilot study on these topics, there will be two main objectives within this research project:

I. Execute a first screening of variation in resistance for *H. annosum s.s.* in the ongoing Swedish breeding programme for Scots pine where superior genets are cultivated for seed production.

II. Analyse the genome of Scots pine for presence and variation of known resistance QTLs found in Norway spruce and if possible, relate them to phenotypic variation in Scots pine.

Hopefully the outcome of these objectives could provide some valuable information for forest tree breeders when doing selection for the next generation of Scots pine.

2 Theory

2.1 An Introduction to the Species Complex *H. annosum s.l.*

2.1.1 Species Composition and Distribution

The taxonomy of the *Heterobasidion* genus has historically been confusing and undergone large reconstructions as mycological studies progressed. The species today known as *H. annosum* was originally described as *Polyporus annosus* in 1821 and since then more than 20 different synonyms have been used (Niemelä & Korhonen, 1998). As fungal taxonomy developed, the current name was established in 1888 (ibid). Traditionally, the fungal agent of root rot of conifers was long regarded as the result of one single species, *H. annosum s.l.* (*sensu lato*, in the broad sense) (Gontier & Thor, 2013). However, observed differences in both morphology and host preferences led to the categorisation of *H. annosum s.l.* into three sub-groups based on the main tree species being infected: P-group (pines), S-group (spruces) and F-group (firs) (Niemelä & Korhonen, 1998).

During the last decades of the 20th century, mating experiment of the fungus revealed that the *H. annosum s.l.* was not a single species but rather a complex consisting of five distinct species (Gontier & Thor, 2013). The reason why the recognition of the different species within the complex was not possible before mating tests was due to the close similarities between the groups (Mitchelson & Korhonen, 1998). The species of *H. annosum s.l.* share morphological resemblances, have an overlapping geographic distribution, their host specialization is not absolute and occasionally they form hybrid offspring (ibid). The five species of *H. annosum s.l.* now recognized are distributed throughout the coniferous forests of the northern hemisphere, three of which are found in Eurasia: *H. annosum s.s.* (*sensu stricto,* in the narrow sense)*, H. parviporum* and *H. abietinum* (Gontier & Thor,

2013). The remaining two species, *H. irregulare* and *H. occidentale*, are both found on the North American continent (ibid). One interesting exception from this distribution is a population of *H. irregulare* found in Italy, thought to be introduced via military equipment made of infected wood during the second world war (Gonthier et al., 2004). See figure 1 for the geographical distribution of the species in the *H. annosum s.l.* complex.

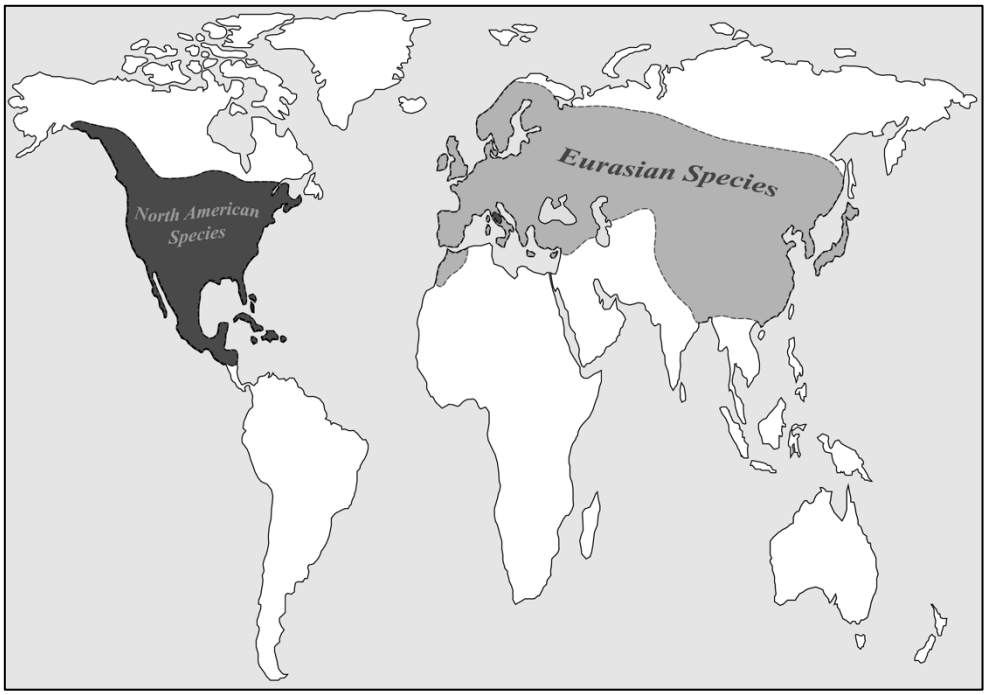

Figure 1. Global distribution of the *H. annosum s.l.* species. The three species found in Eurasian are *H. annosum s.s.*, *H. parviporum* and *H. abietinum* (geographic range in light grey). The two North American species are *H. irregulare* and *H. occidentale* (distribution shown in dark grey). Note the introduced population of the North American species *H. irregulare* established in Italy. Map drawn after Garbelotto & Gonthier (2013) with permission by authors.

Studies by Dalman et al. (2010) have revealed that the geographic distribution of the *H. annosum s.l.* species is not coherent with phylogenetic data which cluster *H. annosum s.s.* with *H. irregulare* in one clade and *H. abietinum*, *H. occidentale* and *H. parviporum* in another one. It appears that the two North American species originate from different ancestral species colonizing the continent independent of each other (ibid). The phylogenetic data also suggest that *H. parviporum* and *H. abietinum* diverged into different species recently and/or that gene flow is still occurring (ibid). From a taxonomic point of view, *H. annosum s.l.* is somewhat complicated, especially in older literature before mating experiments made differentiation of the species within the complex possible. The different groups' abilities to hybridize with each other further blurs the traditional concepts of species. It is

sometimes important to distinguish the different species of *H. annosum s.l.* (in particular when discussing forest tree breeding against these pathogens), but because of the historical confusion the solitary name *H. annosum* will be used in the following parts of the theory chapter unless otherwise specified. It should also be noticed that in addition to the five species of *H. annosum s.l.*, there exist additionally *Heterobasidion* species in Asia and Australasia (i.e. Dai et al., 2007; Tokuda et al., 2009). Those species are however rarely seen on living trees (i.e. Chen et al., 2014; Dai & Korhonen, 2009; Ota et al., 2006) and hence are of minor importance to the forest industry.

2.1.2 Epidemiology and Life Cycle

Due to its aggressive wood-decaying profile, *H. annosum* represents one of the most severe forest pathogens in the northern hemisphere (Asiegbu et al., 2005). The fungus can infect more than 200 species of woody plants with conifers being its main hosts (Korhonen & Stenlid, 1998). *H. annosum* has two routes to invades its host, either through spore infection (primary infection) or via root-to-root transmission (secondary infection) (ibid). The primary infection route begins with the release of basidiospores from the fungus fruiting bodies (Redfern & Stenlid, 1998). The basidiocarps can form on both dead trees and trees that are infected but still alive and they appear on stumps, logs and roots growing just below the surface level rich in litter (ibid). The basidiocarp (figure 2) is of polypore-type, mostly perennial and can reach sizes up to 40 cm when measured across and up to 3.5 cm in thickness (Korhonen & Stenlid, 1998). Production of basidiospores are primarily governed by temperature and humidity and are favoured by mild, stable weather conditions (Redfern & Stenlid, 1998). *H. annosum* is capable of large-scale production of basidiospores, it has been found that a large fruiting body of *H. annosum* can dispose up to 30 000 spores dm^{-2} h^{-1} when measured at 1 m distance (Kallio, 1970). Other studies on the spore production of the basidiocarps of *H. annosum* have revealed that the surface of the polypore can produce over 400 000 spores cm^{-2} hr^{-1} (Kallio, 1973). The basidiospores can travel great distance via air currents, dispersal ranges of 500 km have been recorded (Kallio, 1970).

Figure 2. Basidiocarp of a *H. annosum* species. (Photo: Johanna Boberg, SLU)

Infection by the released basidiospores occurs when the air-borne propagules lands on freshly exposed wood. This could be either an open wound of a living tree, or as in silvicultural circumstances, on a freshly cut stump surface (Korhonen & Stenlid, 1998). Once the spore has germinated and the mycelium has been established the fungal infection proceeds downwards to the lateral roots of the tree (Stenlid & Redfern, 1998). The fungus exudes a variety of extracellular enzymes for degradation of the complex compounds in wood in order to obtain the released nutrients (Asiegbu et al., 1998). *H. annosum* have an aggressive wood-decaying profile and can utilize both lignin, cellulose and various other structural compounds as its carbon source (ibid). When *H. annosum* spreads its mycelium downwards to the root system of the tree or stump, the secondary infection route by mycelium transmission at root contacts can occur (Stenlid & Redfern, 1998). As the fungus in practice is unable to grow through unsterile soil, root contacts are essential for spreading the disease from colonized stumps to nearby healthy trees within the stand (ibid). The fungal growth rate inside the host depends on both the vitality of the tree and the moisture content in the wood, normal spread rates in boreal forests are 10 – 30 cm yr^{-1} but mycelium advances of 2 m yr^{-1} in roots and 1 m yr^{-1} in stems have been recorded (Asiegbu et al., 2005). If compatible mating types were present mating occurs and new basidiocarps emerges from the infect stump, tree or debris and the next generation of basidiospores are released, thus completing the life cycle of *H. annosum* (Korhonen & Stenlid, 1998). An attempt to visualize the life cycle and the infection routes of the fungus are shown in figure 3 on next page.

Except basidiospores, *H. annosum* can also produce both conidiospores and chlamydospores (Redfern & Stenlid, 1998). Conidiospores are asexual produced spores generated from budding of the mycelium (ibid) and chlamydospores are thick-walled cells that occasionally form during senescence of the fungus or when it encounters microbial antagonists (Stenlid & Rayner, 1989). The role of these spore-types is not completely understood but given the large-scale production of basidiospores they are likely of minor importance for the epidemiology and life cycle of *H. annosum* (Redfern & Stenlid, 1998). Chlamydospores do have an important role as long-term survival structures in other fungal species (e.g. Sitton & Cook, 1981) but *H. annosum* have never been observed to produce them in nature (Redfern & Stenlid, 1998). There are also some questions regarding alternative spore-dispersal mechanisms other than wind. It has been suggested that insects and other arthropods might act as vectors for the fungus (e.g. Kadlec et al., 1992) but studies within this topic is difficult since all types of propagules could be carried by the animal, including living mycelium (Redfern & Stenlid, 1998). Since the knowledge is very limited both on the potential vectors for the fungus as well as the role of conidiospores and chlamydospores, they are not included in the illustrated life cycle.

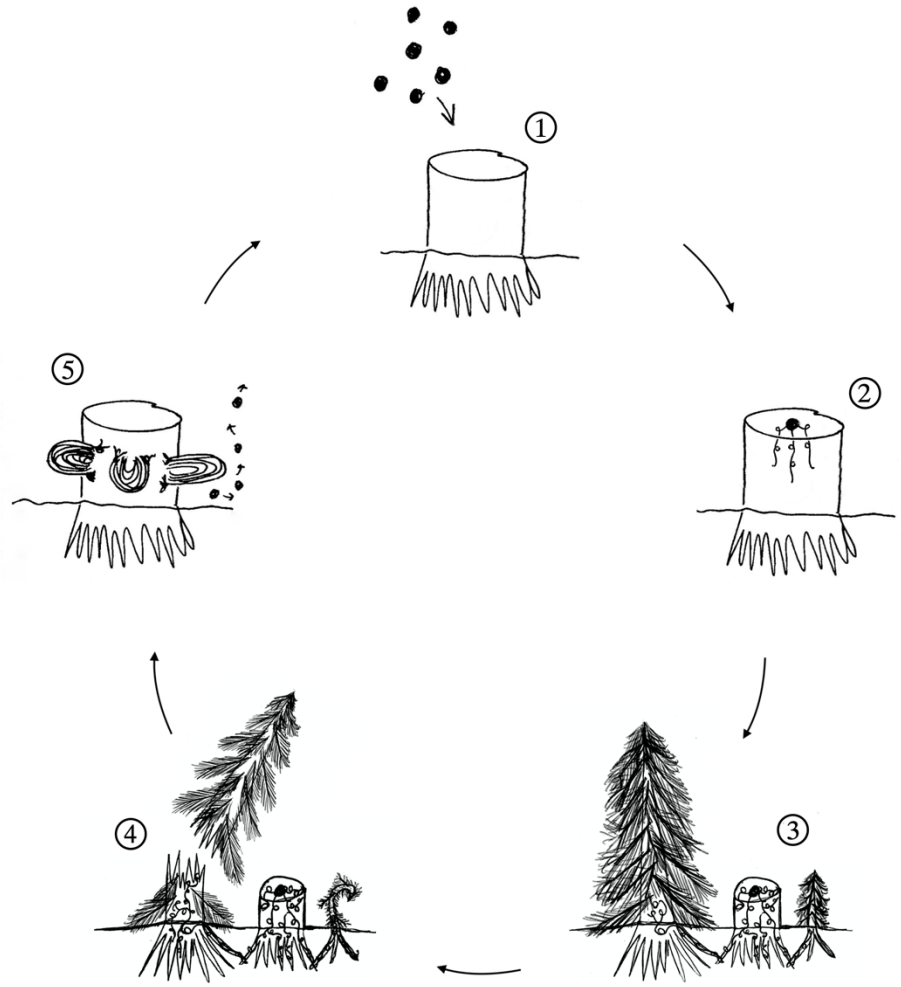

Figure 3. The life cycle of *H. annosum*: (1) Air-borne basidiospores released from the fruiting bodies lands on a freshly cut stump or wound of a living tree. (2) Basidiospores germinates and mycelium start to grow within the stump. Structural compounds of the wood are degraded as the fungus spreads. (3) Secondary infection by root-to-root transmission to nearby trees, hence enabling spread within the stand. (4) Death and decay of adjacent trees as the disease proceeds. (5) New basidiocarps emerges on stumps, roots or stems and releases the next generation basidiospores.

What is well-known about the fungus life cycle is the importance of freshly exposed wood for the primary (basidiospore mediated) infection to occur. *H. annosum* is only able to infect a stump surface within one or two weeks after the tree have been cut down (Korhonen et al., 1998[b]). Due to the strict requirements for fresh wood, *H. annosum* had very limited impact before intensive forest management began with its large-scale felling of stands which created vast numbers of stumps to be infested by the fungus (ibid). The fungus can be very long-lived once the colonization of trees or stumps has been successful. Field studies have revealed that *H. annosum* remained active on stumps from conifers felled more than 60 years earlier (Greig & Pratt, 1976). Similar studies found active fruiting bodies on spruce stumps 46 years after the stand had been cut down by (Piri, 1996). Perhaps *H. annosum* can survive on wooden debris for centuries when established (Korhonen et al., 1998[b]). This implies that not only does stumps serve an infection pathway into the plantation system, but also as a long-term reservoir for inoculum to the next generation forest, hence enabling a spread in both space and time. The fungus persistency to remain vital in stumps and wood debris for decades possess the forestry industries with unique challenges and are discussed in the following chapter.

2.1.3 Silvicultural Aspects and Economical Impact

By breaking down the structural components in wood, the fungus causes large economic impact to the forest industries every year. Not only is the rotten timber useless as construction material, but the growth of the tree is also greatly decreased (Bendz-Hellgren et al., 1998). The infection can also result in death of the tree (ibid). Further economic losses are also caused by increased risk of windthrow due to root decay and costs for control measures (Woodward et al., 1998). Although being specialized on coniferous trees, especially on spruces, pines and firs (Korhonen & Stenlid, 1998), the outcome of the infection by *H. annosum* varies depending both on the fungal species/strain as well as the species of tree being attacked (Bendz-Hellgren et al., 1998). In Norway spruce for instance, stem rot up to 10 m height can occur as a results of *H. annosum* infestation, but death of the host is relatively rare (ibid). The pathogenic profile of *H. annosum* in Scots pine is however very different as the fungus mainly target the root system during the infestation, usually resulting in the tree being killed (ibid). Although the death process in Scots pine can be decades long, the post-infection period is characterized by heavily stunted growth (ibid). Estimations of the damages caused by fungus was is 1998 predicted to exceed €790 millions in the European Union alone (Woodward et al., 1998), equivalent to €1.14 billion today when adjusted for inflation. The economic impact is likely even higher by now due to the fungal disease continuous advancement. Studies of *H. annosum* root rot in Sweden between 1983 – 2001 found a frequency increase of 23% in managed forest stands between those two decades (Thor et al., 2005).

There have been various approaches to control this forest pest with some successful results. Silvicultural actions are likely important means to stop the spread of the disease since the change in forest management is what caused the rise of *H. annosum* to begin with (as discussed earlier in chapter 2.1.1). One concrete silvicultural method to minimize the risk of new infection is by thinning the stand as few times and as late as possible, hence creating a minimum amount of new-cut stumps for the primary infection route (Lygis et al., 2004). One step further to this would be to cultivate one rotation of non-susceptible species in the plantation system which potentially could sanitize the site from *H. annosum* inoculum (Korhonen et al., 1998[b]). Unfortunately, it is not always economical or practically possible to grow deciduous species (ibid). Another silvicultural method that have been under investigation is the effects on root rot diseases by stump removal. The outcome for disease control by this method have in general been positive and also have effect on the secondary infection pathway since inoculum are removed from the site (Cleary et al., 2013). Removal of stumps is however an expensive control measure (Korhonen et al., 1998[b]) although the economical calculation may change to a more cost-effective outcome due to the increased demand for forest-based bioenergy which stumps could become a viable source to (Eriksson & Gustavsson, 2008). There are also negative ecological side effects associated with stump removal such as soil compaction caused by the heavy machinery needed (Thies et al., 1994).

In addition to the silvicultural practices, there has been development of both chemical and biological products to prevent new infections by *H. annosum*. The main substances used for chemical stump treatment are borates and urea since they are inexpensive, easy to handle and have low toxicity (Pratt et al., 1998). The efficiency to prevent new infections have been proven in many studies for both urea (e.g. Oliva et al., 2008) and borates (e.g. Keča & Keča, 2012). As biocontrol agents, the fungus *Phlebiopsis gigantea* (another wood-decaying basidiomycete) have been used as an antagonist to *H. annosum* in Europe for over 60 years (Pratt et al., 2000). By applying *P. gigantea* formulations on newly cut stumps during the felling it efficiently outcompetes *H. annosum* (Holdenrieder & Greig, 1998). Occasionally *P. gigantea* too infects wounds of living trees, but this happens too seldom to have a significant impact (ibid). Further studies have found other microbial antagonists to be effective against *H. annosum* as well (Nicolotti et al., 1999).

Although preventive stump treatment by chemical or biological agents in many ways have been fruitful, they do not solve the issue of the continuous infection in already colonized stands (Malin Elfstrand, personal communication). Furthermore, both stump treatment methods have their own costs and side effects. Equipping a modern harvester needed for large scale felling with system for chemical application can be both complicated and expensive (Pratt et al, 1998). Also, urea is a corrosive

substance which can add significant cost in the maintenance of the machinery and the preparation of biocontrol agents slows down felling time (ibid). It is also the case that *P. gigantea* need to have complete coverage of the stump to prevent infection (Rönnberg et al., 2006). The biocontrol agent can also contaminate timber and cause red streaks on the wood (Holdenrieder & Greig, 1998).

With both the ecological and economical concerns for the control measures described above, alternative means to reduce future damages by *H. annosum* should be developed. Advances in molecular biology in general and plant breeding in particular could entail unique opportunities to achieve this objective. The following chapter highlights the possibilities and challenges for forest tree breeding.

2.2 Host Resistance Against *H. annosum* Infection

2.2.1 A Brief Overview of Forest Tree Breeding

The process of breeding forest trees is quite different from the breeding of agricultural crops. One key difference is the fact that crop plants have been under domestication for much longer time than forest tree species. For comparison, crops have been under man's selection since dawn of agriculture more than 10 000 years ago (Ross-Ibarra et al., 2007). Breeding programme for forest trees on the other hand is a relatively new development. As an example, the official forest breeding programme in Sweden was established in the mid 1930s (Eriksson et al., 2006). Another important difference in the breeding process of crop plant versus forest trees is the time differences between generations. While most of the crop plants are annuals (i.e. offspring are produced every year), forest tree species such as pines requires ≥10 years before starting to reproduce (Owens et al., 2005). These time scales make techniques used for breeding of agricultural crops such as allele transfer by back crossing practically impossible for forest tree species since 7 – 8 generations are required to move the allele of interest into the desired genet (Eriksson et al., 2006). Mutational breeding by chemical means or gene editing techniques could theoretically possess the solution to alter a specific allele without the time and labour demanding process of back crossing, but this method is most suitable for highly bred crops where only a single trait is desired to be altered (ibid). Due to this, mutational breeding is of minor to no importance for forestry breeding today (ibid). However, modern molecular methods developed in recent decades possess great opportunities to the forest breeding programme and are further discussed in the following chapter.

Even though there are some significant differences between forestry and agricultural breeding, there are also similarities. The underlying plant biology is generally the

same, and quite a few important crop plants are also trees or shrubs, especially in the horticultural area. There are also some species grown for biofuel such as *Salix* which are essentially forest trees grown as an agricultural crop, blurring the borders between agriculture and forestry even more. Similarities also exist between agriculture and the cultivation of seed orchards for forestry species. The management of these resembles the management of an agricultural crop more than that of a forest plantation. Finally, it should also be recognized that plants species often faces similar threats from fungal pathogens regardless of their cultivation end-usage.

Ambitious efforts have been made to establish successful forestry breeding programs in all regions where artificial forest regeneration is practiced at a large scale (Ruotsalainen, 2014). The breeding goals usually strike a balance between enhanced wood production and higher timber quality (ibid). There are examples of forestry breeding programmes focusing on enhanced disease protection. In North America for example, breeding of White pine (*Pinus strobus*) and Loblolly pine (*Pinus taeda*) resistant to blister rust and fusiform rust respectively have yielded great success since the beginning more than 50 years ago (Sniezko et al., 2014). Studies of the interaction between *H. annosum* and conifers have provided hopeful results regarding resistance breeding. Both genetic and biochemical markers for *H. annosum s.s.* resistance in Scots pine have been identified (e.g. Mukrimin et al., 2019). Although some research has been done within this topic, it is surprising how little knowledge exist about the possibility for resistance breeding in Scots pine given the severity of the disease. Hopefully this thesis can provide some new insights into this topic.

2.2.2 Possibilities for *H. annosum s.s.* Resistance in Scots Pine

Coniferous plants and their relatives in the gymnosperm clade have among the largest genomes of all organism, typical in the range of 20 – 30 gigabases (Nystedt et al., 2013). Nearly all conifers are diploid (2n = 24), including genera as *Pinus*, *Picea* and *Larix* (Ahuja & Neale, 2005). Genetic studies on Norway spruce have revealed that the huge genome of conifers does not contain a significant larger number of genes than genomes in angiosperms such *as Arabidopsis*, despite having more than a 100-fold larger genome size (Nystedt et al., 2013). The huge genome of coniferous plants seems to be caused by an insufficient mechanism to eliminate transposable elements, resulting in an accumulation of those over time (ibid). Due to the large and complicated genomes of conifers, genetic studies lag behind the research of angiosperms (Pavy et al., 2012). For instance, Scots pine still have not got its genome sequenced (Pyhäjärvi et al., 2020) despite being the widest distributed pine species in the world (Skilling, 1990). Scots pine is an important species with respect to both ecology and economy, covering an area of >28 million hectares in the EU alone which represent at least 20% of the commercial forest area

in the union (Mason & Alia, 2000). Hopefully, there will be many coniferous genomes sequenced in the near future as sequencing techniques are becoming both cheaper and more readily available.

Despite the challenges involved when working with the complex genomes of conifers and the long time between generations, there has been several studies providing optimistic results for breeding conifers with resistance to *H. annosum*. Chen et al. (2018) showed that traits related to wood quality (e.g. density), fibre properties (e.g. length) and growth performance of Norway spruce had no correlation with resistance to *H. parviporum*. This suggest that selection for resistant phenotypes of conifers could be done without adverse effects on quality and growth respectively (ibid). Since resistance to a pathogen is usually associated with an expense of energy resource and hence a lower growth rate of the plant (e.g. Smedegaard-Petersen & Tolstrup, 1985), the result is both noteworthy and optimistic.

It is well known that homogenous environments, such as an agricultural system or an artificial forest, can promote rapid evolution and dispersal of plant pathogens (Möller & Stukenbrock, 2017). This, in combination with the long generation time of coniferous plants when compared to microbials such as fungi, implies that qualitative resistance (major gene resistance) in which only a single gene is causing the effect is not a sustainable solution to address the *H. annosum* issue. Plants' defense system against fungal pathogens with necrotrophic characteristics, such as *H. annosum*, is not based on gene-to-gene interaction but rather build on an array of genes resulting in systemic resistance (Lind et al., 2014) If a single resistance gene would be implemented on a large-scale as a protection to *H. annosum*, it would likely break early on. Rather, a polygenic approach based on quantative trait loci (QTLs) should be used to obtain a durable resistance.

QTLs are mapped within a population by identifying genomic differences by DNA markers and then correlating the markers with the phenotypic variation for the trait of interest (Klug et al., 2014). Single-nucleotide polymorphism markers (SNPs) have become increasingly popular for plant geneticist since they have a genome-wide occurrence and therefore enables researchers to create high-definition genetic maps (Mammadow et al., 2012). SNPs can affect the phenotypic outcome by multiple mechanisms (Shastry, 2009). A change of a single nucleotide in the genetic code can result in change of amino acid codon, modified promotor activities or altered mRNA stability (ibid). Although QTL-mapping have already been widely adapted in the agricultural sector both for crop and animal breeding (Klug et al., 2014), its implication in forestry development is limited. Nevertheless, recent progress in this field sheds light on the possibility for host resistance against *H. annosum* in coniferous species.

PaLAR3 was recently identified in Norway spruce as QTL-marker for resistance to *H. parviporum* (Nemesio-Gorriz et al., 2016). The *PaLAR3* gene encodes for leucoanthocyanidin reductase (LAR), a key enzyme in the flavonoid-biosynthetic pathway (Hammerbacher et al., 2014). Two known variants of *PaLAR3* exist in Norway spruce: *PaLAR3A* and *PaLAR3B*, both of which are involved in the synthesis of the fungistatic compound (+) catechin found in bark (Nemesio-Gorriz et al., 2016). When measuring fungal growth in sapwood (FGS) of *H. parviporum* in Norway spruce, individuals being either homozygous for the *PaLAR3B* or heterozygous for *PaLAR3A*/*PaLAR3B* showed on average a 27% reduction of fungal growth compared to their half-sibling being homozygous for *PaLAR3A* (ibid). The gene expression of *PaLAR3* are governed by the transcription factors (TFs) NAC (abbreviation for No apical meristem, *Arabidopsis* transcription activation factor, Cup-shaped cotyledon) (Dalman et al., 2017), one of the largest TF families and important regulators of both stress responses and developmental programmes (Jensen et al., 2010). Selection of Norway spruce genets carrying the *PaLAR3B* locus could potentially be an important component towards resistance against *H. parviporum* and further studies are currently being conducted on this (Hernán Capador-Barreto, personal communication).

Since the identification of *PaLAR3* locus, more studies of QTL-resistance have been conducted. Chaudhary et al. (under review) found other NAC-genes, the *PaNAC04* and two of its subgroup paralogs, to be highly induced in response to *H. parviporum* infection in Norway spruce. In total, 10 candidate QTLs for *H. parviporum* was reported in this study. Elfstrand et al. (2020) identified 11 QTLs in Norway spruce that correlated with variation in resistance to *H. parviporum*, each QTL explaining between 2.1 – 5.2 % of the phenotypic variation with respect to resistance degree. One of the more promising QTLs was the identification of a laccase-encoding gene, the *PaLAC05*, which exhibit high expression pattern in spruce cells in close proximity to *H. parviporum* (ibid). It is hypothesized that *PaLAC05* could be associated with the formation of the lignosuberized boundary zone in bark adjacent to the inoculation site (ibid). Understanding the mechanism of lignification and suberization is important since both processes are essential in host defence against infection by pathogenic fungi (Asiegbu et al., 1998).

Even though the divergence of the genus *Pinus* from *Picea* occurred approximately 140 million years ago (Wang et al., 2010), there is a strong synteny and collinearity of coniferous plants (Pavy et al., 2012). The mutual genetics suggest it is possible that QTLs associated with *H. parviporum* resistance in Norway spruce also exist as orthologues in Scots pine. If indeed orthologues of these QTLs exist, they would perhaps exhibit similar resistance properties against other species within the complex of *H. annosum s.l.* Identification of the QTLs and selection for alleles

associated with lower fungal growth is a promising mean to achieve durable host resistance in Scots pine. The following chapters of this thesis will focus on this possibility.

3 Materials and Methods

3.1 Selection of Candidate Resistance QTLs

Data of known QTLs identified in Norway spruce for resistance against *H. parviporum* was used based on research by Chaudhary et al. (under review) and Elfstrand et al. (2020) (see chapter 2.2.2. for more details). In those studies, a total of 21 QTL-holding candidate genes associated with resistance in the *H. parviporum*/Norway spruce-pathosystem was reported. Those 21 gene models were BLAST-searched for best matches in other coniferous species at the Norway spruce genome portal (http://congenie.org/). The genomic sequences found in the matches by the BLASTX search were used as a "proxy template" for genomic work with Scots pine. There was a number of constrains when assessing which of the BLASTX matches would be feasible to work with in the Scots pine system. Sequences was with a high frequency and/or very large introns were rejected. No sequence containing un-assembled elements were included as candidate genes since the true size of those sequences would be unknown. Primers were designed for the remaining candidate genes by using the online software "Primer3" (version 4.1.0, http://primer3.ut.ee) with the set requirements of a primer length of 18 – 22 bp, a GC-content in the interval of 20 – 80% and a melting temperature (T_m) of 57 – 63 °C (Appendix I). Primers were obtained from Eurofins Genomics Denmark A/S.

3.2 Identification of SNPs in Scots Pine

3.2.1 Sampling of Plant Material

Buds of Scots pine were collected in beginning of February at Långtora seed orchard plantation (59°42'46.1"N, 17°07'19.0"E) which is approximately 30 km southwest from Uppsala. Långtora is a third-cycle pine seed plantation and was established

between 2012 – 2019 (Curt Almqvist, personal communication). Of all the 46 different genets present in the seed orchard, 20 commonly occurring clones was included in the sampling (table 1). This represented approximately 71% of all genets present in the seed orchard. The buds were dried at 50°C for 3 days right after collection to prevent microbial growth which could result in the plant material being degraded and/or contamination of the samples with foreign DNA.

Table 1. *List of genets included in this project. Genets with a code starting with S01 are plus-trees that originated from wildtype trees with desired properties regarding straightness, branching angels etc. The letter in the plus-trees names indicates clonal origin based on the counties of Sweden (e.g. "W" for Dalarna county). Genets with code starting with S2 can either be a plus-trees from wild type trees or a progeny from a controlled crossing within the breeding programme. The two digits following K indicate the year of selection. The T18-index is a relative measurement of the breeding value of the genet.*

Genet	Number of ramets	Fraction of population (%)	T18-index
S01W4009	269	6.5	4.52
S22K9711417	258	6.2	5.00
S01S6206	207	5.0	2.82
S01X4005	192	4.6	4.45
S21K8310010	181	4.4	–
S01E3003	165	4.0	3.59
S01W6059	163	3.9	5.81
S01W4606	160	3.9	1.45
S01X2010	160	3.9	2.72
S01S3030	156	3.8	4.75
S01W6015	156	3.8	2.95
S01W3184	155	3.7	4.88
S01X4013	137	3.3	3.91
S01P2016	131	3.2	1.14
S21K8810018	101	2.4	–
S22K9711411	94	2.3	5.19
S01U1003	74	1.8	0.73
S22K9711412	74	1.8	4.73
S21K8310011	57	1.4	–
S21K8810020	57	1.4	–
Total:	**2947**	**71.3**	

3.2.2 DNA Extraction, Amplification and Purification

Extraction of DNA was conducted shortly after the buds had been dried and was based on a protocol by Doyle & Doyle (1990). Between 2 – 4 buds from each genet were used for the extraction procedure depending on the size of the buds. The

concentration of DNA as well as level of purity was determined by spectrophotometric measurement (NanoDrop ND-1000, Thermo Fisher Scientific, U.S.) and then diluted to a concentration of 50 ng μl^{-1}. Genet- and gene-wise PCR was conducted to amplify the candidate genes. The PCR were performed in 50 µl reaction volumes using the DreamTaq DNA polymerase with the 10X DreamTaq Green Buffer (both produced by Thermo Scientific, Lithuania). Strips containing the pre-PCR mixture was put in the thermal cycler and ran in the ITS57-programme (cycle conditions: 95°C 5 min, then 35 cycles of 95°C 30 sec; 57°C 30 sec; 72°C 30 sec finished with an extension of 72°C for 7 min). The quality and size of PCR product was then examined by running 5 µl in a SB-gel electrophoresis (1%$_{m/v}$ agarose with added Nancy-520 DNA Gel Stain; Sigma-Aldrich, U.S.). Product size determination was done by comparing the samples with the GeneRuler DNA Ladder Mix (Thermo Scientific, Lithuania).

The result from the gel electrophoresis was used to decide if any amplicons needed additional optimization of the PCR run. Amplicons that had either continuously unstable products or absence of them in the gel electrophoresis even after modification of the PCR runs were discarded for further studies within this project. The gel electrophoresis result was also used as a mean to decide which purification methods would be adequate to obtain pure enough samples for later sequencing. PCR product with very defined products were purified by NaOAc/EtOH wash and samples with defined but somewhat smeared gel bands were purified by spin column extraction. Gel extraction was conducted if a sample had multiple bands in the gel electrophoresis. All of the remaining volume left from the PCR reaction (45 µl) was used for the purification procedures. For samples purified by spin column the E.Z.N.A. Cycle Pure Kit (Omega Bio-tek, Inc, U.S.) was used. Gel extraction was made possible by running a TAE-gel (1%$_{m/v}$ agarose with added Nancy-520 DNA Gel Stain) and then using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, U.S.). After purification, the samples were dried solid at 37°C before Sanger sequencing (conducted by Macrogen Europe B.V., Netherlands).

3.2.3 Analysis of DNA Sequences

The sequences were analysed by using the SeqMan Pro™ software, version 12.1.0 (DNASTAR, Inc, U.S.). running on Microsoft Windows 10 Education (version 1809, 64-bit system). Retrieved sequences were imported in ABI-format and the sequences ends were trimmed with the 'quality' parameter set to 'high'. A standard threshold of ≥80% match was used during the assembly of the contigs. Based on the electropherograms from the Sangers reactions, fragments of the ends of the sequences were also manually removed when the read quality was deemed too low. In some cases, the whole sequenced product of a genet was removed completely

from the contig when the background noise of the electropherograms became apparent, hence obstructing the correct identification of the base-call. Problem with background noise in the electropherograms could be caused by a number of different factors, including pre-PCR contamination, insufficient aneling of the designed primers, inadequate gel purification or that the PCR output consisted of multiple DNA products with similar sizes.

After processing the sequences, the consensus sequence generated by SeqMan Pro™ was BLASTX-searched at the Norway spruce genome portal against Loblolly pine to confirm the obtained product indeed was the expected one. The same procedure was conducted in the cases when PCR products of unexpected sizes appeared during the gel electrophoresis in order to identify those products. SNPs were identified by using the SNP-finder tool in SeqMan Pro™. Only significant SNPs (i.e. SNPs with at least 25% of the sequences being different from the rest at the particular position) were included. Manual verification of the SNPs identified by the software was conducted. In some rare cases, adjustments were done when SeqMan Pro marked a base-call as homozygous even when a top could be observed for another nucleotide but not strong enough for the software to recognize it as a heterozygous allele. An example of this case is shown in figure 4.

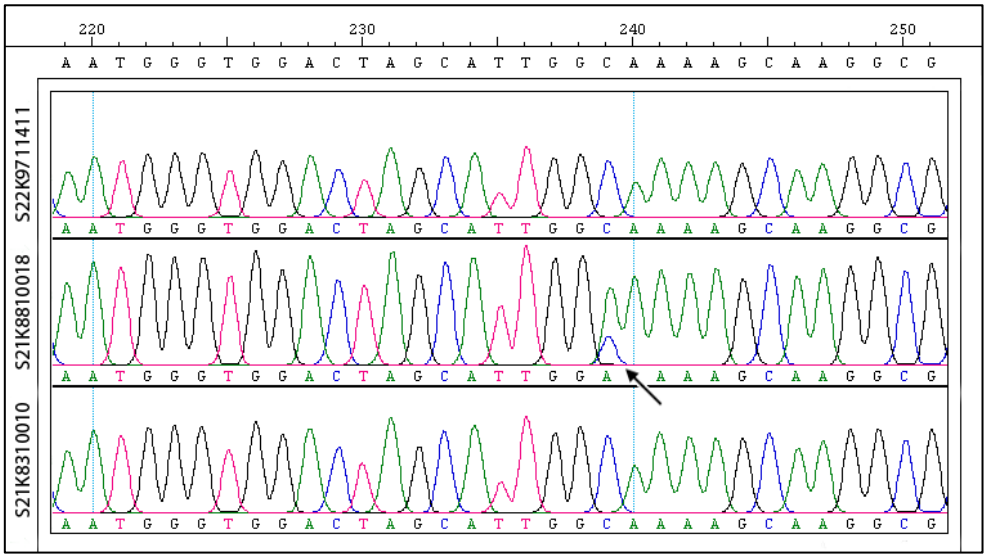

Figure 4. Example of a manual corrected base-call. The software did not recognize a heterozygous genotype for the genet S21K8810018 (middle) at position 239 in the amplicon PITA_000013606 (see pointer). Since other genets showed heterozygosity at this SNP-position, the base-call was manual adjusted to the genotype "AC" rather than "AA" as suggested by SeqMan Pro™.

3.3 Inoculation of *H. annosum s.s.* in Seed Orchard

Four isolates of *H. annosum s.s.* were cultivated on Hagem's agar medium at 25°C to determine which strain would be most suitable for this study. The different strains examined was Mj20, Mj65, Mj87 vx14/p and Sä34 (all collected in 1986; see Stenlid & Karlsson, 1991). It was judged that Sä34 was the best suitable strain since it exhibited aggressive growth and lacked any signs of sectorial behaviour during its cultivation. Infectious dowels were obtained by transplanting wooden fragments from a Scots pine stick (Ø = 5 mm) cut in approximately 5 mm length to the cultivation medium. Before adding the wood fragments to the culture, it was autoclaved twice to ensure a complete sterilisation of the material. Microscopy studies were also conducted in multiple stages during the cultivation of *H. annosum s.s.* Sä34 to confirm that no foreign microbes were present in the Petri dish. Confirmation that it indeed was a heterokaryotic form of the isolate being grown was done by examining the mycelium for presence of clamp connections. The dowels were let to be colonized with the fungus for approximately 3 weeks, after which a thick layer of mycelium and conidiophores could be observed covering the plugs.

Figure 5. The three steps of *H. annosum s.s.* inoculation: wound making, attachment of infectious dowel and finally parafilm wrapping.

Inoculation of Scots pine genets was conducted at end of March at Långtora seed orchard. Infectious dowels were transplanted to the center of the 2018-year growth of the branches by creating a small circular wound (Ø = 5 mm) hence exposing the sapwood to the mycelium of *H. annosum s.s*. A few layers of parafilm was then wrapped around the branch-dowel complex to ensure a fixation of the plug and to reduce the risk of infection from other pathogenic microbes. See figure 5 above for inoculation procedure. The branch internode of 2018-year growth was deemed to be the most suitable site for fungal inoculation considering it had a large enough

diameter for wound making and attachment by the infectious dowels. The 2018-year growth internode was also considered to be of enough length for the *H. annosum s.s*. to grow before being hindered by the branch conjunction of the 2017- or 2019-year growth. Also, the 2018-year internode had a fairly low presence of needles hence facilitating the fixation with the parafilm wrapping. Two infection dowels were transplanted per ramet on different branches and a total of four trees per genet were inoculated, hence 8 inoculations per genet were conducted. The genet S22K97-11412 were not inoculated since no viable plants could be found.

Collection of inoculated branches were done in early May, approximately 7 weeks after the inoculation were conducted. The collected twigs were stored in a freezing container during the field work and afterwards in a cold room storage at 4°C. This was done to slow down any activity of *H. annosum s.s.* present in the twigs until all genets had been measured for fungal growth.

3.4 Estimation of Fungal Spread within the Genets

The spread of *H. annosum s.s.* in the different Scots pine genets were estimated by measuring two variables: lesion length (LL) and fungal growth in sapwood (FGS). LL was determined by cutting the bark of the branch open and then measuring the length of necrotic tissue on the phloem from the infection site. LL was measured for both the upwards and downward direction of the twigs. See figure 6 for appearance of a lesion. FGS was determined by cutting the branches 5 cm upwards and downwards respectively prior to the inoculation site in 5 mm fragments. The pieces were then put on moist filter paper in a Petri dish in a sealed plastic box with elevated air humidity to induce conidiophore formation. The conidiophores of *H. annosum s.s.* could later be detected by using a stereo microscope. Observation of conidiophores (as

Figure 6. Twig with clearly visible lesion (here shown on the sapwood, not on the phloem).

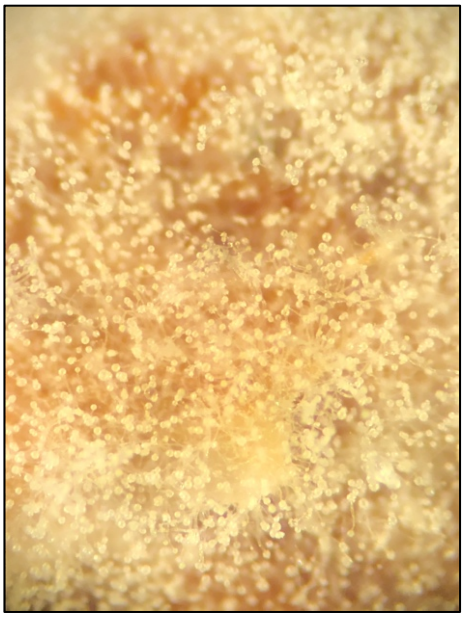

Figure 7. Conidiophores formed at the inoculation site as observed after 11 days incubation in humidity chamber.

seen in figure 7) were done 7- and 11-days post incubation. The reason why FGS measurements were conducted twice was due to the uncertain time required by the fungus to form conidiophores. By observing which fragments had or had not present of conidiophores, the spread of *H. annosum s.s.* within the branch could be concluded. To reduce the risk of propagule contamination during the cutting, the branches were cut in the direction starting from the ends towards the inoculation site. The genets were processed block wise to reduce any biases by potential fungal growth during the processing time. All instruments used during the cutting procedure was also sterilised with EtOH during the change of cutting direction or when assessing the next twig.

4 Results

4.1 BLASTX Matches of Norway Spruce Resistance QTLs

BLASTX searching the previously identified 21 QTLs associated with resistance in the *H. parviporum*/Norway spruce pathosystem returned 19 matched loci found in Loblolly pine (*Pinus taeda*). No description of the matched genes in Loblolly pine were available. With the constrains regarding work feasibility (as discussed in chapter 3.1) there was 13 Loblolly pines sequences remaining as candidate genes for *H. annosum s.s.* resistance in Scots pine (table 1). Two of the Norway spruce QTLs (MA_103386g0010 and MA_86256g0010, both of which being NAC-encoding TFs) got the same match in Loblolly pine (PITA_000042744), suggesting there might be an incomplete structure of the phylogenetic data of Norway spruce. The results of the expected product length derived from using the online software "Primer3" are also shown in the table.

Table 2. *The 13 QTLs associated with resistance of* H. parviporum *in Norway spruce that had both matches by BLASTX and deemed feasible to work within the scope of this study. Abbreviations in description column are found at page 7.*

Resistance QTL in Norway spruce	**Description of gene in Norway Spruce**	**Best BLAST-hit (coniferous spp.)**	**Exp. ampl. length (bp)**
MA_103386g0010	NAC domain-containing 68-like	PITA_000042744	486
MA_10428976g0010	PLAC8 family	PITA_000035261	656
MA_110169g0010	Chaperone domain	PITA_000082678	423
MA_125631g0010	P-loop nucleoside triphosphate hydrolase superfamily with calponin homology domain	PITA_000018556	642
MA_14352g0010	Transcription factor bHLH118	PITA_000038198	888
MA_14707g0010	ATP-dependent zincmetallo-protease FTSH chloroplastic	PITA_000055438	691
MA_18316g0010	IST1 homolog	PITA_000051036	620
MA_24271g0020	Transcription factor MYB39	PITA_000019173	535

Resistance QTL in Norway spruce	Description of gene in Norway Spruce	Best BLAST-hit (coniferous spp.)	Exp. ampl. length (bp)
MA_25569g0020	Hypothetical protein MTR_8g037205	PITA_000014583	458
MA_264971g0010	NAC domain-containing 68-like	PITA_000030354	642
MA_5978g0020	Nuclear factor 1 A-type isoform 2	PITA_000013606	1579
MA_86256g0010	NAC domain-containing 68-like	PITA_000042744	486
MA_942991g0010	Elongation factor chloroplastic-like	PITA_000025860	908

4.2 PCR and Gel Electrophoresis Yield

The result of the PCR amplification based on the gel electrophoresis output are shown in table 3. The gel electrophoresis was also used to determine if any modification of the PCR should be conducted to improve yield and which purification method would be suitable as mentioned in chapter 3.2.2.

Table 3. *Results of PCR amplification and gel electrophoresis.*

Amplicon	Gel electrophoresis result (approx. amplicon length, bp)	Additional PCR optimization	Purification method
PITA_000013606	Expected product found (1600 bp) but to some extent occurrence of smeared PCR products	None	Gel extraction
PITA_000014583	Expected product found (450 bp)	None	Precipitation in NaOAc/EtOH
PITA_000018556	Expected product found (650 bp) but presence of unwanted product at >1000 bp	None	Gel extraction
PITA_000019173	Rejected due to continuous absence of PCR products	–	–
PITA_000025860	Rejected due to continuous absence of PCR products	–	–
PITA_000030354	Expected product found (650 bp) but presence of unwanted product at <500 bp and >1000 bp	Optimization via PCR gradient (57-60-63°C) did not improve yield	Gel extraction
PITA_000035261	Expected product yield (650 bp) for most genets but product absent in some	Multiple PCR runs conducted until sufficient yield for all genets were achieved	Gel extraction
PITA_000038198	Unstable product yield (900 bp)	Higher [$MgCl_2$] had some stabilizing effect	Gel extraction
PITA_000042744	Expected product found (500 bp)	None	Spin column
PITA_000051036	Rejected due to continuous unstable PCR yield	–	–

Amplicon	Gel electrophoresis result (approx. amplicon length, bp)	Additional PCR optimization	Purification method
PITA_000055438	Rejected due to continuous unstable PCR yield	–	–
PITA_000082678	Expected product found (400 bp)	None	Spin column

4.3 Sequencing and Contig Assembly Output

Five out of the eight remaining amplicons was successfully sequenced (at least for the majority of the different Scots pine genets). See table 4 for summary of sequencing results. PITA_000042744 could not be sequenced for any of the genets despite only having one band in the gel electrophoresis. The amplicons PITA_000038198 and PITA_000042744 which yielded multiple bands in the gel electrophoresis could not be sequenced for the expected products for any of the genets, but some of the unexpected products yielded could (shown in next chapter). Failure of amplicon sequencing could be the result of pre-PCR contamination, unspecific primers or insufficient gel extraction as discussed in chapter 3.2.3.

Table 4. *Results of Sangers sequencing the products of expected size and assembly in SeqMan Pro™.*

Amplicon	Genets successfully sequenced	Generated contig length (bp)	Contig coverage of expected length (%)
PITA_000013606	20 (100%)	967	61
PITA_000014583	16 (80%)	305	67
PITA_000018556	19 (95%)	581	90
PITA_000030354	0 (0%)	–	–
PITA_000035261	14 (70%)	614	94
PITA_000038198	0 (0%)	–	–
PITA_000042744	0 (0%)	–	–
PITA_000082678	17 (85%)	372	88

4.4 Gene Identification by BLASTX

The results of BLASTX-searching the assembled consensus sequences through the Norway spruce genome portal can be found in table 5. Judging by the results of BLASTX, it indeed looks like all the amplicons sequenced from Scots pine were the one expected as they match the predicted genes of Loblolly pine.

Table 5. *Results of BLASTX-searching the consensus sequences generated by SeqMan Pro™ (see Appendix III) at the Norway spruce genome portal with the predicted sequence to be Loblolly pine.*

Amplicon	**BLASTX results**			
	Gene predicted	Avr. E-value	Avr. Identity (%)	Gene quality
PITA_000013606	PITA_000013606	3.17 E-90	95.71	High
PITA_000014583	PITA_000014583	1.75 E-24	86.27	Low
PITA_000018556	PITA_000018556	1.76 E-126	97.88	High
PITA_000035261	PITA_000035261	7.19 E-148	98.51	Low
PITA_000082678	PITA_000082678	4.02 E-81	91.74	High

Unexpected PCR products i.e. bands occurring in the gel electrophoresis with too long or too short lengths compared to the expected are shown in table 6. Those products could in most cases be sequenced and hence identified by BLASTX. The fact that unknown products were amplified in these amplicons suggest that the primers used were not specific. PITA_000030354 and PITA_000038198 yielded at least four and three different PCR products respectively, possible even more if the unsuccessful sequencing of the 650 and 350 bp bands was due to multiple different products yielded with similar lengths.

Table 6. *List of PCR products with unexpected sizes found during the gel electrophoresis. Gene quality parameter in BLASTX at the Norway spruce genome portal was set to 'high' for all sequences.*

Amplicon	**Amplicon length (bp)**	**BLASTX results**		
		Gene predicted	Avr. E-value	Avr. identity (%)
PITA_000018556	1000	PITA_000070669	1.15 E-23	40.13
	650 (expected)	PITA_000018556	1.76 E-126	97.88
PITA_000030354	1500	PITA_000016756	2.37 E-04	41.86
	1200	PITA_000030344	7.43 E-22	86.27
	650 (expected)	*sequencing failed*	–	–
	350	*sequencing failed*	–	–
PITA_000038198	900 (expected)	*sequencing failed*	–	–
	800	PITA_000038198	4.13 E-23	71.10
	650	PITA_000038198	2.37 E-24	81.55

The results from the gel electrophoresis, sequencing and BLASTX for the amplicons PITA_000030354 and PITA_000038198 are a bit confusing. The PCR products with the expected length from the gel run was not able to be sequenced, but some of the unexpected products could. The generated consensus sequence for those was matched with the expected genes when BLASTX-searched. Perhaps some introns are present in the Scots pine genome in the regions of those genes but not in Loblolly pine, causing interference with the PCR amplification. Only a few samples of the unexpected products were sequenced so caution should be taken about the

consensus sequence and corresponding BLASTX matches. Also, SNPs could not be identified with high enough confidence for the unexpected products due to the few genets included in the contigs.

4.5 Recognized SNPs in Amplicons

Identified SNPs found in the sequenced amplicons are shown in table 7 below. As mentioned earlier, the PITA_000038198 had too few genets included in the assembled contig to detect SNPs with acceptable confidence. The amplicon PITA_000082678 was sequenced with high quality but did not contain any SNPs. Full matrix of the SNPs composition of specific genets can be found in Appendix II. Consensus sequences of the below listed amplicons are attached in Appendix III.

Table 7. *List of successfully sequenced amplicons and presences of SNPs. SNP-scores are listed accordingly to position in consensus sequences.*

Amplicon	Number of SNPs detected	SNP-positions in consensus sequence	SNP-score (%)
PITA_000013606	2	239, 432	25.0; 55.0
PITA_000014583	5	132, 168, 192, 211, 264	37.5; 31.3; 25.0; 56.3; 25.0
PITA_000018556	1	425	47.4
PITA_000035261	4	48, 230, 386, 440	42.9; 35.7; 35.7; 35.7
PITA_000082678	0	–	–

4.6 Analysis of *H. annosum s.s.* Inoculation and Growth Rate

The results of LL measurements are shown in figure 8 (see next page). There was in general a very low amount of LL for the different genets with mean values ranging from 2.60 mm as lowest (S01S3030) and 6.25 mm as highest (S01W3184). No statistically significant differences of LL between the genets could be detected by doing a Kruskal–Wallis test ($\alpha = 0.05$) although there was a strong trend in the dataset (p-value = 0.054). Variation in LL when assessed on individual genes rather than on genets-level could not be identified either when conducting a T-test with significance level adjusted by Bonferroni correction. It should be mentioned that the LL was sometimes difficult to assess due to the occasionally indistinct borders between the necrotic and the healthy tissue, hence giving a subjective element to this measurement.

Incubation of the twigs for the FGS measurement yielded very low frequencies of conidiophore formation, both when observed after 7- and 11-days in the moisture chamber. Approximately 25% of the collected twigs had conidiophores present at

the inoculation site, varying between 0 – 63 % between the genets (figure 9). Despite the relative frequent presence of conidiophores at the inoculation site, only 11 of the 153 twigs had any further conidiophore formation along the branch. Those 11 branches were distributed over 9 genets and no sample had conidiophores formed more than 10 mm from the inoculation site. No statistical methods with acceptable strength could be conducted for the FGS measurement due to the weak data set. Because of the non-significant differences of neither LL or FGS it was not possible to do any correlation between identified SNPs and potential resistant phenotypes.

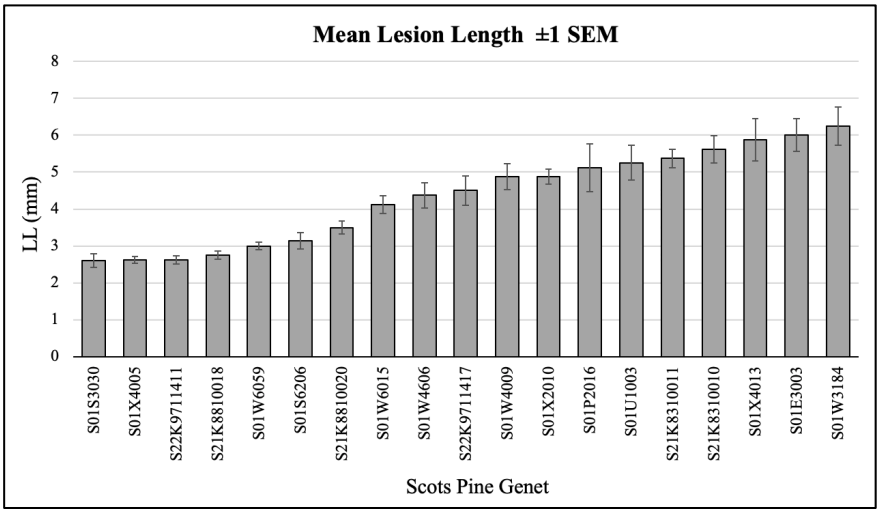

Figure 8. Results of LL measurements. Note that S22K9711412 is not present since not enough vital plants could be found for this genet.

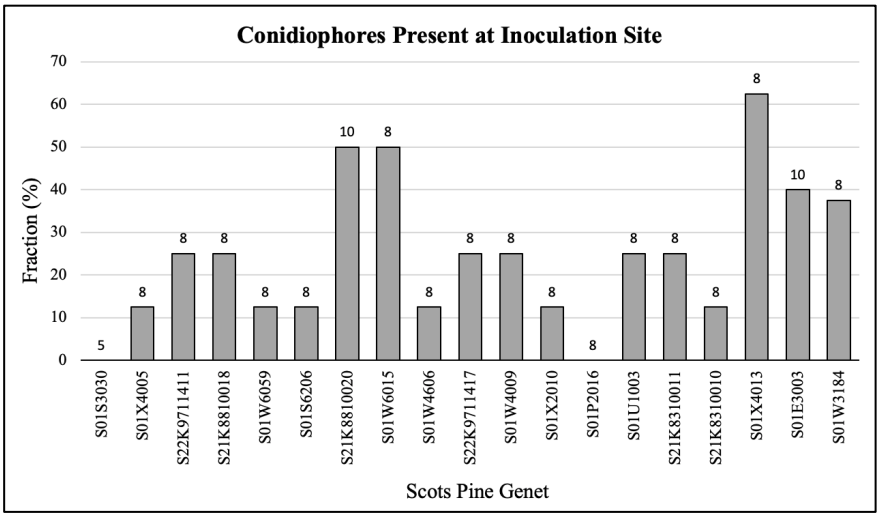

Figure 9. Proportion of twigs with conidiophores found at the inoculation site (number of twigs examined are shown above bars). S22K9711412 was not included, see above.

5 Discussion

The first objective with this thesis was to analyse the genome of Scots pine for genes previously identified in Norway spruce associated with resistance to *H. parviporum*. If orthologues were found, it was also this study intention to analyse them for presence of variation in terms of SNPs. Five out of 13 amplicons were successfully sequenced and designated as orthologues since the BLASTX matched the generated consensus sequences with the expected genes. Furthermore, four of those five orthologues contained SNPs. It is possible that more of the already identified QTL-holding candidates in Norway spruce (see Elfstrand et al., 2020 and Chaudhary et al., under review) could be found in Scots pine if the primers used were further optimized. Improvement of the purification methods used for the PCR products in this study (e.g. refined gel extraction) could also help to identify other potential orthologues in the samples that was not successfully sequenced. However, it is also possible that some of the genes associated with the QTL resistance in Norway spruce are simply not present in the Scots pine genome due to gene deletion occurring after the split between spruces and pines. Furthermore, there are many types of gene duplication events that could arise during the evolutionary pathway and as such result in an increase of gene sizes (Graur & Li, 2000). The occurrence of amplicons with longer lengths than expected found in this study could therefore be explained by PCR amplification of genes within in the same gene family.

If the orthologues genes found within this study have the same or comparable functions in the Scots pine as in Norway spruce, they then have a very diverse roles in the plant according to the BLAST description of gene function (table 1). Some caution should however be taken when drawing conclusion from the BLAST result since the genes might have evolved in different direction following the divergence of spruce and pine. The gene description given by BLAST is also very broad and unspecific which further increases the uncertainty of function within the plant. Nevertheless, some insights of the orthologues could be given from the BLAST description data. PITA_000082678 (orthologue to MA_110169g0010) was described as a chaperone protein, a group that previously have been linked to stress

responses in conifers such as heat shock and insect feeding (Lippert et al., 2007). More studies of this gene and its encoded protein would therefore be interesting. No SNPs were found within this orthologue but all the other genes had them and were the following: PITA_000013606 (orthologue to MA_5978g0020; nuclear factor, two SNPs found), PITA_000014583 (orthologue to MA_25569g0020; gene product only hypothesized, five SNPs identified), PITA_000018556 (orthologue to MA_125631g0010; hydrolase enzyme, one SNP found) and PITA_000035261 (orthologue to MA_10428976g0010; gene in the PLAC8 family, four SNPs found).

Not much information exists about the gene product of PITA_000013606 which was designated as a nuclear factor of "type 1A, isoform 2" by BLAST. It is however known that other nuclear factors can have impact on plant responses to pathogens attacks and abiotic stresses (Oh et al., 2013). Despite that the gene product(s) of PITA_000014583 has not yet been observed, more knowledge about this gene would be desirable since it contained five SNPs making it an interesting candidate were variation in gene expression and function could potentially be found. PITA_000018556 is of interest since some hydrolases are known to inhibit fungal growth in plants (Boller, 1993). Further investigation of this gene would therefore be desirable. The gene family PLAC8 which PITA_000035261 belongs to is a large group of genes found in both plants, fungi and animals (Song et al., 2011). The PLAC8 proteins found in plants have been shown to participate in two very different processes: determination of fruit size and transportation of heavy metals (ibid). It is not obvious if and how this gene could have effect on a fungal invader, but perhaps detailed studies of this orthologue will answer this question. Since four SNPs were identified for this orthologue it can also be a promising candidate gene to find phenotypic variation within. Closer examination of the identified orthologues within this study could give insight in the functions and expressions of the genes within the Scots pine system as well as reveal if they differ from their relatives in Norway spruce. Further investigation if *H. annosum s.s.* and *H. parviporum* responds in different ways when encountering the products of the genes would also be extremely interesting.

The second objective within this thesis was to do a first screening of resistance levels to *H. annosum s.s.* of the Scots pine genets used in the national forest breeding programme of Sweden. If any variation was found, this thesis also aimed to relate the phenotypic variation with the genomic data of the newfound QTL-holding candidates. Unfortunately, genets inoculated with the *H. annosum s.s.* had in general a very low spread of the fungus both when measuring LL and FGS. No significant differences in resistance variation could be found for the genets or on the gene-level although the output from the Kruskal–Wallis test was very close to the threshold (p-value = 0.054). Because the non-significant differences in fungal growth between

the genets, no correlation to the orthologues was conducted. The low fungal spread could possibly be due to the cold spring of 2020 since the growth rate of *H. annosum s.s.* rapidly decreases below its temperature optimum of 17 – 22 °C (Nikolajeva et al., 2012). It was also observed that large amounts of resin were secreted during the inoculation of the genets. Since conifers (especially pines) uses resin exudate as their primary defence against fungal infections (Asiegbu et al., 1998) this could also explain the low fungal spread within the twigs. Furthermore, competition by other fungal species could present yet another cause of the poor growth of *H. annosum s.s*.

To improve future studies were inoculation of *H. annosum s.s.* are conducted under field conditions, it is probably wise to wait until temperatures are more favourable for the pathogen. Prolonging the time for the fungus to grow before measuring the spread within the tree could also be recommended. For instance, in a study by Mukrimin et al. (2019) LL of *H. annosum s.s.* in Scots pine were measured five months post inoculation as compared with the roughly two months of this study (although that study examined the fungal growth in stems rather than in twigs). Trials with other isolates than Sä34 could perhaps be worth testing since variation in growth rate for different isolates have been observed (e.g. Stenlid & Swedjemark, 1988). Considering how close this study was to detect variation in resistance between the genets even under sub-optimal conditions as discussed above, it seems feasible that variation indeed could be found if inoculation would be conducted under more favourable conditions. Recognition of resistance variation would be interesting both to see if the orthologues and their SNPs could be linked to a certain phenotype and as such be designated as QTLs. Determination of the variation would also provide a basis to decide if any genet(s) should be excluded from the breeding programme due to high susceptibility.

The main conclusion of this thesis is that despite the failure to detect variation in the Scots pine with respect to *H. annosum s.s.* resistance, the recognition of orthologues of QTLs in the *H. parviporum*/Norway spruce pathosystem is still interesting and brings some hope when considering future breeding prospects. The fact that the orthologues also contained SNPs suggest that they might have genetic diversity that could be exploited for developing Scots pine varieties with enhanced resistance. Future studies could hopefully detect resistance variation in Scots pine and correlate the data with the new insights of the Scots pine genome brought by this study!

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Appendix I

Table 8. *Primer pairs used for the PCR and Sangers sequencing reactions.*

Amplicon	**Direction**	**Sequence (5' → 3')**	**T_m (°C)**	**GC-content (%)**
PITA_000013606	Forward	GCACAGACAGCTTCAGTTCC	59.4	55.0
	Reverse	TTCGAATACCCCTCCTCAGC	59.4	55.0
PITA_000014583	Forward	GCGAATGATCACCTCCGTTC	59.4	55.0
	Reverse	CCATCCATTGCCCCTTTCAG	59.4	55.0
PITA_000018556	Forward	AGACCACCAACTCATGCTCA	57.3	50.0
	Reverse	CGGTGGCTTCTTGATCAGTG	59.4	55.0
PITA_000019173	Forward	CAGCAGCCATGATACAGCAG	59.4	55.0
	Reverse	TGGCTTCTTGAATGCTGCTG	57.3	50.0
PITA_000025860	Forward	GCCCGGGGAAAATTTGAGAG	59.4	55.0
	Reverse	GCCAAAACCATTCCCCTCTC	59.4	55.0
PITA_000030354	Forward	CGTCTTGCCGAAGTTACCAG	59.4	55.0
	Reverse	CACGTTGTAGCCTGCTTGAG	59.4	55.0
PITA_000035261	Forward	GGGTTTGTGCGTTGTTGTTG	57.3	50.0
	Reverse	AACGTTTCACATCACAGGCC	57.3	50.0
PITA_000038198	Forward	CTTCACGCCCAAAGTTCCAA	57.3	50.0
	Reverse	ACGGTGACCTTAACAGTGGA	57.3	50.0
PITA_000042744	Forward	CCCTCTCCGAATTGCAGAAC	59.4	55.0
	Reverse	CCTCGGTAAGTAGCCTGGAG	61.4	60.0
PITA_000051036	Forward	GCTCCACATGTTTCCAAGCA	57.3	50.0
	Reverse	CCAGGGGTAGGAAAGTGGTT	59.4	55.0
PITA_000055438	Forward	TGGAACAGTGGCAATTGTGG	57.3	50.0
	Reverse	ATCATTGCCTCCGCCAATTC	57.3	50.0
PITA_000082678	Forward	GCACACAGGATAGTCTTGAGG	59.8	52.4
	Reverse	AATTCAACCCTGACCCCTGG	59.4	55.0

Appendix II

Table 9. *Matrix of all SNPs found in the orthologues. Position in matrix marked with"–" means that the amplicon for that particular genet could not be successfully sequenced.*

SNP position →	**PITA_000013606**		**PITA_000014583**				**PITA_000018556**			**PITA_000035261**		
Genet ↓	**239**	**432**	**132**	**168**	**192**	**211**	**264**	**425**	**48**	**230**	**386**	**440**
S01W4009	AC	CC	CG	CC	TT	AG	TT	GG	CC	TT	CT	GG
S22K9711417	AC	CC	–	–	–	–	–	GG	CT	AT	TT	AG
S01S6206	CC	CG	CG	CC	CT	AG	CT	–	CG	TT	CC	GG
S01X4005	CC	CG	–	–	–	–	–	GG	TT	AA	TT	GG
S21K8310010	CC	CG	CC	CC	TT	GG	TT	AA	–	–	–	–
S01E3003	CC	CG	CG	CC	TT	AG	TT	AG	CC	TT	CT	AG
S01W6059	CC	CG	CC	CC	TT	GG	TT	AG	–	–	–	–
S01W4606	CC	CG	CC	CC	TT	GG	TT	AG	–	–	–	–
S01X2010	AC	CC	CC	CC	TT	GG	TT	AG	TT	AA	TT	GG
S01S3030	CC	CG	CG	CC	CT	AG	CT	AG	CG	TT	TT	AA
S01W6015	CC	GG	CG	CT	TT	AG	TT	AG	CT	AT	TT	GG
S01W3184	CC	CC	CC	CC	TT	GG	TT	GG	CC	TT	CT	AG
S01X4013	CC	CG	CG	CT	TT	AG	TT	GG	CC	TT	CT	GG
S01P2016	CC	GG	CG	CT	TT	AG	TT	GG	CC	TT	TT	GG
S21K8810018	AC	CC	CC	CC	TT	GG	TT	GG	CT	AT	TT	AG
S22K9711411	CC	CG	–	–	–	–	–	GG	CG	TT	TT	GG
S01U1003	AC	CC	GG	CT	CT	AA	CT	GG	–	–	–	–
S22K9711412	CC	GG	–	–	–	–	–	GG	–	–	–	–
S21K8310011	CC	CG	CG	CC	CT	AG	CT	AA	–	–	–	–
S21K8810020	CC	CG	CG	CT	TT	AG	TT	AG	CC	TT	TT	GG
Major allele	**CC**	**CG**	**CG**	**CC**	**TT**	**AG**	**TT**	**GG**	**CC**	**TT**	**TT**	**GG**

Appendix III

Assembled consensus sequences are shown below for all orthologues that were successfully sequenced. Positions where SNPs were found are marked with a grey box (▒). Genetic codes according to IUPAC standards. All sequences are written in 5' to 3' direction.

PITA_000013606:
ATCTTCACAGCATTGCAGCAAGCTTCTATTTAGAAGAAGCTGATTTGTGCAAGATGTCAACCTCCACCTGTT
TCCAGTCCCCCAAAGCTTATTTGGAAATAGTAATCTTTACAGGAAGGCAGGAACCTTGCTGTGGCAGTAGC
AGTAGTAAGCGGATTGGGACCTTCAGATTGCAAGTGGGACCAGAATGGAGAGAGGTAAGGACAGTGCAGC
TTCACAATGGGTGGACTAGCATTGGCAAAAGCAAGGCGCGGGGTGGAATTCCTGAGGTGGAGTTCCATGT
AAGAGTCATGGTTGAGCCTGATCCCCGATTAATCTTTCAATTTGATGATGAAACTGTATTGAACCCCAGGA
TTGTGCAACTGCAAGGCAGTGTGAGGCAGCCTATTTTCAGCTGTAAATTCAGCCAAGGATCACGGTTAGTA
ATCAGCSTATCTAAGAATTAAGCTTGTTTAGCACAGTGTGTTTTTGGAATGAATGTTACATATGAAACCTAT
CAAACTCGGCCAACTTATAATCCAAGTCTCTATGCCTGCTGGGGTTTGATTCTTGAATCATTGGACCACAGG
CAGTGGACTGCTTAGCATAGCTTTTTGCTGTTACAAACTATGGTTTATTAATATTTACCTAAAAGAATGATT
AGAAACTCACACCATTTGTGGCAGTTAACTTACACAATTTGTGACAGTTGCATCTGTCCAAAAATGAAACG
GAGATGTTCTCATAGCTAACTAAAGATCAGTTTATTTAATATTGATTTTTGCCATGACAAACAATGGTTCAA
TTGCTTGAAACAATGAATATTAACGTATGCCATTGGTAGCATTTACTTCTATCAAAAATGAAATCCAGATCT
TCTCAACTTGACGTTTCAATTCTGGTTGACATTATAGTCTTATGGTTGTCTCCTCTCTCCCAGTGTCTCTCAA
CTTGATAGCATTGCCACTGGTCGGGCTGGTTCTCAAAa

PITA_000014583:
TG--AGGGATCTGATACAGCCACTGT-GTTTGACA-TTTTTGAGGAAGACAAGCACCAGCTCCTGCCCGACGA
GGAATGT-GTCCAAAAAGGCGTTCATGACCACATTCCCATGCCCAGGCCTCAATTGCACCTTGACACAGCC
AAGGTTACTATGTCACTAGGGATCAGTATAATGTCCAGTGTTTTAGATAAATTAAATTTGCATTTGRAAAA
TATGGTTTAATTGGTGGTTTAGATTGGTTACTTGGCTACCTACTAATTTCATTCTGTCATGTCATCTTCTTTT
TTTGCAGACTAAAATCT

PITA_000018556:
TCCTCTTGTGTTTGGCAATGGTTTAGGTGAAATGAAGAAAGCAGTGCGCCGACCATCTCCAGATAGAAGAT
TGACATTGGACCATTGTAGTCAAACAAAGAACAAAATTAAGCATGATATCTTTGAAGAGCAAGATAGGAG
AAATTGGCAGGACAAGGTGATGATGAGCAAATATTTACCCATCCAGGGTCCATCTTTGCAAGATGACGTAA
TGGGATTGTGGGAAGGCCATGAACTGGAAAAACTAGACAATCCTTCTGAAATTTATAACCAAATGTACCAT
GCCAATCAAAGAAAGATTCATCCAGATTGCGAAGAAGAGAAATTGTTACGGCAGCAGCTTCATGAGATGC
AGGGGGTAATTAAAAGCAGACCTGTTAGAAAAGACAAACGTGAACATGATGGGCAAAGAACTCCTTATGA
AGCTGCAACTACTGATGAGTCAGAGGTTGAAATTGATGCAAGAAGCAAGGATGACTATCTGACAAGTGAT
TCATCAGAAGCAGATCTTTTGTGGCAATTCAAGGCACCAAATGTGGCTGCTGTGACCCCAGTTGAACATGT
CTCACTGATCAgAAA-A

PITA_000035261:
GCTGGCCTATATCTCATCCTCAGTCC-GCTCGGAAAGGATTACAAAGCTGAAAGT-GATTTGGAGGCTCTCG
TAGATCTTACTCAGATGGGTGATAACTCTGTAAAGGGCAAAGCTGCATCCGTTCTGCTTGAAAAGAGAGAC
ACTTTTCATGCAA-GAGAGAGTAAAAGAATGGAACCTAAACCGGAATGGCAGGGGGGTCTCTTTGAT-TGCT
ACAGTGATCCT-TCTGTTGCATTTTTTTCAACATTTTGTGGGTTCTGTGTTTTTGGGTGGAATATGGATAGAC
TAGGATTTGGGAACAAGTATGTGCACATAGTGACCTTCATTTTGTTTTGCATGGCCCCTTATTGGGTTTTCA
CCTTGGCTGCTGTGAATATTGATAATGAGAATATTCGGAAAGGCTTTCATATCACTGGGATCATTCTTTGCG
TGCTTGGGCTTCTTTACGGCGGGGTTTGGAGGATTCAGATGCGGAAGACGTTTGGCCTACCTGGGAATGAC
TGGTGTTGTGGTCAGCCTAATGTGACAGATCTAATGCAGTGGCTTTTCTGCAGTGTTTGTTCTCTCTGTCAG
GAGGTTCACACCGGAAATTTCTACGAG-GCCTGTGATGG

PITA_000082678:
ATTGGATTTCGTC-GGATGGATTCAAGCAATCTGAACCTCCTACGAAAATCGAAGGAGCAAATTTTGAAAA
TAATGATTTACTGAATGAGGAGATGGGCGAGGAATGGTGGGGTGATGAT-TCATTCTATGATTTATTGAATG
ATGAGGAGATGGCAGAGGAATGGTTGGGTGATGATACATTCTATGGCCTCTATTCTAACATTCATGAAGAT
AACGATCTATATTCTGCCATTCAAAGCCACCATCAAATTACACCAGAGGAGAAAGATCTGTTTATAAACAA
TAAGAACAAGAAACCCAGAGCTCATATTCGAGAAGGAACTGGCTTTGAAATTTATTCTTTCAGCCAG-GGG
TCAGGG-TTG-AATTA

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