Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2022:3
Faculty of Forest Sciences

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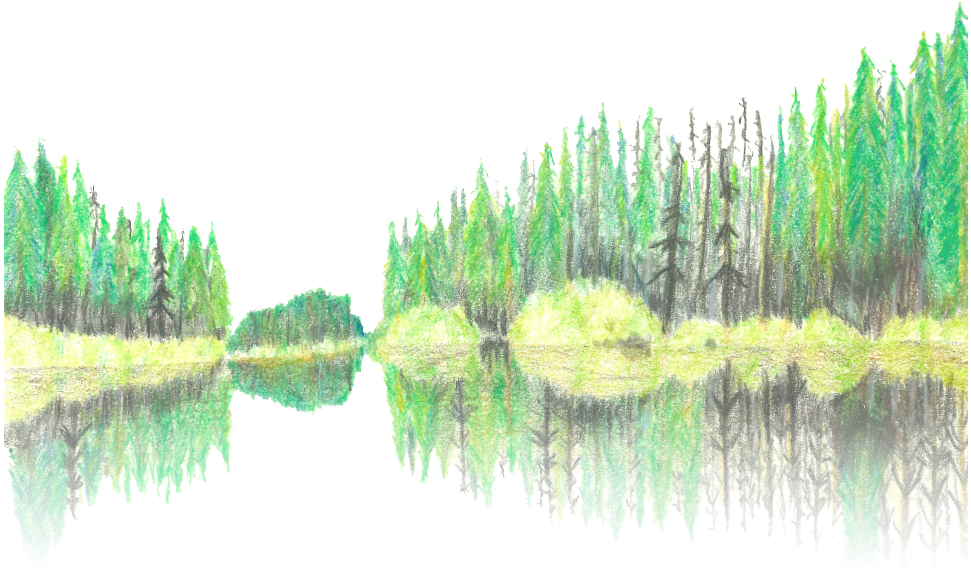
A look in the past with an eye to the future

Hernán Dario Capador-Barreto



Genetics of disease resistance in Norway spruce (*Picea abies*)

A look in the past with an eye to the future

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DOCTORAL THESIS

Uppsala 2022

Acta Universitatis Agriculturae Sueciae
2022:3

Cover: A forest mirrored in water, as seen by the author (Hernán Dario Capador-Barreto)

ISSN 1652-6880
ISBN (print version) 978-91-7760-881-3
ISBN (electronic version) 978-91-7760-882-0

Uppsala
Print: SLU Service/Repro, Uppsala 2022

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Abstract

Trees have evolved strategies to fight enemies and survive during their million-year history. These strategies have been shaped by natural selection and are reflected in their genomes today. Tree planting is a priority for governments, but there is a risk that trees selected by humans will lack alleles important for disease resistance. Norway spruce (*Picea abies*) is a characteristic species in the Swedish landscape and one of the most important trees for the forest industry. Therefore, the overall aim of this thesis was to study the genetic variation of resistance traits in Norway spruce to *Heterobasidion parviporum* and *Heterobasidion annosum s.s.,* two fungal pathogens causing root and stem rot in conifers.

In the first two papers, the genomic basis of resistance traits was studied with genome-wide association studies (GWAS). Associations between single nucleotide polymorphisms (SNPs) and resistance traits led to the discovery of several variants, with relatively small effects, associated with resistance to each pathogen. Correlation of resistance traits to these two species was dependent on the environment but using GWAS pleiotropic SNPs associated with resistance to both pathogens were found. Synergistic pleiotropic SNPs are genes that could provide multiple disease resistance in trees.

In the third paper, signatures of selection in *PaLAR3* were studied. This gene is associated with defence against pathogenic fungi in Norway spruce. Genomic analyses demonstrated that variation in *PaLAR3* has been likely maintained by balancing selection in Norway spruce. Moreover, it seems that this process started before Norway spruce isolated reproductively from white spruce (*Picea glauca*).

In the fourth paper, resistance in the bark was studied in ten Norway spruce genotypes varying in susceptibility, inoculated with five *Heterobasidion* isolates varying in virulence. Both host and pathogen influenced the length of lesions in the bark. Using differential gene expression and co-expression networks, it was shown that Norway spruce genotypes with relatively high resistance had a robust response, which included the expression of pathogen recognition genes. In contrast, in a more susceptible host, the response was dependent on the virulence of the *H. annosum s.s.* isolate.

Overall, the thesis advances the knowledge on disease resistance in Norway spruce. This knowledge will support the Swedish Norway spruce breeding program decision making in selecting healthier trees in the future.

Keywords: genome wide association study (GWAS), pleiotropy, balancing selection, gene evolution, quantitative disease resistance, RNA-seq

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Genetics of disease resistance in Norway spruce (*Picea abies*): A look in the past with an eye to the future

Sammanfattning

Träd har utvecklat strategier för att bekämpa fiender och överleva under miljoner år. De här strategierna har formats av det naturliga urvalet och återspeglas i deras genom idag. Att plantera skog prioriteras av många styrande organ, men det finns en risk att träd som valts ut av människor saknar alleler som är viktiga för resistens mot patogener. Gran (*Picea abies*) dominerar i det svenska skogslandskapet och är ett av de viktigaste trädslagen för skogsindustrin. Därför var det övergripande syftet med denna avhandling att studera den genetiska variationen som kontrollerar resistensegenskaper mot *Heterobasidion parviporum* och *Heterobasidion annosum* s.s., två arter av rotticka som båda orsakar rotröta i gran.

I avhandlingens två första studier analyserades den genomiska kontrollen av resistensegenskaper med genomomfattande associationsstudier (GWAS). Associationer mellan resistensegenskaper och singelnukleotidpolymorfismer (SNP) ledde till upptäckten av flera loci, med relativt små effekter, associerade med resistens mot rotticka. Den statistiska korrelationen mellan resistenserna mot de olika rottickearterna berodde på miljön testet utfördes i, men via GWAS identifierades flera synergistiska pleiotropa loci, dvs gener som kan ge träden resistens mot båda svamparna samtidigt.

I den tredje studien studerades selektionsmönster i *PaLAR3*, en gen i försvaret mot olika skadesvampar i gran. Genomiska analyser visade att variation i *PaLAR3* i gran sannolikt har upprätthållits genom balanserande selektion. Resultaten tyder på att *PaLAR3* var under balanserande selektion innan gran och vitgran (*Picea glauca*) isolerades reproduktivt.

Slutligen studerades resistensen i barken i tio grankloner med olika känslighet för rotticka. De inokulerades med fem *Heterobasidion*-isolat med

olika virulens. Resultaten från försöket visar att både gran och rotticka påverkar hur långa nekroser som utvecklas i barken. Genuttrycksmönstren av gran och rotticka studerades via RNA-sekvensering av prov tagna bredvid nekroserna. Analyser av differentiellt uttryckta gener visade att responsen i granar med relativt hög mottaglighet beror på svampens virulens medan granar med relativt hög resistens hade ett robust svar, inklusive uttryck av gener som styr igenkänning av patogenen.

Arbetet i den här avhandlingen bidrar till förståelsen för resistens mot skadesvampar i gran och kan stödja urvalet av robusta träd det svenska granförädlingsprogrammet.

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Keywords: genomomfattande associationsstudier (GWAS), pleiotropi, balanserande selektion, genevolution, kvantitativ sjukdomsresistens, RNA-sekvensering

Dedication

To my parents, Martha Helena and Luis Dario

A mis padres, Martha Helena y Luis Dario

"Insight into universal nature provides an intellectual delight and sense of freedom that no blow of fate and no evil can destroy"

Alexander von Humboldt

Contents

List of publications

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

I. M. Elfstrand; J. Baison; K. Lundén; L. Zhou; I. Vos; **H. D. Capador**; M. Stein Åslund; ZQ Chen; R Chaudhary; Å Olson; HX. Wu; B Karlsson; J. Stenlid; MR García-Gil (2020). Association genetics identifies a specifically regulated Norway spruce laccase gene, PaLAC5, linked to *Heterobasidion parviporum* resistance. Plant, Cell & Environment, 43, 1779– 1791.

II. **H. D. Capador-Barreto**, C. Bernhardsson, P. Milesi, I. Vos, K. Lundén; HX Wu, B Karlsson PK Ingvarsson, J. Stenlid, M. Elfstrand. Killing two enemies with one stone?: Genomics of resistance to two sympatric pathogens in Norway spruce. Molecular Ecology, 30 (18), 4433– 4447.

III. **H. D. Capador-Barreto**, PK. Ingvarsson, K. Ihrmark, X. Wang, J. Stenlid, M. Elfstrand. Balancing selection maintains variation in *PaLAR3*, a disease resistance associated gene in *Picea abies*. (Manuscript).

IV. **H. D. Capador-Barreto**, G. van Iersel, M. Brandström Durling, J. Stenlid, M. Elfstrand. Transcriptional regulation of genotype-by-genotype interactions between Norway spruce and *Heterobasidion annosum s.s.* (Manuscript).

The contribution of Hernán Dario Capador-Barreto to the papers included in this thesis was as follows:

I. Performed experiments in the greenhouse, contributed to data analysis and interpretation. Contributed to writing and conceptualization.

II. Planned and performed disease resistance experiments. Was the lead author in data analysis, visualization, and writing.

III. Contributed to conceptualize and design the project. Was the lead author in data analysis, visualization, and writing.

IV. Contributed to conceptualize and design the project. Planned and performed disease resistance phenotyping, was the lead author in data analysis. Contributed to writing and visualizing.

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Abbreviations

LGM	Last Glaciation Maxima
bp	Base pair
LL	Lesion length
SWG	Sapwood growth
QTL	Quantitative Trait Loci
PP	Polyphenolic Parenchyma cells
LSZ	Ligno-suberized zone
RZ	Reaction zone
RLK	Receptor Like Kinase
NLR	Nucleotide Binding Leucin Rich Repeat
SNP	Single Nucleotide Polymorphism
EBV	Estimated Breeding Value
PCA	Principal component analysis
GWAS	Genome wide association studies
dpi	Days post inoculation
QDR	Quantitative disease resistance
LD	Linkage disequilibrium

1. Introduction

The forest cover in Europe has increased in the last decades accompanied by a higher frequency of forest disturbances, likely driven by both increased wood production and natural events (Senf and Seidl 2020). Because forests are important for human wellbeing, production of goods and biodiversity, reforestation is a clear strategy for sustainable forest management in Europe. Indeed, tree planting is a priority for governments and a recurrent issue in the environmental agenda. In fact, the new European Union forest strategy includes a pledge to plant more than 3 billion trees by 2030 (European Commission 2021).

Tree planting can change the genetic composition of a forest because trees are planted in areas they might never reach naturally. Changes in the genetic composition occur because different evolutionary forces, that otherwise change due to natural processes, are altered. For example, gene flow is affected through assisted migration and imports of seeds, mating is affected by controlled crosses in breeding programs, as well as selection for desired traits (Adams *et al.* 1992). The balance of these evolutionary forces will determine the success of planting and growing the right tree in the right place, for the right purpose. Therefore, the study of genetic composition of trees used for reforestation, and how this affects their performance in the field is a key aspect to achieve the current reforestation goals (Hall, Hallingbäck, & Wu, 2016).

Because natural selection has shaped resistance mechanisms in trees for millions of years, a risk of reforestation is that the altered genetic composition of forests fails to include genes contributing to resilience and resistance to pests and diseases. Therefore, the aim of this thesis is to study

the genetic variation of resistance traits in Norway spruce - a widely planted tree species native to European forests - to *Heterobasidion annosum s.l.,* a complex of fungal pathogens causing root and stem rot in conifers. This approach allows me to take a look in the past and learn how disease resistance has evolved in Norway spruce. In the end, knowledge generated in this thesis will support the Swedish Norway spruce breeding program decision making in selecting healthier trees in the future.

2. Background

2.1 Norway spruce (*Picea abies*)

Norway spruce (*Picea abies* (L.) Karst.) is a long-lived coniferous tree dominant in boreal forests and in much of Europe. Its distribution ranges from the polar tree line in the North to the Ural Mountains in the East and as south as to the Carpathian Mountains in Romania (Figure 1).

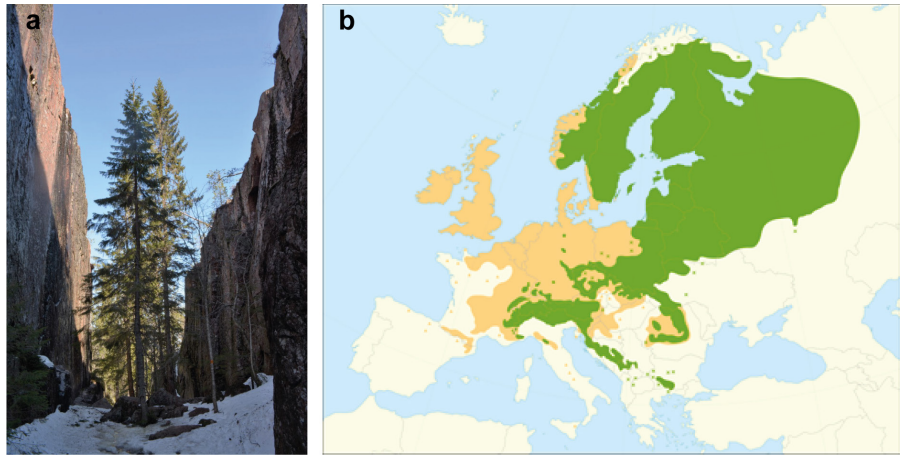


Figure 1 a) Norway spruce trees, Skuleskogens National Park, Sweden. b) Distribution map of Norway spruce (*Picea abies* (L.)H. Karst). Green: native range. Orange: introduced areas. Crosses and triangles denote isolated populations (Caudullo *et al.* 2017)

In Norway spruce, there is evidence for extensive population structure throughout its natural distribution, likely reflecting its recent evolutionary

history. Currently, it is believed that Norway spruce, just like other plants in Europe, went through a strong bottleneck during the last glaciation maxima (LGM), about 20.000 years ago (Petit *et al.* 2003; Clark *et al.* 2009). During the LGM, it is believed that Norway spruce survived in refugia south of the Ural mountains, the Balkans and Scandinavia (Tsuda *et al.* 2016). Thereafter, as the global temperature increased, Norway spruce expanded into available land in Europe. Then, through mixture between the different refugia and adaptation to the local conditions, the current populations are believed to have formed (Chen *et al.* 2019, 2021; Milesi *et al.* 2019).

2.1.1 The genus *Picea*

Just as Norway spruce, trees in the genus *Picea* are common in northern hemisphere ecosystems in Asia and America. The genus is phylogenetically complex, with hybridizing zones between species, and phylogenetic trees that vary depending on the genes they are based on (Lockwood et al. 2013; Ran et al. 2015; Feng et al. 2018; Sullivan et al. 2018). The most recent view suggests that species in North America belong to two lineages: lineage IV: including *P. glauca* (White spruce) and *Picea sitchensis* (Sitka spruce), among others, and lineage III, where *Picea mariana* (Black spruce) belongs (Feng *et al.* 2018). Norway spruce is part of lineage II along with other species distributed through Eurasia and is phylogenetically closest to the lineage of White spruce (Lineage IV) (Feng *et al.* 2018).

Studies in Norway spruce and White spruce have led to a general consensus that genes in *Picea* are largely conserved in sequence variation and genome organization (Pavy et al. 2013; Bernhardsson et al. 2019), and are expected to have low linkage disequilibrium (LD) as a result of high recombination rate due to outcrossing (Larsson et al. 2013; Nystedt et al. 2013), large introns (Nystedt et al. 2013), and an excess of rare alleles explained by a current expansion after the LGM both in Europe and America (Holliday et al. 2010; Namroud et al. 2010; Larsson et al. 2013).

2.1.2 The Norway spruce genome

The Norway spruce genome was published almost 10 years ago. It has a large genome size (19.5 x 109 base pairs (bp)), with a similar number of genes to other plants, but large introns, intergenic spaces, and repetitive regions (Nystedt et al. 2013). The current assembly of the genome is shattered in 10 M scaffolds with a median length of 700 bp, which is worse compared to other plant genomes (Sun et al. 2021).

Because the genome is so large and fragmented, an exome-capture genotyping method was developed to circumvent these difficulties (Vidalis *et al.* 2018). Exome capture is a cost effective and targeted sequencing method based on available genomic information (Clark *et al.* 2011). For Norway spruce, 40,018 synthetic DNA probes were designed based on the sequences of 26,219 genes (Vidalis *et al.* 2018). These probes were then used to "capture" selected DNA fragments in given trees to later sequence them (Vidalis *et al.* 2018). The development of this technique allowed the genotyping of thousands of trees, which has improved the understanding of Norway spruce recent evolutionary history (Chen *et al.* 2019; Milesi *et al.* 2019), the location of gene models in the genome (Bernhardsson *et al.* 2019), and the association of gene models with phenotypic traits (Baison *et al.* 2019; Milesi *et al.* 2019; Chen *et al.* 2021). Nonetheless, it is important to mention that probes only cover ~39% of the predicted genes (Vidalis et al. 2018), and because probes are designed on exons, most variants will be located in coding DNA, which will give a partial view of variation in the genome.

2.2 Norway spruce in Sweden

Norway spruce is a characteristic species in the Swedish landscape and together with Scots pine (*Pinus sylvestris*) the most important tree for the forest industry. For centuries, forests have been utilized in Sweden to sustain industrial activities such as mining, iron making, and from the mid-19th century sawtimber and pulp, which led to a depletion of forested areas by the end of the 19th century (Royal Swedish Academy of Agriculture and Forestry 2015). Since then, Norway spruce has expanded: in the beginning of the 20th century by natural regeneration, but lately due to government policies to encourage reforestation and industrialization of the forest practices

(Lindbladh *et al.* 2014; Royal Swedish Academy of Agriculture and Forestry 2015).

Today in Sweden, 68% of the land is covered by forests, out of which 84% are production forests (23.5 million of hectares), and Norway spruce encompasses 39.7% of the tree cover (Department of Forest Resource Management (SLU) 2021). These production forests in Sweden are supported by planting of trees and supported by research at universities and Skogforsk, the forest research institute of Sweden. In 2013, 216 million Norway spruce seedlings were planted. These seedlings were sourced from genetically superior trees in seed orchards in Sweden (a result of the Norway spruce breeding program), foreign and native forest stands and to a lesser degree foreign seed orchards (Haappanen *et al.* 2015).

Table 1 Origin of sold Norway spruce plants for planting in Sweden in 2013. Adapted from (Haappanen *et al.* 2015)

Origin (2013)	**Amount (%)**
Swedish seed orchards	69
Swedish stands	12
Foreign stands	13
Foreign seed orchards	5

2.2.1 The Swedish Norway spruce breeding program

"*The general objectives of the Swedish breeding programmes are to: Efficiently improve traits of high economic value; conserve adequate genetic variation; and prepare for possible climatic and other changes"* (Rosvall 2011).

Towards the mid-20thcentury, the breeding program for Norway spruce started in Sweden. Generally, “plus trees” (or trees with desired traits) were selected from commercial stands to establish the initial breeding populations. Because Sweden has a cline in temperature and daylight, 22 breeding populations have been established (Rosvall 2019). “plus trees” in the populations are crossed in a double mate paring design, where each tree is mated to two other individuals in the population. These crosses are evaluated in field trials (often clonal field trails, where trees are cloned and evaluated in different environments), and “breeding values” based on the traits measured are used to select candidates that will form the breeding population of the next cycle (Rosvall 2011). The best candidates of each population are selected to mass seed production in seed orchards.

The 3rd generation of Norway spruce seed orchards in Sweden are divided in 14 zones composed of trees from different breeding populations. A seed orchard is typically composed of more than 25 clones, and their frequency is determined by their breeding values, where the most frequent genotype will be the best, according to the breeding objectives (Rosvall and Ståhl 2008). The estimates for economic gain in these orchards is expected to vary between 16 and 28% (Lindgren *et al.* 2008).

Since forest trees in Sweden have long rotation times, the interval between selection and harvest is long and therefore selection of traits is performed with a long-term view (Rosvall 2011). For selecting trees and calculating the breeding values, an index is used, where traits are combined and given different weights depending on their importance and correlation. For Norway spruce, these include growth, survival, wood quality, and vitality (Rosvall 2011). In the latter clones are scored in a scale from 0 to 3, where 0 = dead individual, 1 = severely damaged individual with low survival ability, 2 = moderately damaged individual with rather good survival ability, and 3 = healthy individual (personal communication, Torgny Persson and Curt Almqvist). Therefore, although resistance to specific pests and pathogens has not been implemented in the breeding program yet, a diffuse selection for disease resistance can be expected from the vitality score.

2.3 Root and stem rot caused by *Heterobasidion annosum s.l.*

Heterobasidion annosum s.l. is one of the most studied forest pathogens because its conifer hosts are economically important in the Northern Hemisphere and the pathogen affects wood production and quality (Garbelotto & Gonthier, 2013). In the forest, the disease can spread long distances by basidiospores landing in fresh wood (Rishbeth 1951) and by short distances through the spread of vegetative mycelia through root connections (Stenlid 1985). Once in a tree, the fungus will grow vegetatively through the vascular tissues, using necrotrophic abilities to kill host cells.

Infections can be detrimental for the tree since it will consume resources and likely affect growth or ultimately (Bendz-Hellgren 1997; Garbelotto and Gonthier 2013). Furthermore, windthrow is reported to be more frequent in *Heterobasidion* infected trees (Oliva *et al.* 2008). Once a tree is dead, *Heterobasidion* can grow saprophytically in the dead tissue and produce basidiocarps. Notably, basidiocarps are also produced when the tree is still alive (Garbelotto and Gonthier 2013).

H. annosum s.l. is a species complex composed of five different species (Niemela and Korhonen 1998). In Sweden, *H. annosum s.s*. and *H. parviporum* live in sympatry with a geographical overlap in the mid-southern area and the ability successfully infect Norway spruce (Korhonen *et al.* 1998a) (Figure 2). These species diverged 60 million years ago (Dalman *et al.* 2010) and have partially specialized in different hosts and display somatic and sexual incompatibility (Stenlid & Karlsson, 1991). *H. annosum s.s.* has a stronger pathogenic lifestyle and can infect more hosts than *H. parviporum* (Korhonen *et al.* 1998a; Daniel *et al.* 1998). *H. annousm s.s.* is commonly found infecting trees in the *Pinus* genus, where it grows preferably on non-heartwood tissues (Oliva *et al.* 2013) (Figure 3). Conversely, *H. parviporum* has low pathogenicity on *Pinus* and displays more of a saprotrophic lifestyle, where it avoids living tissues in the tree and grows in heartwood within the trunk, preferably in Norway spruce (Oliva *et al.* 2013)

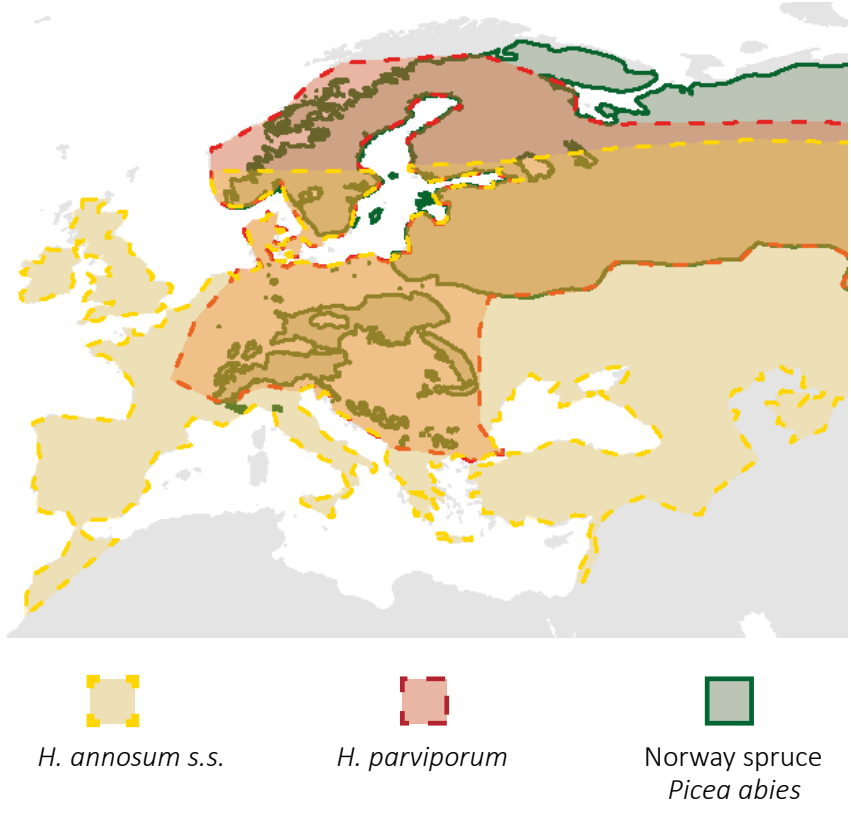


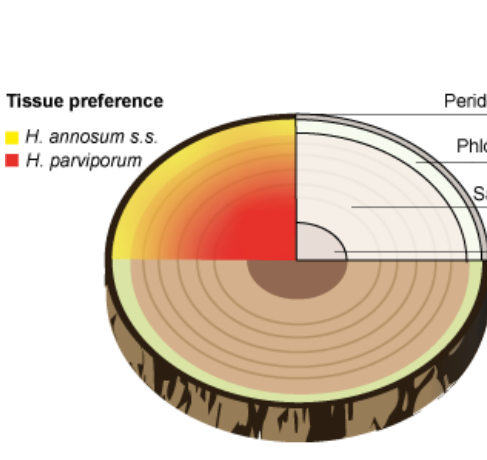
Figure 2. Distribution of *H. annosum s.s.*, *H. parviporum* and *Picea abies* in Europe. Based on (Garbelotto and Gonthier 2013) and (Caudullo *et al.* 2017).

2.4 The challenge of being a tree: Defence strategies in Norway spruce

Pests and pathogens have a substantial impact on tree populations in forest ecosystems, which is evidenced in recent epidemics (Coker et al., 2019; Ennos, 2015). At the same time, trees have specific life history traits, such as long generation time and secondary growth, which pose specific challenges when it comes to interaction with pathogens (Loehle 1988; Eyles *et al.* 2010). For instance, trees must cope with the attack of several pathogens during their lifetime, and this could sometimes happen at the same time, as coinfections in the same or different tissues (Tobias and Guest 2014;

Ennos 2015). Furthermore, secondary growth, large size and longevity demand investment in protection strategies for the stem to ensure longevity and reproduction success (Loehle 1988; Krokene 2015).

Given these challenges, Norway spruce has evolved a structured but flexible defence strategy, with pre-formed defences organized in different tissues that can be induced in response to attack (Figure 3). For example, the periderm (or outermost part of the bark, Figure 3) is a preformed defence strategy in the stem and roots that is effective against the invasion of diverse threats, such as fungi and small insects (Franceschi *et al.* 2005; Krokene 2015). If this layer is breached, there are cells prepared with preformed defences in the inner bark (Figure 3), which are able to recognize danger and induce a stronger response to limit the spread of the pathogen, compartmentalize the area and ultimately heal it (Franceschi *et al.* 2000; Solla *et al.* 2002; Krokene 2015). Even if the inner bark is breached, induction of defence can also occur in the sapwood (Krokene, 2015; Oliva et al., 2015). For example, a reaction zone (RZ) rich in lignans and with high pH is usually formed when pathogens like *H. parviporum* have already reached the core of the tree (heartwood, Figure 3) and spread into the inner sapwood (Shain 1971; Oliva *et al.* 2015; Nagy *et al.* 2022).



	Chemical	Structural	Induced	Constitutive
Periderm (outer bark)	+	+++	+	+++
Phloem (inner bark)	+++	++	+++	+++
Sapwood (Xylem)	++	++	++	++
Heartwood	+++	++	-	+++

Figure 3. Structured and flexible defence strategy in the stem. Tissue preference by *H. annosum s.s.* and *H. parviporum* (left) and morphology defence strategy of Norway spruce in the stem (right).

The induction of a defence response implies that recognition needs to occur for it to be triggered. Induction happens within hours after infection (Karlsson *et al.* 2007), and it is expected to occur after the tree recognizes molecular patterns: molecules that can be derived from damage of self, such as plant cell wall fragments, or molecular patterns in the pathogen, such as chitin (Salzer *et al.* 1997; Boller and Felix 2009). For recognition, plant use receptors bound to the cell membrane (typically "receptor like kinases" or RLK: proteins with an extracellular receptor domain with Leucin Rich Repeats and an intracellular signalling domain), and cytoplasmatic receptors (typically, proteins with nucleotide binding domain and Leucin Rich Repeats domain: NB-LRR or NLR) which collectively can defined as "R genes"(Ellendorff *et al.* 2009; Thomma *et al.* 2011). Trees such as Norway spruce have expanded and diversified R gene families compared to other plants (de Vries *et al.* 2018; Van Ghelder *et al.* 2019). Actually it has been hypothesized that a high R gene abundance is a characteristic feature of long-lived trees (Tobias and Guest 2014; Plomion *et al.* 2018).

2.4.1 The genetics of disease resistance in Norway spruce to *Heterobasidion* root and stem rot

The genetic component of disease resistance traits in Norway spruce to *H. parviporum* has been studied extensively: with artificial inoculations full-sib families (Arnerup *et al.* 2010; Lind *et al.* 2014; Skrøppa *et al.* 2015), half-sib families (Steffenrem *et al.* 2016; Chen *et al.* 2018b), clonal trials (Swedjemark and Karlsson 2004) and in naturally occurring infections in clone trials after 20 years of establishment (Karlsson and Swedjemark 2006). However, the variation in response to *H. annosum s.s.*, which is also present in most of Norway spruce distribution in Europe, has been much less studied.

The genetic component of disease resistance in Norway spruce to *H. parviporum* is quantitative (Swedjemark and Stenlid 1997; Karlsson and Swedjemark 2006; Arnerup *et al.* 2010; Chen *et al.* 2018b), with a variety of responses that go from very resistant to very susceptible. Responses in the host have been investigated with two phenotypic traits: sapwood growth (SWG) and lesion length (LL) (Figure 4). The longitudinal growth of the pathogen in the sapwood provides a measure of how well constitutive defences and the induced defence in the sapwood can control the spread of

the fungus (Figure 3 and Figure 4A&B). On the other hand, LL refers to the size of the discernible necrotic tissue closest to the wound or progressing infection in the bark and is a measure of how induced defences and wound healing responses interact to control the spread of necrotrophic pathogens (Figure 3 and Figure 4A&C).

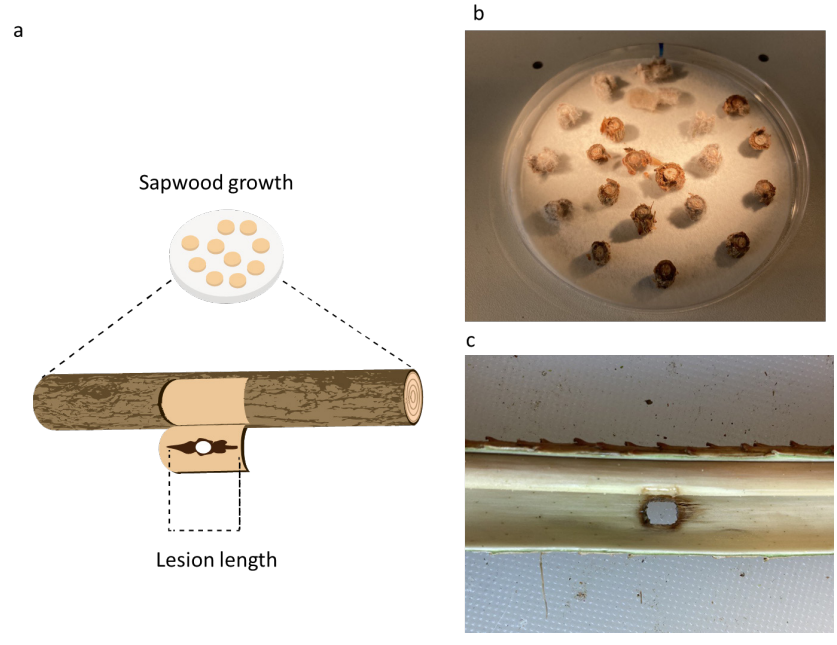


Figure 4. Resistance traits to *Heterobasidion.* A&B) Sapwood growth (SWG): Inoculated stem is cut up into 5-mm discs and placed on moist filter paper in Petri dishes. After seven days in incubation under humid conditions the presence of *Heterobasidion* is evaluated under the microscope. A&C) Lesion length (LL): length of the discernible necrotic tissue in the inner bark.

These resistance traits are genetically controlled with moderately high heritability values which indicate that there is potential for selection in this trait (Karlsson *et al.* 2008; Arnerup *et al.* 2010; Skrøppa *et al.* 2015; Steffenrem *et al.* 2016; Chen *et al.* 2018b). Additionally, it is encouraging for the breeding program that most of the reported traits do not correlate strongly with growth or wood quality traits, and therefore are not in conflict with the main breeding objectives for Norway spruce (Skrøppa et al. 2015; Chen et al. 2018b).

Through the advancement of gene sequencing techniques and the release of the genome of Norway spruce (Nystedt *et al.* 2013), quantitative trait loci (QTL) have been associated with resistance traits to *H parviporum* (Lind *et al.* 2014; Mukrimin *et al.* 2018). The best studied candidate gene is *PaLAR3,* a gene encoding for an enzyme that forms the last step in the synthesis of catechin (Hammerbacher *et al.* 2014). This gene is located on a region in the genome associated to SWG (Lind *et al.* 2014) and individuals carrying the *PaLAR3B* allele have on average of 27% lower pathogen spread in the sapwood compared to half-siblings homozygous for the *PaLAR3A* allele (Nemesio-Gorriz *et al.* 2016) (Figure 5). The transcription factor *PaNAC03* interacts with the promoter of *PaLAR3*, and differences in the promotor sequences, including NAC-binding sites, are thought to be the reason why differential expression depends on the plant genotype (Dalman *et al.* 2017).

2.4.2 The induced response of Norway spruce to *H. annosum s.l.*

The induction of disease responses in Norway spruce has been studied using chemical and transcriptional methods. Typically, transcriptional changes in Norway spruce are characterized by the activation of the jasmonate and ethylene hormone signalling (Arnerup *et al.* 2011, 2013; Lundén *et al.* 2015), and recently the role of hormone abscisic acid has been highlighted (Kovalchuk *et al.* 2019). Even though there are similarities between the transcriptional responses to infection with *H. annosum s.l.*, wounding , and other non-pathogenic fungi (Arnerup *et al.* 2011; Pepori *et al.* 2019), it is clear that fungal pathogens can induce distinct transcriptional responses in Norway spruce (Hietala *et al.* 2004; Fossdal *et al.* 2012; Hammerbacher *et al.* 2014; Chaudhary *et al.* 2020). For example, genes can show induction of expression at the edge of the lesions formed in response to *H. parviporum* compared to only a few cm away (Hietala *et al.* 2004; Arnerup *et al.* 2013; Chaudhary *et al.* 2020). Additionally, gene expression can also vary depending on the genotype of the trees, just as seen in *PaLAR3* (Nemesio-Gorriz *et al.* 2016) (Figure 5).

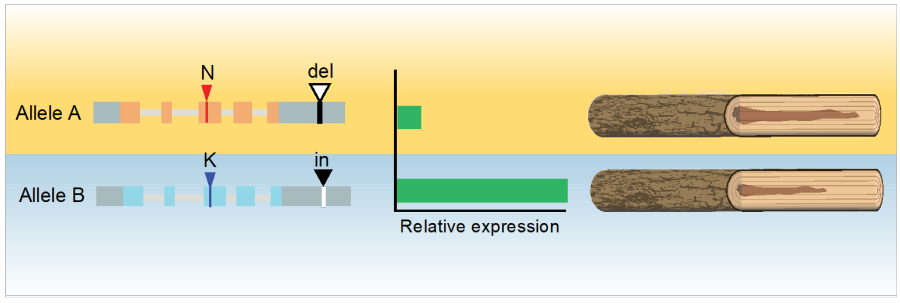


Figure 5. (**paper III**). *PaLAR3* allele structure and effect on resistance to *H. parviporum*. *PaLAR3* has two allele lineages in Norway spruce defined by one amino acid change (N175K) and an indel in the 3' UTR. Difference in expression levels appear to explain difference in resistance in the sapwood. Based on Nemesio-Gorriz and collaborators 2016 (Nemesio-Gorriz et al., 2016).

In the bark, Norway spruce is equipped with polyphenolic parenchyma cells (PP) which are key players for disease resistance, since they are normally produced during development (as pre-formed defence) and are induced upon attack by pathogens (Nagy *et al.* 2004; Li *et al.* 2012; Krokene 2015). Additionally, Franceschi and collaborators (2000) suggest that PP cells are involved in the formation of an induced structural barrier, rich in lignin and suberin (Franceschi *et al.* 2000), analogous to scar tissue in humans. In Norway spruce, a successful formation of this barrier - or so called lignosuberized zone (LSZ) (Woodward 1992; Solla *et al.* 2002) - can be seen as a later stage in the structured response, where the tree walls away the damaged tissue together with the pathogen, leading to eventual exclusion and closing of the wound (Franceschi *et al.* 2000). It has been observed that *Heterobasidion* is able to penetrate through the LSZ, and that formation of this structure does not always exclude the pathogen, but there is reportedly genetic variation in this response (Solla *et al.* 2002). The molecular mechanisms controlling this process are still largely unknown.

2.5 Resistance breeding: a feasible management practice for controlling *Heterobasidion* root and stem rot

Current forest management strategies increase the incidence of *Heterobasidion* root and stem rot, with forest thinning being undoubtedly a major source of infections (Piri and Korhonen 2008), since fresh stumps left after harvesting represent an infection gateway (large wounds that have breached the structured defence strategy of the tree). Currently, there are management practices in place to decrease the extent of infections, such as the use of biological control agents or thinning during low spore production seasons (Holdenrieder and Greig 1998; Korhonen *et al.* 1998b). However, the problem remains because in areas previously infected with *Heterobasidion*, new trees planted will likely be infected. Alternatives such as planting other species such as birch have been suggested (Lygis *et al.* 2004), but *H. annosum* can also infect birch (Piri 2003). Hence, even when management practices are in place, *Heterobasidion* root and stem rot is still a large problem for reforestation in suitable forest land.

Therefore, the use of resistance breeding for *Heterobasidion* root and stem rot is promising strategy, since through planned mating, selection, and migration in the breeding program, the genetic composition of the population could shift to healthier and more resilient trees that could perform well in *Heterobasidion* infected sites. Furthermore, the development of disease resistance in forest trees is advantageous compared to other strategies that can be costly, need to be repeatedly used, or are detrimental to the environment (Sniezko & Koch, 2017). Although there are successful examples of deployment of disease resistance trees in some commercial tree plantations in North America (Alfaro *et al.* 2013; Sniezko *et al.* 2014), it still remains an infrequent practice due to long generation times of trees, inconsistent funding from public agencies, and hesitation from stakeholders (Buggs 2020).

3. Objectives and Hypotheses

The main objective of this thesis is to understand how genetic variation in Norway spruce affects disease resistance, mainly to the two species of *H. annosum s.l.* present in Sweden. The specific objectives were:

- To understand the genetic control of disease resistance traits to both species of *Heterobasidion annosum s.l.* present in Sweden.
  Hypotheses:
  - Norway spruce has variation in its resistance traits to *Heterobasidion annosum s.s.* (**paper II**)
  - Resistance to *Heterobasidion annosum s.s.* is correlated to resistance to *Heterobasidion parviporum* (**paper II & IV**)

- To identify genomic variation correlated with disease resistance traits to both species of *Heterobasidion* present in Sweden.
  Hypotheses:
  - QTLs associated with *Heterobasidion parviporum* are expressed upon inoculation in Norway spruce (**paper I**)
  - QTLs could explain multiple-disease resistance to *Heterobasidion annosum s.l.* in Norway spruce (**paper II**)

- To study signals of selection in *PaLAR3*, a gene associated to disease resistance in Norway spruce
  Hypotheses:
  - *PaLAR3* has an excess of balanced polymorphisms compared to other regions in the Norway spruce genome (**paper III**)

- Balanced polymorphisms are not maintained by overdominance or local adaptation in *PaLAR3* (**paper III**).
- Shared polymorphisms in *LAR3* in *Picea* species have been maintained by balancing selection in Norway spruce (**paper III).**

- To investigate the variation in gene expression between different genotypes of Norway spruce in response to different isolates of *Heterobasidion annosum s.s.*

  Hypotheses:
  - Variation in Norway spruce and *Heterobasidion annosum s.s.* affects disease symptoms (**paper IV**)
  - Norway spruce genotypes respond differently in gene expression patterns to *Heterobasidion annosum s.l.* isolates varying in virulence (**paper IV**)

- To contribute with knowledge to the Norway spruce breeding program (**paper I – IV**)

4. Materials and methods

4.1 Plant material and fungal isolates

In all projects, Norway spruce plant material was provided by Skogforsk, an important ally in this project. In **paper I & II**, mother trees part of the southern Norway spruce breeding population were genotyped and their progenies phenotyped for resistance to *H. parviporum* and *H. annosum s.s.* In **paper I & IV,** grafted saplings originating from a field trial naturally infected by *H. annosum s.l.*(Karlsson and Swedjemark 2006) were used to study gene expression in greenhouse trials. For **paper III**, we obtained seeds from Norway spruce and the North American Black spruce (*Picea mariana*) and Sitka spruce (*Picea sitchensis*), which were planted in field trials in Sweden. Additionally, seeds from White spruce (*Picea glauca*) were obtained from the Canada Seed Tree Centre. Finally, we also used branches of trees planted in seed orchards owned by Stora Enso to measure disease resistance traits in the field.

4.2 Disease resistance phenotyping

The standard *Heterobasidion* inoculation and resistance phenotyping was used ((Swedjemark *et al.* 1997), Figure 4). Briefly, fungal isolates were grown on Hagem media (Stenlid 1985) for three weeks prior the experiment together with 5 mm Norway spruce wood plugs. At inoculation time, bark was removed with a 6-mm diameter corkborer and then a wooden plug

colonized by the fungus was placed at the wound and covered with Parafilm®.

For **paper I & II**, inoculations were performed in the main stem of two-year-old seedlings that were grown outside in a plant nursery. In **paper IV**, inoculations were performed in branches of 5-year-old, grafted saplings inside a greenhouse. Additionally, inoculations were also performed in the field, in branches of trees standing in three seed orchards in central Sweden: Gårdskär (60.6 N, 17.5 W), Nässja (60.2 N, 16.8 W) and Ön (60.2 N, 16.7 W). Since orchards varied in time of establishment, plants were of different age. Genotypes repeated in more than one orchard (n=6) were also inoculated with *H. Parviporum* Rb175 and *H. annosum s.s.* Sä 16-4. Nine ramets per genotype were inoculated at each orchard. One branch per ramet was inoculated. Inoculations were divided in three blocks separated by one week starting on week 19 (May 2021). Every week, three ramets per genotype were inoculated in each seed orchard. At the end of the experiment, branches were collected for phenotyping ~10 cm below the infection point to ensure no pathogen was left in the trees.

At harvest, LL above and below the edge of the inoculation point was measured. SWG was measured according to Arnerup and collaborators (2010): The inoculated stem was cut up into 5-mm discs and placed on moist filter paper in nine cm Petri dishes together with the original colonized wooden plug. To avoid contamination, the stem was cut from the tip to, and from the base to the point of inoculation, respectively. After seven days incubation under humid conditions, the presence of *H. parviporum* and *H. annosum* on the discs was determined by observation of characteristic conidiophores under a stereo-microscope (Swedjemark *et al.* 1997; Arnerup *et al.* 2010).

4.3 DNA and RNA sequencing

In **paper I & II**, DNA was sequenced to genotype trees part of the southern Sweden breeding population with exome capture probes (Vidalis *et al.* 2018). Sample collection, DNA extraction, read mapping and initial variant calling is described in detail by Baison and collaborators (2019) (Baison *et al.* 2019). In **paper II,** variants were filtered according to

Bernhardsson et al. (2020) with minor modifications(Bernhardsson *et al.* 2020). Briefly, only biallelic single nucleotide polymorphisms (SNPs) within the extended probe regions were included. SNPs with depth 6–40, GQ < 15, mean depth between 10–30, 20% missing data, minor allele count 1, and a p-value= >1e−10 for excess of heterozygosity were retained to avoid collapsed reads. Individuals with more than 30% missing variants after filtering were excluded from analysis. Missing variants in the remaining individuals were imputed with beagle 4.1 (Browning and Browning 2007). **In paper III**, we also used exome-captured sequences from an expanded population (compared to **paper I and paper II)**, together with 34 fully re-sequenced trees (Bernhardsson *et al.* 2020; Wang *et al.* 2020) and a Sanger sequenced specific DNA region from haploid megagametophytes from four *Picea* species and *Pinus sylvestris*. In **paper IV**, we extracted RNA from the edge of the lesions in the bark and sequenced it at Sci Life Lab in Uppsala, Sweden in an Illumina NovaSeq 6000 system.

4.4 Estimated breeding values (EBV) and heritability

A key aspect of this project was to estimate the genetic component of resistance to *Heterobasidion* in Norway spruce. In **paper I & II,** mixed models were used to estimate the proportion of the variation in the disease resistance traits to *Heterobasidion* that could be explained by the genetic identity of the mother trees, using the following model:

$$y_{ijkl} = \mu + B_j + D_{ijkl} + F_k + e_{jkl}$$

Where y_{ijkl} is each observation on the *l*th seedling from the *k*th family in the *j*th block, μ is the general mean and B_j is the fixed effect of the *j*th block. The variable F_k is the random effect of the *k*th family, e_{ijkl} is the random residual effect and D_{jkl} is a covariate for diameter at inoculation point. Based on this model, variance partitioning could be performed, and the proportion of variance explained by the genotype, the phenotype and the residual error could be estimated. These estimations allowed for calculating narrow sense heritability, a measurement of how much of the variation can be explained by additive genetics or put simply: how much of the studied trait is inherited

by the progenies from their parents. The individual-tree narrow-sense heritability was estimated using the equation:

$$\hat{h}_i^2 = \frac{\hat{\sigma}_a^2}{\hat{\sigma}_p^2} = \frac{4 \times \hat{\sigma}_f^2}{\hat{\sigma}_f^2 + \hat{\sigma}_e^2}$$

where h_i^2, $\hat{\sigma}_a^2$, $\hat{\sigma}_f^2$, $\hat{\sigma}_e^2$, and $\hat{\sigma}_p^2$ are narrow-sense heritability, additive genetic effect, family, residual, and phenotypic variance components, respectively.

Once these models were built, Estimated Breeding Values (EBV) were calculated. These are measurements of resistance for the mothers, based on the resistance of their progeny. The advantage of using mixed models is that the systematic effects captured in the experiment design, such as the effect of the environment in different blocks, will be subtracted reflecting a more accurate estimate of resistance for the mother tree.

4.5 Genome wide association studies (GWAS)

After EBVs were calculated, associations between EBV and DNA sequence variation measured with exome capture was performed. The results of these associations are the additive effect of a locus (Fisher 1919), or how much the EBV changes for every unit change in the DNA sequence, measured as a change from homozygote for one allele (aa), to heterozygote (Aa) to homozygote for the other allele (AA). If the effect size = 0, it means that the variation in DNA sequence has no effect on the EBV, and therefore is not involved in the variation of the trait. For this purpose, LASSO (Least absolute shrinkage and selection operator) regressions were used in **paper I**, while in **paper II** we use single-trait and multi-trait mixed models in GEMMA (Zhou and Stephens 2012) for all the variants identified with exome-capture sequencing. Principal component analysis (PCA) was used in **paper I & II** to correct for population structure.

4.6 Population genomics statistics

In **paper III**, we studied genomic signatures of selection in *Picea*. Tajimas' D, nucleotide diversity, allele frequencies, and linkage disequilibrium (r2), were calculated with VCFTOOLS (Danecek et al. 2011) in the 34 re-sequenced Norway spruce trees. Allele coalescence and time since the most recent ancestor was calculated in the 34 re-sequenced individuals with ARGweaver (Rasmussen et al. 2014) and BALLET (DeGiorgio et al. 2014).

4.7 Gene expression analyses

Total RNA was isolated according to the protocol by Chang, Puryear, and Cairney (1993) (Chang *et al.* 1993). For **paper I,** we estimated relative expression from the threseshold cycle using the 2ΔΔCT-method (Livak and Schmittgen 2001) by using the geometric mean of Phosphoglucomutase (Vestman *et al.* 2011) and elongation factor 1-α (ELF1α) (Arnerup *et al.* 2011) to normalize transcript abundance. For **paper IV**, quality controlled and trimmed illumina reads were aligned to the Norway spruce genome (v 1.0 gene models only) (Nystedt *et al.* 2013) using STAR default settings (Dobin *et al.* 2013). Unnormalized gene counts from STAR were used as an input to perform differential gene expression analysis in DESeq2 (Love *et al.* 2014) and gene co-expression network analysis in WGCNA (Langfelder and Horvath 2008) in R (R Core Team 2020).

5. Results and discussion

The main objective of this thesis was to understand how genetic variation in Norway spruce impacts disease resistance traits. Even though it is known that resistance traits in Norway spruce vary in response to *H. parviporum*, it is unknown if resistance in Norway spruce varies in response to different member the *H. annosum s.l.* species complex, which genes contribute to variation in these traits, how much they contribute and how they have evolved.

5.1 The genetic architecture of disease resistance to *Heterobasidion*

In **paper I**, we measured disease resistance to *H. parviporum* Rb175 in 466 different half-sib families that were part of the Norway spruce breeding program and correlated these traits with genomic variation in the mother trees to those half-sib families using GWAS.

In **paper II,** we measured the same resistance traits as in **paper I**, but this time in response to *H. annosum s.s.* Sä 16-4 in a slightly different population, where 226 half-sib families were overlapping with families from **paper I**, which allowed us to compare the genetic component of resistance to both species in the *H. annosum s.l.* species complex. In **paper II**, we show that resistance traits to these two closely related forest pathogens, considered to cause the same disease in their host, are not necessarily correlated in Norway spruce. When we performed individual GWAS for resistance traits to both pathogens separately, we encountered that the SNPs associated with either

pathogen were different, which is not surprising given that the resistance traits to *H. annosum s.s.* and *H. parviporum* were not correlated.

For both pathogens we found that resistance traits were polygenic, which is characteristic of quantitative disease resistance traits. In **paper I,** we found 11 SNPs significantly associated with resistance traits to *H. parviporum,* with relatively small contributions to the variation in the phenotype (3-5%). In **paper II**, no variants were significantly associated with the traits, so a suggestive threshold of $p < 1x10\text{-}5$ was used. After this threshold, we found 21 SNPs significantly associated with resistance traits to *H., annosum s.s*. These variants had relatively small contributions to the variation in the phenotype (4-6%) and were located in 7 different linkage groups. Therefore, our results suggest that the genetic architecture of disease resistance traits to *H. parviporum* and *H. annosum s.s.* is characterized by several genes with small effects, distributed in different locations in the genome. Importantly, the exomic probes used cover only ~39% of the predicted gene models in the spruce genome (Vidalis *et al.* 2018). Therefore, this is a likely representative, but still only a partial view of the genetic architecture of resistance traits to members of the *H. annosum s.l.* species complex.

5.2 The breadth of resistance in Norway spruce

An advantageous breeding objective in plants is to have resistance to multiple diseases at the same time (Wisser *et al.* 2011), and examples of this phenomenon in crops have been described before (Risterucci *et al.* 2003; Schweizer and Stein 2011; Wiesner-Hanks and Nelson 2016). For trees, this is an important trait, since they are expected to face multiple attackers during their life span (Tobias and Guest 2014). Specifically for Norway spruce and sympatric members of the *H. annosum* s.l. species complex, the concept of multiple-disease resistance is relevant. From a theoretical point of view, the nature of disease resistance to multiple pathogens could be based on the distance between the genes causing this effect. For example, unlinked genes can be effective against different diseases independently and provide resistance to multiple diseases in the organism. Also, clusters of linked genes, at the same genomic location, can be effective against different diseases. Otherwise, this can be observed in individual pleiotropic genes,

where the same gene contributes to resistance to multiple diseases (Wisser *et al.* 2011; Wiesner-Hanks and Nelson 2016; Nelson *et al.* 2018), as observed in **paper II** (see section 5.3).

Resistance to *H. parviporum* is not correlated to resistance to *Endoconidiophora polonica* (the fungus associated with the bark beetle (*Ips typographus*) (Skrøppa et al., 2015), and as we show in **paper II**, neither it is to *H. annosum s.s*., suggesting that at the organism level, multiple disease resistance is not there, or at least we are not able to detect it yet. Arguably, the method of measuring resistance in half-sib families, which was both used in (Skrøppa *et al.* 2015) and in **paper I & II** could have introduced variation that did not allow to see significant correlations. Likewise, another limitation of **paper II** was that the resistance traits were measured in different years and the different environment could have influenced the outcome of infections leading to a lack of correlation (Capador-Barreto *et al.* 2021). To understand better the cause for the observed results in **paper II,** I designed two additional inoculation experiments with the same fungal isolates: experiment #1 in greenhouse conditions (**paper IV**) and experiment #2, in three different seed orchards (in the field). In experiment #1 (**paper IV**) I decided to include *H. parviporum* Rb175 along with five different *H. annosum s.s.* isolates. There, I tested whether resistance to both pathogens was consistent in the same environment (greenhouse), after inoculation in ten different clonally propagated Norway spruce hosts. At 21 days post inoculation (dpi), there was a significant difference in lesion length between the five fungal isolates. Pairwise post-hoc comparisons revealed that this difference was between *H. annosum s.s.* isolates, and *H. parviporum* Rb175 was not significantly different from *H. annosum s.s.* Sä 16-4 in the greenhouse, under the same environment (Table 2). Therefore, lesion length in the 10 different evaluated clones was the same between these two pathogens.

Table 2. Differences in lesion length in Norway spruce between four *H. annosum s.s.* and isolates and *H. parviporum* Rb175 at 21 days post inoculation.

Pathogen isolate	Mean Lesion length	Standard Deviation	Significance group
H. annosum s.s. Rb_28-20	4.3	4.6	NA
H. parviporum Rb175	4.6	5.4	a
H. annosum s.s. 87087/8	5.4	6.9	a
H. annosum s.s. Sä 16-4	7.5	8.6	ab
H. annosum s.s. L12-1	7.6	6.0	b

In the field experiment (#2), I tested if (1) correlation of disease resistance traits in the same environment was also true in the field and (2) if different environments would affect this correlation (genotype-by-environment interactions). To do so, we infected branches in three different Norway spruce seed orchards, where the same genotypes were replicated several times. First, I confirmed that when measured under the same environment (in this case in the same seed orchard), resistance traits between the two pathogens tend to be correlated (Figure 6 A&B). This occurred only in two of three seed orchards evaluated, so under certain environments these traits are not correlated. Interestingly, for SWG some genotypes varied in response to *H. annosum* (698, 931, 887, 1171) depending on the seed orchard, while others varied in resistance to both pathogens (2026 and 696) (Figure 6C). For LL, the picture is similar, with the exception that genotype 931 varies much more for *H. parviporum*, and 969 does not vary at all for *H. annosum* (Figure 6D).

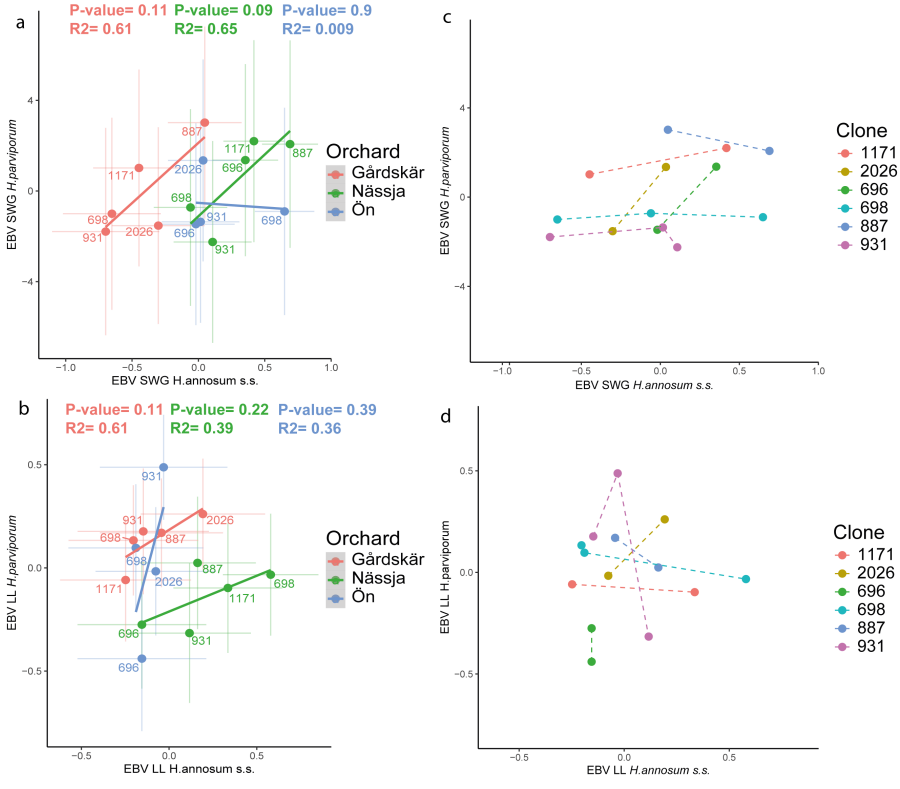


Figure 6. Relative resistance *H. parviporum* and *H. annosum* s.s**.** Estimated Breeding Values (EBV) in resistance traits to *H. annosum* s.s. are plotted on the horizontal axis and Estimated Breeding Values (EBV) in resistance traits to *H. parviporum* on the vertical axis. The relationship for relative resistance in the sapwood (SWG) is shown in figure A&C. and relative resistance in the bark (LL) in figure B&D. Numbers labels refer to the genotype ID. Pearson correlations (R^2 and P-values, and regression lines) between resistance, calculated per orchards are shown (A&B). Dashed lines connect EBV between orchards for the same clone (C&D).

To sum up, our results suggest that under the same environment (in the greenhouse and in the field) resistance to *H. annosum s.s.* and *H. parviporum* can be correlated. However, as seen in **paper II** and in experiment #2, the environment plays an important role in this correlation, since it seems that under a different environment, resistance can change in magnitude depending on the species of *H. annosum s.l.*, the trait measured, and the genotype of the tree (Figure 6). Genotype-by-environment interactions in disease resistance traits to *H. annosum s.l.* in Norway spruce should be

researched further, especially since genotype-by-environment effects have been detected for vitality scores in Scots pine in Sweden (Calleja-Rodriguez *et al.* 2019).

5.3 Novel gene models associated with resistance to *Heterobasidion*

In **paper I** we found that two of the variants significantly associated with LL were located in two gene models in the same genomic scaffold. These two gene models are a good example of the level of fragmentation of the Norway spruce genome, since in the initial genome assembly they were separated. However, they are actually one single gene that was originally isolated from lignin-producing Norway spruce suspension cultures (Koutaniemi *et al.* 2015). Transcriptome analyses suggest that this gene (from now on *PaLAC5*) is associated with the activation of stress associated lignin production (Laitinen *et al.* 2017). We also showed that *PaLAC5* was induced after inoculation with *H. parviporum* (**paper I**), and in response to *H. annosum s.s.* (**paper IV)**. Additionally, we confirmed this pattern by inoculating branches of saplings in the greenhouse and observed that *PaLAC5* was induced specifically by *H. parviporum* compared to wounding, and this induction was localized (**Paper I**). Hence, it is probable that *PaLAC5* expression is driven by specific cell types (such as PP, as suggested by Franceschi and collaborators (2000) (Franceschi *et al.* 2000)) in the bark adjacent to the inoculation site in response to *H. parviporum*. The LSZ is characterized by deposition of phenolics and suberin, and development of a discernible LSZ is crucial in stopping fungal invasions (Bodles et al., 2007; Solla, Tomlinson, & Woodward, 2002; Woodward et al., 2007). However, it needs to be proved that *PaLAC5* is involved in the LSZ formation and if genetic variation associated with *PaLAC5* influences the formation of the LSZ and therefore resistance to *H. annosum s.l.*

Additionally, in **paper II** we performed multi-trait associations with resistance traits for both *H. parviporum* and *H. annosum s.s.* to study if there were pleiotropic variants in the genome that could have an effect on both pathogens. The SNPs were classified as belonging to two main categories: (1) those with the same effect size direction for both pathogens (synergistic

pleiotropy) (Figure 7, upper-left and lower-right), and (2) those with opposite effect sizes, (antagonistic pleiotropy) (Figure 7, lower-left and upper-right). Interestingly, *PaLAC5* had a synergistic pleiotropic effect for LL in both pathogens. Therefore, this confirms that variation in *PaLAC5* is likely to play a role in resistance to both members of the *H. annosum s.l.* species complex. Genes with synergistic pleotropic effects are examples of how one single gene can provide multiple-disease resistance in trees. For breeding programs, these loci could be used as one "stone" to select trees with improved resistance against both enemies. In contrast, SNPs with antagonistic pleiotropic effects could explain why these pathogens have evolved to inhabit different niches when infecting conifers. One example is a secoisolariciresinol dehydrogenase-like gene (Figure 7, lower-right quadrant), which encodes for an enzyme involved in the production of matairesinol (Suzuki and Umezawa 2007). Matairesinol is a characteristic lignan synthesized in the reaction zone in the inner sapwood in response to infection by *H. parviporum* (Nagy *et al.* 2022) which inhibits the activity of extracellular enzymes produced by a *Heterobasidion* isolate *in vitro* (Popoff *et al.* 1975; Johansson *et al.* 1976). The role of variation in matairesinol synthesis in resistance to these two pathogens in the sapwood is an exciting research avenue.

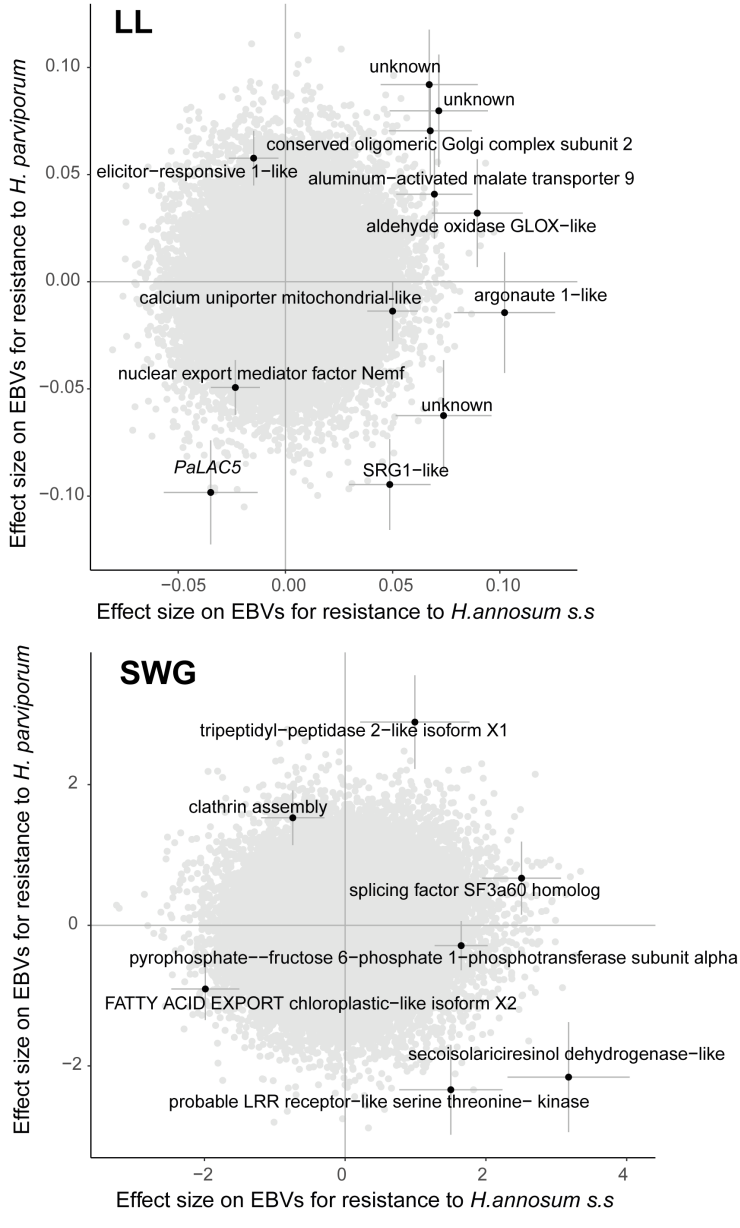


Figure 7. (**paper II**) Effect size of significant SNPs in the multitrait GWAS for estimated breeding values (EBVs) for resistance traits (LL, lesion length, SWG, sapwood growth) to *H. annosum s.s.* and *H. parviporum*. Dark points represent SNPs significant after the suggested threshold and the bars behind the standard error. EBVs for LL are in logarithmic scale.

In **paper IV,** we used transcriptomics to understand how different Norway spruce genotypes respond to different isolates of *H. annosum s.s.* There, we found several nucleotide binding leucin rich repeats genes (NLR) that are differentially expressed between genotypes, respond specifically to *Heterobasidion annosum s.s.* in comparison to wounding, and are correlated to changes in expression in the pathogen (Figure 8). The NLR protein family is a large family of immune receptors in plants with a central role in stress tolerance in conifers (Van Ghelder *et al.* 2019; Weiss *et al.* 2020; Tamborski and Krasileva 2020), and transcripts of these genes have been shown to accumulate after abiotic and biotic stress treatments in conifers (Fossdal *et al.* 2012; Kovalchuk *et al.* 2019; Van Ghelder *et al.* 2019). Therefore, it is possible that some of the NLR which respond specifically to *H. annosum s.s.*, and vary between Norway spruce genotypes, allowed the plants to recognize the presence of the pathogen and respond with appropriate and robust transcriptional programs to control the spread of the pathogen (Poland *et al.* 2009; Delplace *et al.* 2020). Given that an expansion of NLR genes has been proposed as a strategy used by long-lived trees, further research on perception of pathogens mediated by NLR is essential for understanding the mechanisms behind disease resistance in trees. Furthermore, because NLR have been used in resistance crop breeding for many years with varying levels of success (McDowell and Woffenden 2003), they have a high potential for resistance breeding in forestry.

5.4 A look in the past: how has natural selection shaped variation in resistance traits and genes?

In nature, many interactions between species are mediated by quantitative traits (just like between Norway spruce and *H. annosum s.l.*.) and therefore, they are central to understand co-evolution between species (Thompson 1999). The genome of Norway spruce has been shaped by natural selection and adaptation (Wang et al., 2020), and since pests and pathogens are a threat for tree survival and fitness, their role in the evolution of the Norway spruce genome is likely important, but understudied.

5.4.1 Difference in resistance between geographical origins

In **paper II**, we use the exome-captured genomic SNPs to attribute the progenies of Norway spruce to their geographical origin, which enabled us to show that resistance to *H. annosum s.s.* (but not to *H. parviporum*) in the bark follows a latitudinal cline. This is the first time that a difference between tree origins has been observed in the interaction between a conifer and *H. annosum s.l.* Bodles an collaborators (2007) tested this hypothesis in Sitka spruce, but did not find an effect (Bodles *et al.* 2007). Our results suggest that resistance to *H. annosum s.s.* might be locally adaptive or has trade-offs with locally adaptive traits (such as phenology or growth rate). Although it seems likely that trade-offs are driving the pattern we observe, further experiments are needed to confirm this observation.

Both in **paper I & II** we used kinship and population structure between trees to correct for the effect of family relationships and demographic processes. This type of corrections are routinely used to lessen the effect of demography in the significance of SNPs that are different between geographical clusters and involved in the architecture of the trait (Zhao *et al.* 2007; Milesi *et al.* 2019). Then, it is likely that some of the variants that we found to be significantly associated with resistance traits will not explain differences between geographical origins. In the future, the study of SNPs significantly associated with resistance traits and different between geographical clusters could enlighten the molecular mechanisms behind the pattern we observed.

5.4.2 Ancient evolution of a disease resistance associated gene in *Picea*

A typical gene in Norway spruce is expected to have low linkage disequilibrium (LD), large introns, and an excess of rare alleles due to the recent population expansion (Larsson *et al.* 2013; Nystedt *et al.* 2013). Interestingly, observations in the disease resistance associated gene *PaLAR3*, showed short introns, a considerably long LD block and an unusual allele dimorphism (Nemesio Gorriz et al., 2016). These striking features, together with the fact that variation in *PaLAR3* was originally identified using a SNP chip designed for variants in *P. glauca* (Lind *et al.* 2014; Nemesio-Gorriz *et*

al. 2016), suggested that *PaLAR3* was not evolving neutrally, or at least differently to the rest of the genome.

Indeed, in **paper III,** we demonstrated that *PaLAR3* has high Tajima's D values in comparison to the rest of the Norway spruce genome, which confirms that this gene has an excess of mid frequency alleles (Figure 8). Thereafter, we tested if the balanced SNPs were due to alleles being favoured differently across the geographical range of the species and therefore appeared to be "in balance" in Norway spruce, or whether heterozygote individuals were more frequent (overdominance). In the end, we rejected the two last hypotheses: different geographical clusters had even allele frequencies and homozygotes for allele A were more common than heterozygotes or homozygotes for allele B.

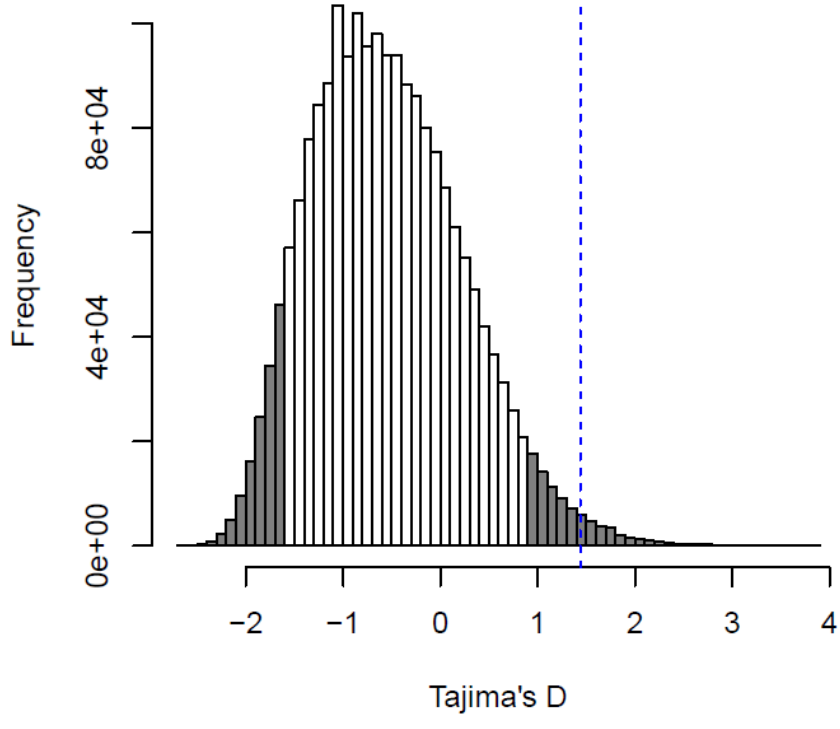


Figure 8. (**paper III**) Genome-wide estimate of Tajima's D in the 34 re-sequenced *Picea* abies trees. Blue dotted line represent value for the coding region of *PaLAR3* =1.43. Grey bars represent the 0.05 quantiles.

Because genes under balancing selection are often maintained for a long time, variants shared between closely related species are consider a hallmark signature of balancing selection (Karasov *et al.* 2014; Fijarczyk and Babik 2015). Hence, in **paper III** we studied variation in *LAR3* in three other spruce species and calculated the time since most recent common ancestor between the two alleles. Shared polymorphisms are frequent in *Picea* (Feng *et al.* 2018), and they have been reported repeatedly in nuclear loci between Norway spruce (*P. abies*) and White spruce (*P. glauca*) (Bouille and Bousquet 2005; Chen *et al.* 2010). In *LAR3,* we found shared polymorphisms together with an inconsistent phylogeny (Figure 9): *LAR3* in Black spruce (*P. mariana*) was the most similar to Norway spruce, but *P. glauca* and Norway spruce shared the largest number of polymorphisms (three in total). This variation pattern can be partly explained by the recurrent introgression events suggested in *Picea* (Sullivan *et al.* 2017; Feng *et al.* 2018).

Taking into account the estimate since the most recent common ancestor, it is likely that allele A is the ancestral state and that allele B appeared about 1.5 MYA, after the common ancestor of *P. abies*, *P. glauca* and *P. sitchensis* diverged from *P. mariana*. Since then, it is possible these alleles have been maintained by balancing selection in *P. abies*, and likely in *P. glauca* too, where a recent gene duplication has also been reported (Warren *et al.* 2015). However, additional tests are needed to confirm if *PgLAR3* is also under balancing selection*,* and if this gene has indeed been maintained in both species by balancing selection or by other processes such and incomplete lineage sorting, which could also explain the existence of shared polymorphisms in *Picea* (Feng *et al.* 2018).

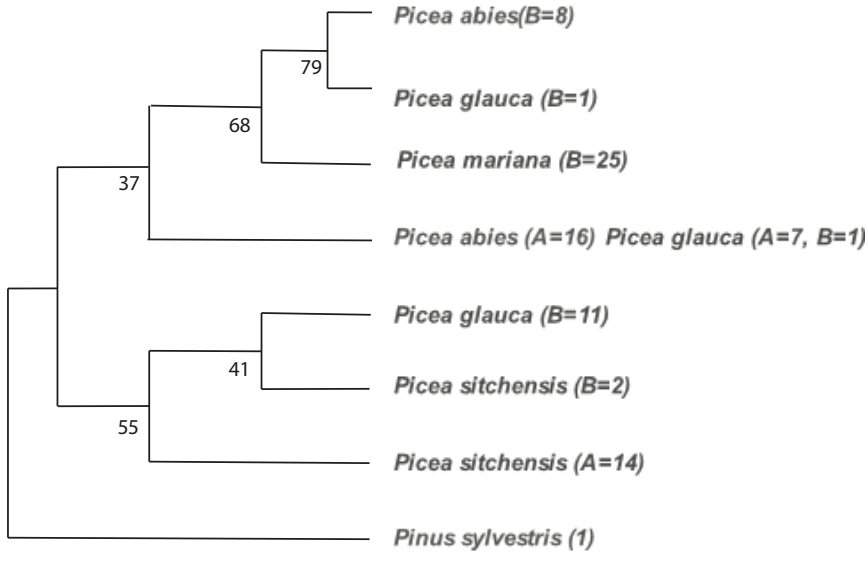


Figure *9*. (**Paper III**) Phylogenetic tree of *LAR3* in *Picea*. Tree is based on 537 positions (excluding indels) spanning introns and exons in *LAR3*. The percentage of trees in which the associated taxa clustered together after 100 bootstraps is shown next to the branches. The number of megagametophytes included in each branch is shown in parenthesis next to the taxa name, as well as the allele distribution based on the N175K non-synonymous substitution.

Because catechin, synthesized by *PaLAR3* (an its paralogs) is an effective defence strategy against different biotic stressors (Hammerbacher 2011; Hammerbacher *et al.* 2014, 2019; Nemesio-Gorriz *et al.* 2016), this gene is another example of diffuse disease resistance evolving under balancing selection (Huard-Chauveau *et al.* 2013; Karasov *et al.* 2014). Genes evolving under balancing selection such as *PaLAR3* involved in non-specific resistance have been described in tomato and Arabidopsis, and their mechanisms of evolution is thought to be ruled by trade-offs between growth and defence, rather than arms-race dynamics (Huard-Chauveau *et al.* 2013; Karasov *et al.* 2014).

Indeed, disease resistance is likely costly for plants, especially when it involves the synthesis of molecules like catechin, which are carbon rich molecules that demand carbon sources (Warren and Mackenzie 2001; Yu *et al.* 2018). For example, in different plant species, individuals with lower anthocyanin production (and therefore no flower pigmentation) generally

have higher fitness under favourable growing conditions while plants producing higher amounts of anthocyanin have a fitness advantage during drought stress (Warren and Mackenzie 2001). Thus, it is possible that variation in *LAR3* has evolved to regulate production of catechin and that allelic variation is maintained by trade-offs between growth and defence.

5.5 Regulation of gene expression in response to different *H. annosum s.s.* isolates depends on the host genotype.

Disease symptoms arise from interactions between diverse molecular pathways in both host and pathogen in an advantageous environment (Delplace *et al.* 2022). However, it is not known if Norway spruce regulates its responses differently depending on the virulence of pathogen isolates, and whether this modulation is dependent on the host genotype and disease progression. Thus, the overall objective of **paper IV** was to study if variation in virulence in *H. annosum s.s.* would induce different responses in Norway spruce.

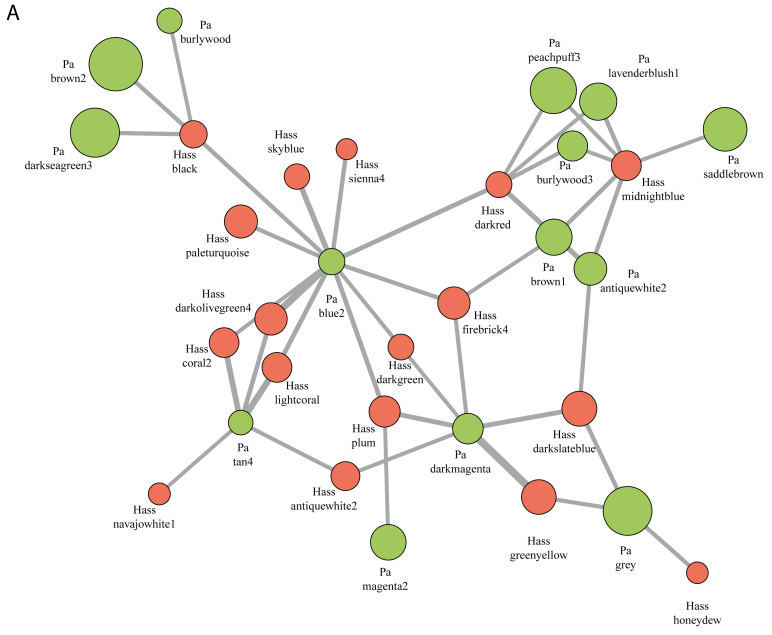
To do so, we used transcriptomics to understand how different Norway spruce genotypes would respond to different pathogen isolates. In general, variation in the host was the main driver of the (LL) extension. Likewise, 11495 genes (22.5% of the gene models in Norway spruce) were differentially expressed between host genotypes. Indeed, we could observe differences between the expression patterns in the three hosts we studied: The more resistant hosts (8590 and 0427) had a more robust response to all pathogen isolates in comparison to wounding, with a few and similar genes differentially expressed (Table 3). In contrast, host 1977, which was predicted to be relatively susceptible to *H. annosum* s.l. (Karlsson and Swedjemark 2006), allowed the longest lesions at 21 dpi, especially in response to *H. annosum* s.s. Sä16-4 and 87087/8, but less markedly to L12-1. Likewise, this host had many differentially expressed genes in comparison to wounding, which depended on the pathogen isolate (Table 3). Altogether, the more resistance hosts had a robust response to all three isolates, while the

more susceptible host genotype might differ in more than one component resulting in a less robust response to the three pathogen isolates.

Table 3 (**Paper IV**). Number of differentially expressed genes between host and pathogen combinations compared to wounding. dpi = days post inoculation.

	Pathogen (5 dpi)			Pathogen (21 dpi)		
Host	**Sä16-4**	**87087/8**	**L12-1**	**Sä16-4**	**87087/8**	**L12-1**
1977	122	11	8	4320	1550	19
8590	18	14	66	16	16	12
0427	39	16	36	45	23	57

Therefore, it is possible that a robust response allowed the more resistant clones to restrict the spread of all three *H. annosum* s.s. isolates. Robustness is a characteristic of QDR, where different components with low functional redundancy and a decentralized response allow plants to maintain their functions against external variation, such as pathogen virulence (Delplace *et al.* 2020). Using gene co-expression network analysis, we identified co-expression modules that correlated to gene expression in the pathogen, and were therefore likely involved in interaction to *H. annosum s.s.* (Figure 10A). The gene models in these modules were enriched in domains associated with NLR genes (Figure 10B). In fact, we also observed that most of these genes were downregulated in the more susceptible host 1977 in comparison to the other hosts. This led us to hypothesize that susceptibility may be related to a lack components in the recognition of infection by specific *H. annosum s.s*. isolates. This clearly warrants more detailed investigation in the future.



B

Module (# of genes)	log(Lesion length)	Host combinations: 0427 vs 1977	Host combinations: 8590 vs 1977	Host combinations: 8590 vs 0427	Pfam domains
brown2 (12212)	−0.59 (6e−08)	0.45 (7e−05)	0.37 (0.002)	−0.15 (0.2)	PF02984 Cyclin, C-terminal domain PF17207 MCM OB domain PF00091 Tubulin/FtsZ family, GTPase domain
peachpuff3 (3391)	−0.26 (0.03)	0.98 (3e−50)	−0.22 (0.07)	−0.98 (1e−52)	PF00931 NB-ARC domain PF13855 Leucine rich repeat PF01582 TIR domain
lavenderblush1 (705)	0.13 (0.3)	−0.27 (0.02)	−0.41 (4e−04)	−0.18 (0.1)	PF00657 GDSL-like Lipase/Acylhydrolase PF14368 Probable lipid transfer PF07993 Male sterility protein
saddlebrown (2192)	0.23 (0.06)	−0.98 (2e−54)	−0.99 (1e−61)	−0.49 (1e−05)	PF01582 TIR domain PF00931 NB-ARC domain PF13855 Leucine rich repeat
antiquewhite2 (321)	−0.096 (0.4)	−0.84 (4e−20)	0.58 (1e−07)	0.97 (4e−44)	PF00931 NB-ARC domain PF00560 Leucine Rich Repeat PF11204 Protein of unknown function (DUF2985)
burlywood3 (203)	0.31 (0.007)	−0.98 (1e−48)	−0.98 (7e−50)	0.95 (6e−37)	NB-ARC domain Protein of unknown function (DUF1365) TIR domain
burlywood (85)	0.43 (1e−04)	−0.3 (0.01)	−0.29 (0.01)	−0.014 (0.9)	PF00931 NB-ARC domain PF07103 Protein of unknown function (DUF1365) PF01582 TIR domain
darkseagreen3 (5694)	0.54 (1e−06)	−0.52 (3e−06)	−0.47 (3e−05)	0.038 (0.8)	PF08031 Berberine and berberine like PF00182 Chitinase class I PF00069 Protein kinase domain

Figure 10 (**Paper IV**)**.** Gene modules differently expressed between hosts are correlated with pathogen gene modules. A) Correlation of host gene expression modules (green) and pathogen gene expression (orange). The size of the nodes reflects the size of the modules and the thickness of the edges the strength of the correlation between modules. B) Correlation between selected modules and traits based on supplementary material Figure S8. Weighted Pearson correlation values per combination and Student asymptotic p-value in parenthesis are shown, together with the 3 most enriched Pfam domains per module. The number of genes per module are in parenthesis after the name of the module. Colours from red to blue correspond to the correlation coefficient and grey squares have a P value > 0.01.

6. Conclusions and future perspectives

Altogether, the results of this thesis signify a leap in the understanding of the genetic variation in disease resistance traits in Norway spruce. Furthermore, they contribute to the understanding of the genetic and molecular mechanisms of disease resistance in trees and have a potential to be applied in the Norway spruce breeding program in Sweden. The main conclusions of this thesis are:

- Resistance to *H. annosum s.s.* and *H. parviporum* is quantitative, under genetic control and associated with variation in some genes with involvement in defence responses (**paper I & II**).

- *PaLAC5* is associated to resistance traits against *H. annosum s.s.* and *H. parviporum* and expressed in the bark adjacent to the infection site in response to *H. parviporum* (**paper I & II).**

- Correlation of resistance traits in Norway spruce against *H. annosum s.s.* and *H. parviporum* is dependent on different genetic mechanisms of resistance and genotype-by-environment interactions (**paper II**).

- Resistance in bark is significantly affected by the geographic origin of the trees following a latitudinal cline to *H. annosum s.s.,* but not in *H. parviporum* (**paper II**).

- Resistance traits to *H. annosum s.s.* and *H. parviporum* are associated with genomic variants with antagonist and synergistic pleiotropic effects (**paper II**).

- *PaLAR3* has an excess of mid-frequency variants compared to the rest of the Norway spruce genome, which are likely maintained by balancing selection and not overdominance or local adaptation (**paper III**).

- *PaLAR3*A is likely the ancestral state and allele B appeared after the common ancestor of Norway spruce and its North American congeners *P. glauca* and *P. sitchensis* diverged from *P. mariana*. Under this scenario, both alleles would have been maintained by balancing selection in *P. abies* (**paper III**).

- Variation in both Norway spruce and *H. annosum s.s.* influence the size of the lesions in the bark (**paper IV**).

- Norway spruce genotypes with relatively high resistance to *H. annosum s.l.* are likely to respond in similar ways to different *H. annosum s.l.* isolates, whereas more susceptible genotypes will respond differently depending on the isolate virulence (**Paper IV**).

- Norway spruce genotypes vary in the expression of NLRs induced after infection with *H. annosum s.s* and these NLRs are correlated with gene expression in the pathogen (**Paper IV**).

A clear contribution of this thesis to the Swedish Norway spruce breeding program is the association between disease resistance traits and SNPs in **paper I & II,** which can be used in genomic selection models**.** Genomic selection is feasible in trees, with the attractiveness of reducing time in the breeding cycle (Resende et al. 2012b). This method could be used for traits such as growth (Resende et al. 2012b; Chen et al. 2018a), and resistance to insect pests and pathogens (Resende et al. 2012a; Stocks et al. 2019; Lenz et al. 2020). Given that breeding for polygenic traits such as growth is feasible

with genomic selection in Norway spruce (Chen *et al.* 2018a), it is likely that quantitative disease resistance traits, like resistance to *H. annosum s.l.* in Norway spruce, can also be employed. However, since population- and environment-specific genome predictions will drive the application of genomic selection in tree breeding (Resende *et al.* 2012; Chen *et al.* 2018a), the effect sizes of the variants we identified in **paper I & II** should be evaluated under different environments in the future. This is especially relevant when it comes to multiple disease resistance, since according to this thesis correlation of disease resistance traits can vary depending on the environment (**paper II** and seed orchard inoculations).

Even though genomic selection is an attractive breeding strategy, it might take some years before it is fully operational in Sweden. Today, vitality scores are considered, and it is therefore reasonable to expect that a component of disease resistance is present in the breeding population. However, branch inoculations as performed in this thesis can be used to survey the disease resistance of "plus trees" in seed orchards. If significantly susceptible genotypes are found, their frequency in the orchards could be decreased, or they could be replaced with other genotypes. Removing susceptible trees from the population could improve the health composition of reforestation material in the future. The benefits and costs of such strategy should be modelled.

Both in **paper II & III,** we found evidence that points to the existence of trade-offs between disease resistance and other traits, such as phenology. In the future, it is essential to understand if trade-offs are present and under which conditions they are more explicit.

As I have highlighted above, the effect environment in the control of disease resistance traits is an important variable that I did not study systematically in this thesis, but its relevance became evident in every project. Because the time interval between selection and harvest is so long in Sweden and changes in the climate are expected in the coming century, it is crucial to understand the effect of future climatic conditions in disease resistance traits.

The understanding that we have today about the regulation and evolution of *PaLAR3* (**paper III)** is the result of many years of work by different research groups (Danielsson *et al.* 2011; Hammerbacher *et al.* 2014; Lind *et al.* 2014; Nemesio-Gorriz *et al.* 2016; Dalman *et al.* 2017; Edesi *et al.* 2021). Throughout this thesis I have identified several gene candidates, which function as the foundation stones for future studies to broaden the understanding of the molecular regulation and evolution of disease resistance in trees like it has occurred with *PaLAR3*. Examples are *PaLAC5* (**paper I**), the gene models containing pleiotropic SNPs (**paper II**) and the genes differentially expressed in **paper IV.**

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Popular science summary

Throughout their million-year history, trees have evolved strategies to fight enemies and survive. These strategies have been shaped by natural selection and are reflected in their genes today. Tree planting is a priority for governments, but if humans select which trees are planted in a forest, there is a risk that trees will lack gene variants important for disease resistance. Norway spruce is a characteristic species in the Swedish landscape and one of the most important trees for the forest industry. Therefore, the overall aim of this thesis was to study the genetic variation of resistance traits in Norway spruce to *Heterobasidion parviporum* and *Heterobasidion annosum s.s.*, two fungal pathogens causing root and stem rot in conifers.

In the first two papers, we studied associations between variation in the genomes of the trees and their resistance to both fungal pathogens. Through these associations, we discovered several loci with relatively small influence associated with resistance to each pathogen. This finding is important to understand how many genes and how much each of them contributes to disease resistance in the host. Correlation between resistance traits against these two pathogens was dependent on the environment they were measured. Additionally, we found loci associated with resistance against both pathogens. These loci are examples of genes providing resistance to more than one pathogen in trees.

In the third paper, we investigated signatures of natural selection in *PaLAR3*. This gene is associated with resistance to different enemies in Norway spruce. Analyses of this gene demonstrated that variation in *PaLAR3* has been maintained by balancing selection in Norway spruce. Genes evolving under balancing selection usually have few alleles at even frequencies and are stable for a long time. Indeed, it seems that this process

started before Norway spruce isolated from the North American white spruce (*Picea glauca*).

Lastly, in the fourth paper, we studied resistance in the bark in ten Norway spruce genotypes varying in resistance, inoculated with five *Heterobasidion* isolates varying in their aggressiveness. Both host and pathogen influenced the size of the lesions in the bark. We analyzed how much genes were expressed in Norway spruce in response to the pathogen to show that Norway spruce genotypes with relatively high resistance had a consistent reaction in response to different pathogens. The consistent response included the expression of genes involved in recognition of the pathogen. In contrast, in a more susceptible host, the response depended on the aggressiveness of the pathogen.

Overall, the thesis advances the knowledge on disease resistance in Norway spruce. This knowledge will support the Swedish Norway spruce breeding program decision making in selecting healthier trees in the future.

Populärvetenskaplig sammanfattning

Träd har utvecklat strategier för att bekämpa fiender och överleva under miljoner år. De här strategierna har formats av det naturliga urvalet och återspeglas i deras gener idag. Att plantera skog prioriteras av många styrande organ, men det finns en risk att träd som valts ut av människor saknar alleler som är viktiga för resistens mot patogener. Men om människan väljer ut vilka träd som planteras i en skog baserat på enbart tillväxt finns det en risk att träden i skogen saknar gener eller alleler som är viktiga för trädens förmåga att försvara sig mot sjukdomar, s.k. sjukdomsresistens. Gran dominerar i det svenska skogslandskapet och är ett av de viktigaste trädslagen för skogsindustrin. Därför var det övergripande syftet med denna avhandling att studera den genetiska variationen som kontrollerar resistensegenskaper mot *Heterobasidion parviporum* och *Heterobasidion annosum* s.s., två arter av rotticka som båda orsakar rotröta i gran.

Först studerade vi det statistiska sambandet mellan genetisk variation i granar och deras uppmätta resistens mot de båda arterna av rotticka. Med statistiska associationsmetoder identifierade vi flera, hittills okända, alleler av gener i gran associerade med resistens mot de olika arterna av rotticka. Varje genvariant bidrar relativt lite till resistensnivån men tillsammans kan de ge bättre resistens mot rotticka. Den statistiska korrelationen mellan resistenserna mot de olika rottickearterna berodde på miljön testet utfördes i, men analyserna identifierade ändå flera genvarianter som associerar med resistens mot båda arterna av rotticka, dvs gener som kan ge träden resistens mot flera sjukdomar.

I den tredje studien studerades selektionsmönster i *PaLAR3*. Den här genen är associerad med försvar mot olika skadesvampar i gran. Genomiska analyser visade att variation i *PaLAR3* i gran sannolikt har upprätthållits genom balanserande selektion. Att en gen är under balanserande selektion

betyder att den har förhållandevis få alleler med relativt jämna (balanserade) frekvenser som är stabila under lång tid. Resultaten tyder på att *PaLAR3* var under balanserande selektion innan gran och den nordamerikanska vitgranen separerade från varandra och blev olika arter.

Slutligen studerades resistens i barken i tio kloner av gran med varierande resistens. De inokulerades (smittades) med fem rottickeisolat som varierar i hur aggressiva de är när de infekterar träd. Både granklonen och rottickeisolatet påverkade hur stora nekroser som bildas i barken. Vi jämförde hur aktiva olika gener var i barken i granklonerna och rottickeisolaten i det här försöket. Det visade att grankloner med relativt hög resistens reagerade på ett robust sätt, bland annat med hög aktivitet av gener involverade i igenkänning av patogener. Däremot berodde genaktiviteterna i den mer mottagliga granklonen på hur stora nekroser rottickan orsakade.

Den här avhandlingen ökar kunskaperna om sjukdomsresistens i gran. Kunskaperna kommer att stödja det svenska granförädlingsprogrammet i arbetet med att välja bättre träd för framtiden.

Acknowledgements

Undoubtedly this PhD has been the biggest task I have had to complete in my life. Spending time in nature and being around so many special people made this journey so much nicer. I feel incredibly fortunate to have spent these years working in such an interesting topic. When I look back, I realize how much I have grown as a scientist and as a person. And for this, I must thank my main supervisor **Malin**. Thank you so much for believing in me in all the moments I did not. You have been extremely encouraging and kind. I have learned so much about trees with you. Also, you have given me personal advice that I will always be grateful for. Tack så mycket! Thanks also to **Jan, Pelle** and **Eva** for your support.

Additionally, I want to thank all my colleagues at the department of Forest Mycology and Plant Pathology, especially **Katta, Maria J,** and **Matilda.** You are amazing at your job. Many of the experiments we performed would have not been possible without you. Thanks to all the previous and current PhD students too. You made lunch break, coffee (thanks also to Per and Pernilla, lol), *fika* and after-work so much fun. **Laura B,** you are the best friend, colleague, and *partner in crime* anyone could wish for. I will always be thankful for your support in the good and the bad times. I am certain that the bond we built in Uppsala will last forever. A word of appreciation goes to all my collaborators and co-authors too.

In Sweden I have made many friends who have inspired me and been with me in the most difficult moments. You know who you are. Thank you so much for being there when I needed you the most. I will be forever grateful!!! I also want to thank you, **Rob**. Your love and support have been unconditional, particularly during the last months where I was under so much

pressure. You are incredible and I feel fortunate to be by your side. I love you so much amor.

Finally, I want to thank all my friends and family in Colombia. Desde la distancia he sentido el apoyo constante de todos ustedes. ¡Muchas gracias! A mis padres, **Martha** y **Luis D**, estoy inmensamente agradecido. Para los tres ha sido difícil estar tan lejos durante estos años, pero yo siempre los he sentido cerca. En cada momento de felicidad por un logro alcanzado y los momentos de frustración ustedes han estado allí, incondicionalmente. Gracias por los consejos, su comprensión y el amor que siempre me han brindado. Los quiero mucho.

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Received: 7 October 2019 | Revised: 21 March 2020 | Accepted: 28 March 2020

DOI: 10.1111/pce.13768

ORIGINAL ARTICLE

Plant, Cell & Environment WILEY

Association genetics identifies a specifically regulated Norway spruce laccase gene, *PaLAC5*, linked to *Heterobasidion parviporum* resistance

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Funding information
Swedish Foundation for Strategic Research, Grant/Award Number: RBP14-0040; The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning, Grant/Award Number: 2017-0040; VINNOVA, Grant/Award Numbers: 2015-02290, 2016-00504

Abstract

It is important to improve the understanding of the interactions between the trees and pathogens and integrate this knowledge about disease resistance into tree breeding programs. The conifer Norway spruce (*Picea abies*) is an important species for the forest industry in Europe. Its major pathogen is *Heterobasidion parviporum*, causing stem and root rot.

In this study, we identified 11 Norway spruce QTLs (Quantitative trait loci) that correlate with variation in resistance to *H. parviporum* in a population of 466 trees by association genetics. Individual QTLs explained between 2.1 and 5.2% of the phenotypic variance. The expression of candidate genes associated with the QTLs was analysed in silico and in response to *H. parviporum* hypothesizing that (a) candidate genes linked to control of fungal sapwood growth are more commonly expressed in sapwood, and; (b) candidate genes associated with induced defences are respond to *H. parviporum* inoculation. The Norway spruce *laccase PaLAC5* associated with control of lesion length development is likely to be involved in the induced defences. Expression analyses showed that *PaLAC5* responds specifically and strongly in close proximity to the *H. parviporum* inoculation. Thus, *Pa*LAC5 may be associated with the lignosuberized boundary zone formation in bark adjacent to the inoculation site.

KEYWORDS

genome-wide association study (GWAS), lignosuberized boundary zone, mitochondrion, sapwood, secretory and endosomal trafficking pathways, suberin, TOM40

1 | INTRODUCTION

The importance of trees and forests for sustaining terrestrial life and biodiversity can probably not be exaggerated (Petit & Hampe, 2006).

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Pathogen and pest attacks on trees negatively impact the health and biodiversity of native forest ecosystems as well as forest plantations, which can have large economic, ecological and societal consequences (Cubbage, Pye, Holmes, & Wagner, 2000; Garbelotto & Gonthier, 2013; Pautasso, Schlegel, & Holdenrieder, 2015; Woodward, Stenlid, Karjalainen, & Hüttermann, 1998). Therefore, it is important to increase the understanding of interactions between the tree and a pathogen in order to incorporate traits that confer to increased resistance into forest tree breeding programs.

Norway spruce [*Picea abies* (L.) Karst.] is economically important for the forest industry in Europe. Its major pathogens are fungi in the species complex *Heterobasidion annosum* sensu lato (s.l.), which causes stem and root rot in Norway spruce and several other conifer tree species (Garbelotto & Gonthier, 2013; Woodward et al., 1998). Under natural conditions, airborne spores of *H. annosum* s.l. can infect stumps created after harvesting and thinning operations. Once the stump is infected, surrounding trees or stumps can be infected by secondary spread when *H. annosum* s.l. mycelium enters neighbouring trees through root grafts and contacts (Oliva, Bendz-Hellgren, & Stenlid, 2011; Redfern & Stenlid, 1998). In Norway spruce, resistance to the spruce-infecting congener *Heterobasidion parviporum* is quantitative in its nature (Arnerup, Swedjemark, Elfstrand, Karlsson, & Stenlid, 2010; Chen et al., 2018; Karlsson & Swedjemark, 2006; Steffenrem, Solheim, & Skrøppa, 2016), and classical interval mapping-based quantitative trait locus (QTL) analysis for resistance to *H. parviporum* identified 13 QTL linked to host resistance (Lind et al., 2014). *PaLAR3*, on the QTLs associated with control of fungal spread in the sapwood, has been validated and the function of the variation at the locus described (Nemesio-Gorriz et al., 2016).

A feature that Norway spruce has in common with all tree species is that a large fraction of the biomass is invested in the sapwood in the trunk (Petit & Hampe, 2006). The primary function of the sapwood is to transport water and nutrients to the crown and it is dominated by dead cells that have a limited capacity to respond to biotic or abiotic stress (Johansson & Theander, 1974; Oliva et al., 2015; Shain, 1971). To protect the sapwood, the trunk of a tree is clad in an impermeable barrier, bark. The term "bark" commonly refers to all tissues external to the vascular cambium of trees. The outer bark is highly suberized and lignified, making it extremely resistant to mechanical and chemical degradation. Only a few pathogenic microorganisms are capable of directly penetrating the outer bark (Lindberg & Johansson, 1991). Therefore, a common mode of entry for fungi that cause stem cankers and decays is via mechanical wounds, exposing the cortex, secondary phloem tissues or the xylem (Woodward & Pocock, 1996). The speed at which the tree is able to seal off the tissues exposed by wounding with wound periderm is critical in avoiding damaging infections and subsequent loss of water transport capacity. The process to heal the bark begins with rapid necrosis of cells closest to the wound or progressing infection. It then continues with programmed death of cells adjacent to the necrosis, forming the lignosuberized boundary zone (LSZ), and de-differentiation of cells next to the LSZ followed by differentiation of the wound periderm (Bodles, Beckett, & Woodward, 2007; Mullick, 1977; Woodward, Bianchi, Bodles, Beckett, & Michelozzi, 2007).

The trait control of lesion length extension (LL, with reported heritability values of 0.14–0.33) is measured as the size of the discernible necrosis cells closest to the wound or progressing infection (Arnerup, Lind, Olson, Stenlid, & Elfstrand, 2011; Chen et al., 2018; Steffenrem et al., 2016). It could be argued that LL provides a measure of how the induced defences and wound healing responses interact to control the spread of the necrotrophic pathogen (Arnerup et al., 2011; Chen et al., 2018; Danielsson et al., 2011; Lind et al., 2014; Steffenrem et al., 2016). The trait control of fungal spread in the sapwood (fungal sapwood growth, SWG) can be considered to provide a measure of how well the combination of constitutive defences and the induced defence responses in the parenchymatic cells can control the spread of *H. parviporum* in the exposed sapwood (Johansson & Stenlid, 1985; Oliva et al., 2015). The narrow-sense heritability of SWG has been estimated to vary between 0.11 and 0.42 depending on the material studied (e.g., experimental cross, natural population) (Arnerup et al., 2010; Chen et al., 2018).

To date, the main focus of practical breeding in Norway spruce has been on climatic adaptation, growth and wood quality traits (Skrøppa, Solheim, & Steffenrem, 2015). In contrast, breeding for replantation material with improved resistance to *H.annosum* s.s. and *H. parviporum* is an overlooked objective because of limited information about genetic variation in resistance to these pathogens and the lack of reliable selection techniques (Skrøppa et al., 2015). There are, however, clearly sufficient phenotypic and genetic variation for resistance to *H. parviporum* in Norway spruce to allow for breeding (Arnerup et al., 2010; Chen et al., 2018; Karlsson & Swedjemark, 2006; Steffenrem et al., 2016), and no adverse correlations between resistance to *H. parviporum* and growth or wood properties traits (Chen et al., 2018; Steffenrem et al., 2016). Hence, the selection for *H. parviporum* resistance in breeding programmes could lead to considerable gain without compromising other breeding achievements (Chen et al., 2018).

To gain a deeper understanding of the heritability and genetic architecture of, for example, disease resistance traits, including the number, location, effect and nature of the loci involved, quantitative and molecular genetic approaches can be used to analyse the relationships between DNA polymorphism and phenotypic variation (Bartholomé et al., 2016; Neale & Savolainen, 2004). The two main approaches to detect QTLs: Interval mapping (IM) in experimental crosses or linkage disequilibrium (LD) mapping, commonly known as genome-wide association studies (GWAS) (Neale & Savolainen, 2004). GWAS, relying on historical recombination in the mapping population, overcomes the limited resolution of IM in experimental crosses (Baison et al., 2019; Neale & Savolainen, 2004). If enough markers can be analysed, this should be especially advantageous in conifers that have particularly short average distances of maintained LD, often even confined within genes (Namroud, Guillet-Claude, Mackay, Isabel, & Bousquet, 2010). The effects of LD are also influenced by the extreme physical distances separating genes in conifers (Nystedt et al., 2013).

It is likely that the Norway spruce genome harbours additional, yet undetected loci, to the 13 QTLs already identified by (Lind et al., 2014) controlling resistance to *H. parviporum* (Chen et al., 2018; Hall, Hallingbäck, & Wu, 2016). Identification of further loci would support the initiation of a breeding programme for the resistance to the pathogen in Norway spruce and, just as importantly, improve the understanding of the interactions between trees and necrotrophic pathogens. The short maintained LD and the polygenic nature of the traits controlling resistance suggest that GWAS could be a powerful method to identify further QTL regions associated with *H. parviporum* resistance in Norway spruce. Consequently, in this study, we aimed to identify Norway spruce loci that correlate with variation in resistance to *H. parviporum* in a population of 466 Norway spruce trees by GWAS. We identified candidate genes associated with the QTLs and analysed the expression patterns of the candidate genes in response to *H. parviporum* hypothesizing that (a) candidate genes linked to the SWG trait would be expressed in sapwood while candidate genes linked to LL are expressed in more peripheral tissues, and; (b) candidate genes that are part of the induced defence are induced in response to *H. parviporum* inoculation.

2 | MATERIALS AND METHODS

2.1 | Phenotyping of resistance traits in the progeny of 466 Norway spruce mother trees

We used the currently available largest Norway spruce resistance phenotyping dataset to perform the GWAS. The material, inoculation method and genetic analyses are described in detail in (Chen et al., 2018). On average ten 2-year-old, open-pollinated progenies derived from 466 tested plus trees in the Swedish breeding population were inoculated with *H. parviporum* Niemelä & Korhonen strain Rb175. A wooden dowel colonized by *H. parviporum* was fixated at a wound on the stem of the plant with Parafilm. The inoculated plants were kept under ambient light and temperature in the forest tree nursery and harvested 21 days post-inoculation. The induced defence responses (LL) in the phloem and inner bark were estimated by measuring the discernible lesion spread upwards and downwards from the edge of the inoculation point on the inside of the bark. SWG was estimated using established protocols (Arnerup et al., 2010; Stenlid & Swedjemark, 1988) (Table 1). The seedlings were cut up into five mm discs and placed on moist filter papers in Petri dishes. Plates were incubated in darkness under moist conditions at 21°C for 1 week to induce conidia formation. Thereafter, the presence or absence of *H. parviporum* conidia on each individual disc was determined under a stereomicroscope. For each seedling, the sum of the discs where conidia were observed multiplied by 5 (mm) was noted as SWG. Plates where no conidia could be observed on the discs, the inoculation point and on the inoculation plug, and that showed total lesion length of 2 mm or shorter, were treated as inoculation failures and were discarded (Lind et al., 2014). Chen et al. (2018) reported narrow-sense heritability values of 0.33 and 0.42, respectively, for LL and SWG and moderate phenotypic (0.48) and genetic (0.47) correlations between LL and SWG in this material.

TABLE 1 Summary statistics of the phenotype data used in the trait-marker association study (Details can be found in Chen et al. (2018))

Inoculation study	Acron.	Unit	N[a]	Mean
Diameter[b]	D	mm	4,628	4.0
Lesion length[c]	LL	mm	4,547	7.6
Fungal growth[d]	FG/SWG	mm	4,554	32.5
Vitality[e]	Vitality	Classes	4,376	1.9

[a]*N*: total number of progenies with valid recording of the trait.
[b]Diameter of the progenies at the inoculation site.
[c]Length of the necrotic lesion in the phloem and inner bark.
[d]Fungal growth in the sapwood of the progenies.
[e]Vitality of the progenies where score 1 was given to fully vital and worst score 3 was given to plants showing a pronounced loss of vitality.

2.2 | Norway spruce genotyping and SNP annotation

Dormant buds were collected from each of the mother trees. Total genomic DNA was extracted from the buds, using the Qiagen Plant DNA extraction kit (Qiagen, Hilden, Germany), and the DNA was quantified using the Qubit® ds DNA Broad Range (BR) Assay Kit (Oregon, USA). The generation and evaluation of exome capture for Norway spruce are described elsewhere (Vidalis et al., 2018). Sequence capture on the mother tree DNA was performed using 40,018 previously evaluated diploid probes (Baison et al., 2019; Vidalis et al., 2018). Probe design and sequence capture were done by RAPiD Genomics (Gainesville, FL, USA). In brief, Illumina sequencing compatible libraries were amplified with 14 cycles of PCR and the probes were then hybridized to a pool comprising 500 ng of eight equimolarly combined libraries following Agilent's SureSelect Target Enrichment System (Agilent Technologies). These enriched libraries were then sequenced to an average depth of 15x using an Illumina HiSeq 2,500 (San Diego, USA) on the 2 × 100 bp sequencing mode.

Read mapping and initial variant calling as well as the recalibration of the quality of SNP calling were then applied to filter the raw variants, described in detail in Baison et al. (2019). In brief, the variant calling was made using GATK HaplotypeCaller v.3.6 as per the best practices protocol (Auwera et al., 2013) in gVCF output format. To increase accuracy, hard filters in the form of minor allele frequency (MAF) and "missingness" of <0.05 and >20%, respectively, were then performed on the final dataset.

3 | GWAS

The LASSO model as described by Li et al. (2014) was applied to the *H. parviporum* resistance trait data for the detection of QTLs.

The LASSO model:

$$\min_{(\alpha_0,\alpha_j)} \frac{1}{2n}\sum_{i=1}^{n}\left(y_i-\alpha_o-\sum_{j=1}^{P}x_y-\alpha_j\right)^2+\lambda\sum_{j=1}^{P}\alpha_j, \quad (1)$$

where y_i is the estimated breeding values (EBV) of an individual i (i = 1,...,n; n is the total number of individuals) for each trait, α_0 is the population mean parameter, x_{ij} is the genotypic value of individual i and marker j coded as 0, 1 and 2 for three marker genotypes AA, AB and BB, respectively, α_j is the effect of marker j (i = 1,...,n; n is the total number of markers) and λ (>0) is a shrinkage tuning parameter. A fundamental idea of LASSO is to utilize the penalty function to shrink the SNP effects towards zero, and only keep a small number of important SNPs that are highly associated with the trait in the model. The stability selection probability (SSP) of each SNP being selected to the model was applied as a way to control the false discovery rate and determine significant SNPs (H. Gao et al., 2014; Li & Sillanpää, 2015). For a marker to be declared significant, an SSP inclusion ratio (Frequency) was used with an inclusion frequency of all traits. This frequency inferred that the expected number of falsely selected markers was less than one, according to the formula of Bühlmann, Kalisch, and Meier (2014). Population structure was accounted for in all analyses by including principal components based on the genotype data as covariates into the model (Baison et al., 2019). An adaptive LASSO approach (Baison et al., 2019; Zou, 2006) was used to determine the percentage of phenotypic variance (PVE) (H^2_{QT}) of all the QTLs. The analyses were all performed in RStudio (Team, 2015).

3.1 | Identification of candidate genes associated with the QTLs

To assess putative functionality of SNPs with significant associations, a gene enrichment analysis of putative genes and their associated orthologs was performed against the *P. abies* v1.0 genome (http://congenie.org), collecting PFAM and GO term annotations and *Populus* and *Arabidopsis* orthologues. The position of the detected QTLs in Norway spruce genome was estimated by searching an ultra-dense genetic map (Bernhardsson et al., 2019) for markers derived from the same probes as the SNP markers holding the QTLs, identified based on tblastn sequence homology for the SNP array sequences in the Lind et al. (2014) study, as described by (Bernhardsson et al., 2019).

Information on the expression pattern of the putative candidate genes associated with the QTL, in the Norway spruce clone Z4006 (the clone sequenced in Nystedt et al. (2013)) and in wood, were collected from three sources. Firstly, expression data were downloaded from the publicly available *P.abies* exAtlas (https://www.congenie.org) and NorWood v1.0 (http://norwood.congenie.org) databases, respectively. Both these databases are comprised of expression profiles from approximately 50-year-old ramets of the genotype "Z4006." Then, we examined an RNAseq study of bark and phloem samples harvested at seven dpi proximal (0–5 mm from the wound) and distal to the inoculation site (10–15 mm away from the wound) from two Norway spruce genotypes (S21K0220126 and S21K0220184) inoculated with *H. parviporum* (Chaudhary et al., submitted manuscript). In brief, two-year-old branches on clones of S21K0220126 and S21K0220184 were inoculated and sampled as described above using wounding as a control. A total RNA from three biological replicates of each clone per treatment were sequenced on the Illumina HiSeq 2500 at the SNP&SEQ Technology Platform (SciLifeLab, Uppsala). Quality filtering was done using Nesoni 0.97 (http://www.vicbioinformatics.com/nesoni-cookbook/index.html#). Differential gene expression was identified using the Tophat-cufflinks pipeline (Trapnell et al., 2012, 2014; Trapnell et al., 2013) and the "*P. abies* v1.0-all-cds.fna" gene catalogue as a reference (Chaudhary et al., submitted manuscript).

3.2 | Branch inoculation with *H. parviporum*

We performed an inoculation experiment on six-year-old grafted cuttings of the Norway spruce genotype S21K7820222. Branches on healthy-looking potted plants were inoculated with wooden dowels colonized by *H. parviporum* Rb175 fixated to a wound on a two-year-old branch with Parafilm. Control treatment branches were wounded and covered with Parafilm. The inoculated plants kept at ambient light and temperature conditions in a greenhouse. At 7 days post-inoculation (dpi), bark surrounding the wounds and inoculation sites were cut into two sections and samples were collected at the inoculation site 0–5 mm around the wound and distal to the inoculation site 10–15 mm from the wound. The bark samples were frozen separately in liquid nitrogen and stored at −80°C until further use.

3.3 | Quantitative PCR analysis of expression patterns in response to H. parviporum inoculation

The total RNA was isolated according to the protocol by Chang, Puryear, and Cairney (1993). To eliminate genomic DNA contamination, samples were treated with DNase I (Sigma-Aldrich) according to the manufacturer's instructions. RNA integrity and quantity were analysed by using the Agilent RNA 6000 Nano kit (Agilent Technologies Inc.). The 1 μg of total RNA was reverse transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad) in a total reaction volume of 20 μl according to the manufacturer's instructions, followed by a two-fold dilution of the cDNA and storage at − 20°C.

Quantitative PCR (qPCR) reactions were performed with the SsoFast™ EvaGreen® Supermix (Bio-Rad) according to the instructions in the manual, using 0.3 μM of each primer (Table S1 in Data S1) and Norway spruce cDNA equivalent to 25 ng of total RNA. The qPCRs were carried out in an iQ5™ Multicolor Real-Time PCR Detection System thermocycler (Bio-Rad) using a program with a 30 s initial denaturation step at 95°C, followed by 40 cycles of 5 s denaturation at 95°C and 10 s at 60°C. Melt curve analyses were used to validate the amplicon. Four biological replicates were used per treatment and two

technical repetitions per standard, sample and negative control were run.

The relative expression was calculated from threshold cycle (Ct) values using the 2ΔΔCT-method (Livak & Schmittgen, 2001) by using the geometric mean of *Phosphoglucomutase* (Vestman et al., 2011) and *elongation factor 1-α* (ELF1α) (Arnerup et al., 2011) to normalize transcript abundance. The gene expression experiments were performed with four biological and two technical replicates. One-way ANOVA with Dunns Post-test (GraphPad Prism 5.0) was used to detect differences in expression levels between treatments.

4 | RESULTS

4.1 | Trait association mapping identifies novel QTLs for resistance to *H. parviporum*

From an average of 1.5 million paired end sequence reads per individual, 197,399 high confidence SNPs from 23,837 probes were identified. The majority of the SNPs were missense (61%) and silent (36%), the highest percentage being either upstream or downstream variants (68% total).

Employing a Stability Selection Probability (SSP) on the estimated breeding values (EBVs) for SWG and LL of the offspring on the 466 trees, we identified six SNPs with significant associations for SWG and five SNPs associating with LL (Table 2). The QTLs for control of sapwood growth of *H. parviporum* (SWG) explained similar fractions of the observed phenotypic variation (H^2_{QTL}) 2.4 to 5.2% (Table 2). The five QTLs for control of the LL development in bark explained between 2.1 and 4.4% of the observed phenotypic variation (Table 2).

To investigate if the identified QTLs are independent from previously identified QTLs for resistance to the same isolate of *H. parviporum* using IM (Lind et al., 2014), we searched an ultra-dense genetic map (Bernhardsson et al., 2019) for the probes the SNP markers originated from. This allowed us to estimate the position of the detected QTLs and the original IM-based QTLs in the Norway spruce genome. We could estimate the position in the Norway spruce genome for six of the SNPs/probes (Table S2.I and Figure S2.II in Data S1). All of the identified SNPs/probes were positioned >30 cM away from the original IM-based QTLs in the genetic map. Given that the maintained LD is estimated to only 109 bp across all the tagged genomic sequences in this study (Table S2 in Data S1), it is likely that they are independent. The SNP MA_53835_9763, associating with the trait SWG, presented a potential exception as the probe MA_14663 is positioned 4 cM away from MA_53835 in the map (Bernhardsson et al., 2019). The probe MA_14663 corresponds to the SNP array sequence for an IM-based QTL for infection prevention (Lind et al., 2014; Chaudhary et al., submitted manuscript).

On the scaffolds holding the SNPs associated with the resistance traits, a total of 14 gene models were identified, including 11 high- or medium-quality Norway spruce gene models (Table 3). On the scaffolds holding more than one gene model, the SNPs were positioned in MA_5978g0020, MA_25569g0020 and MA_97119g0010. Seven of the candidate genes associated with SWG QTLs and seven with LL (Tables 2 and 3). PFAM and GO term annotations and *Populus* and *Arabidopsis* orthologues were collected from *P. abies* v1.0 genome portal (Table 3). These metrics suggested that the gene models MA_97119g0010 and MA_97119g0020, found on the scaffold harbouring the SNP MA_97119_12277, indeed represented one gene. BlastN searches against the NCBI database essentially confirmed this suggestion as both gene models match JX500691.1 (*Picea abies*

TABLE 2 Significant association in the GWA study

Phenotype[a]	QTL	SNP[b]	Allele[c]	SNP feature[d]	Frequency[e]	PVE (%)[f]
SWG_tot	8675	MA_5978_21,011	T/C	Missense	0.71	4.83
	26756	MA_17884_58584	A/G	Upstream variant	0.72	3.41
	54184	MA_53072_3732	G/A	Synonymous	0.551	2.88
	54695	MA_53835_9763	G/A	Upstream variant	0.567	2.40
	56105	MA_56128_7752	C/A	Upstream variant	0.545	5.21
	71928	MA_84091_11329	C/A	Upstream variant	0.534	2.23
LL_tot	21105	MA_14352_27165	G/A	Missense variant	0.603	3.82
	27795	MA_18316_3165	G/T	Upstream variant	0.618	2.11
	31060	MA_19645_22184	C/T	Missense	0.682	2.73
	37057	MA_25569_28091	T/C	Upstream variant	0.667	2.77
	81488	MA_97119_12277	T/C	Upstream variant	0.742	4.39

[a]Phenotype specifies the trait upon which the marker associate.
[b]SNP: The SNP name was composed of the contig (MA_number) and SNP position on contig. For example, the first SNP MA_5978_21011 was located on contig MA_5978 at position 21011 bp.
[c]Allele indicates the biallelic SNP.
[d]SNP feature allelic variation associated with the SNP.
[e]Frequency, stability selection probability inclusion ratios for markers declared significant.
[f]PVE, phenotypic variance explained, only values larger than 1.0% are displayed.

TABLE 3 Candidate Norway spruce gene models associated with the QTL markers

SNP[a]	Candidate gene[b]	Description (Blast2Go)[c]	PFAM-Description/GO term[d]	Orthologs populus/ Arabidopsis[e]
MA_5978_21,011	MA_5978g0010	Phenylcoumaran benzylic ether reductase	PF00106-short chain dehydrogenase, PF01073-3-beta hydroxysteroid dehydrogenase/isomerase family PF01118-Semialdehyde dehydrogenase, NAD binding domain, PF01370-NAD-dependent epimerase/ dehydratase family, PF02719-Polysaccharide biosynthesis protein, PF03435-Saccharopine dehydrogenase, PF03807-NADP oxidoreductase coenzyme F420-dependent, PF05368-NmrA-like family, PF07993-Male sterility protein, PF08659-KR domain, PF13460-NADH(P)-binding	Potri.009G118100.1/ AT1G75280.1
	MA_5978g0020	Nuclear factor 1 A-type isoform 2	PF06219-Protein of unknown function (DUF1005)	Potri.013G071000.3/ AT5G17640.1
MA_17884_58584	MA_17884g0010	Mitochondrial import receptor subunit TOM40-1	PF01459-Eukaryotic porin	Potri.007G000200.1/ AT3G20000.1
MA_53072_3732	MA_53072g0010			
MA_53835_9763	MA_53835g0010	Probable tocopherol O-chloroplastic	PF01209-ubiE/COQ5 methyltransferase family, PF01728-FtsJ-like methyltransferase, PF02353-Mycolic acid cyclopropane synthetase, PF03059-Nicotianamine synthase protein, PF05175-Methyltransferase small domain, PF05891-AdoMet dependent proline di-methyltransferase, PF07021-Methionine biosynthesis protein MetW, PF08003-Protein of unknown function (DUF1698), PF08241-Methyltransferase domain, PF08242-Methyltransferase domain, PF12847-Methyltransferase domain, PF13489-Methyltransferase domain, PF13578-Methyltransferase domain, PF13649-Methyltransferase domain, PF13659-Methyltransferase domain, PF13679-Methyltransferase domain, PF13847-Methyltransferase domain	Potri.013G077000.1 AT1G64970.1
MA_56128_7752	MA_56128g0010			Potri.006G130600.1
MA_84091_11329	MA_84091g0010			
MA_14352_27165	MA_14352g0010	Transcription factor bHLH118	PF00010-Helix–loop–helix DNA-binding domain	Potri.015G134300.1/ AT4G25400.1

TABLE 3 (Continued)

SNP[a]	Candidate gene[b]	Description (Blast2Go)[c]	PFAM-Description/GO term[d]	Orthologs populus/ Arabidopsis[e]
MA_18316_3165	MA_18316g0010	IST1 homologue	PF03398-Regulator of Vps4 activity in the MVB pathway	Potri.019G087400.1/ AT1G34220.2
MA_19645_22184	MA_19645g0010			
MA_25569_28091	MA_25569g0010		*GO:0005618-cell wall*, *GO:0016020-membrane*, *GO:0044444-cytoplasmic part*	Potri.002G054900.1/ AT1G03230.1
	MA_25569g0020			Potri.001G266500.1
MA_97119_12277	MA_97119g0010	Laccase	PF07732-Multicopper oxidase	Potri.019G124300.1 / AT2G30210.1
	MA_97119g0020	Laccase 12	PF00394-Multicopper oxidase, PF07731-Multicopper oxidase	Potri.010G183500.1 / AT5G05390.1

[a]SNP: The SNP name was composed of the contig (MA_number) and SNP position on contig.
[b]Candidate gene.
[c]Description (Blast2Go).
[d]PFAM-Description or GO terms when PFAM descriptions were missing.
[e]*Populus*/*Arabidopsis* orthologs identified in the *P. abies* v1.0 genome portal.

laccase LAC5a) with E = $4*10^{-135}$ and E = 0 and 99.62 and 99.71% identity, respectively. This laccase, *PaLAC5*, was originally isolated from lignin-forming Norway spruce suspension cultures. Apart from MA_97119, two other QTL holding scaffolds (MA_5978 and MA_25569) harboured more than one gene model (Table 3). Both of these scaffolds appear to hold two different gene models as judged by the PFAM annotations and *Populus* or *Arabidopsis* orthologs (Table 3). MA_5978g0010 appears to encode a phenylcoumaran benzylic ether reductase (PCBER) with similarity to PicglPPR21 (Porth, Hamberger, White, & Ritland, 2011). The gene model MA_14352g0010 may belong to the *basic helix–loop–helix (bHLH) DNA-binding* superfamily since the PFAM-ID PF00010 (Helix–loop–helix DNA-binding domain) is associated with the gene model. The candidate gene MA_18316g0010 is associated with PF03398 (regulator of Vps4 activity in the MVB pathway), indicating that this gene too may be involved in regulatory activities. The gene model MA_53835g0010 appears to encode a protein with methyltransferase capacities based on its PFAM annotation and its *Arabidopsis* orthologue (Table 3), and based on its PFAM annotation (PF01459) and the annotation of the *Arabidopsis* orthologue, AT3G20000.1 (Table 3) which encodes β-barrel protein, TOM40, forming channels in the outer mitochondrial membranes, it is likely that the candidate gene MA_17884g0010 encodes a Norway spruce TOM40-like protein.

4.2 | A majority of the candidate genes associated with SWG are expressed in stem and wood forming tissues

To gain a better understanding of the functionality of the candidate genes, we assessed the expression in silico using available resources

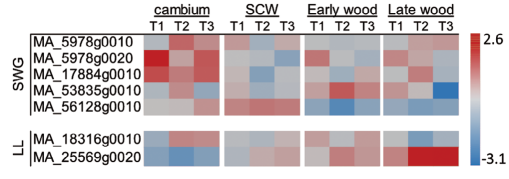

FIGURE 1 Relative expression levels of candidate genes associated to *H. parviporum* resistance QTLs through different stages of xylem development including cambium and expanding early wood (cambium), secondary cell wall-forming xylem (SCW), first dead early wood cells (Early wood) and the previous year's latewood (late wood). Data collected from NorWood v1.0 (http://norwood.congenie.org) database, T1-T3 represent the expression level in each of the three analysed trees (Jokipii-Lukkari et al., 2017). The bar to the left indicates the relative expression level of the candidate gene in the heat map

such as NorWood and *P. abies* exATLAS databases. It predicted that the candidate genes linked to SWG would more commonly be expressed in sapwood than genes linked to LL. Only seven candidate genes (MA_5978g0010, MA_5978g0020, MA_17884g0010, MA_53835g0010, MA_56128g0010, MA_18316g0010 and MA_25569g0020) were expressed in any of the libraries in NorWood (Figure 1). Of the expressed candidate genes, five were linked to SWG. This indicated a trend (Chi-square = 3.233, p = .07) where candidate genes linked to the SWG QTLs were expressed more often in wood compared to candidate genes linked to LL.

NorWood is a database of transcript abundances in high spatial resolution section series throughout the cambial and woody tissues of Norway spruce (Jokipii-Lukkari et al., 2017). Three of the five candidate genes associated with control of SWG (MA_5978g0010, MA_5978g0020 and MA_17884g0010) showed the highest transcript

levels in the cambial region. MA_56128g0010, also associated with SWG, appeared to be more active in the expanding early wood and secondary cell wall-forming tissues (Figure 1). One of the two candidate genes associated with the LL extension in the phloem and inner bark that were detected in the NorWood libraries, MA_25569g0020 showed very high activity in the samples collected at the visual appearance of dead early wood cells and in latewood (Figure 1). The inspection of the expression patterns in the *P. abies* exATLAS indicated that all candidate genes but MA_84091g0010 and MA_19645g0010 were expressed in at least one tissue of the clone Z4006 (Figure S3 in Data S1). Apart from the candidate genes that were also detected in the NorWood database, several candidate genes (MA_14352g0010, MA_25569g0010, MA_97119g0010 and MA_97119g0020) associated with LL were found to be expressed in samples derived from stem tissues (Figure S3 in Data S1).

4.3 | The transcriptional responses to *H. parviporum* inoculation identifies candidates responding specifically to the pathogen

If the candidate gene models associated with QTLs contribute to the control of the *H. parviporum* infection, they may be involved in either the constitutive or induced defence in the tissue (or both) (Arnerup et al., 2011; Danielsson et al., 2011; Oliva et al., 2015). Assuming that genes associated with the induced defences respond to inoculation with the pathogen, it is relevant to assess the candidate genes expression pattern in response to *H. parviporum* (Arnerup et al., 2011; Danielsson et al., 2011; Oliva et al., 2015). We used an RNASeq study of transcriptional responses in bark and phloem response to wounding and *H. parviporum* inoculation (Chaudhary et al., submitted manuscript). Five candidate genes showed constitutive expression at seven dpi irrespective of the treatment: MA_5978g0020, MA_17884g0010, MA_53835g0010, MA_56128g0010 and MA_25569g0020 (Figure 2). Most of these showed moderate expression levels, but MA_17884g0010 expression was relatively high in all samples. Four gene models associated with LL were differentially expressed at seven dpi: MA_14352g0010, MA_18316g0010, MA_97119g0010 and MA_97119g0020 (Figure 2). Interestingly, the two candidate gene models, (MA_97119g0010 and MA_97119g0020, i.e., *PaLAC5*) that showed the largest induction in response to the inoculation treatment compared to the wounding control proximal to the inoculation site, were not induced but rather downregulated distally at seven dpi (Figure 2). To validate the transcriptional responses estimated from the RNAseq data, we set up a separate inoculation experiment in a single Norway spruce genotype for qPCR validation of the expression patterns at seven dpi. The qPCR verified the transcriptional regulation patterns between *H. parviporum* inoculation and wounding treatment for most genes (Figures 2 and 3). This included the absence of a transcriptional activity of the candidate genes MA_53072g0010, MA_84091g0010, MA_19645g0010 and MA_25569g0010. The repression of the putative bHLH transcription factor MA_14352g0010 in response to *H. parviporum* was not detected in the qPCR experiment. The qPCR did confirm that *PaLAC5* (MA_97119g0010 and MA_97119g0020) is strongly and specifically upregulated in close proximity to the *H. parviporum* inoculation site (Figure 3d). Two of the candidate genes linked to the SWG QTLs with detected expression in the Norwood database, MA_17884g0010 and MA_53835g0010, were shown to be induced in response to *H. parviporum* compared to the control (Figure 3f,g). None of the tested candidate genes, including MA_17884g0010 and MA_53835g0010, were differentially expressed between H. *parviporum* inoculation and wounding in sapwood in early interactions (Table S4 and Method Section in Data S1).

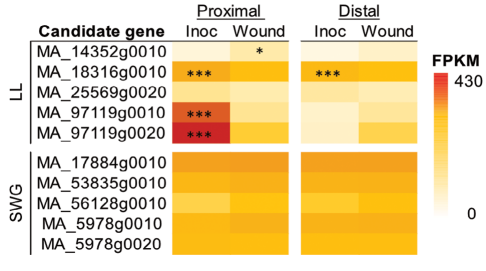

FIGURE 2 Expression profile of candidate genes for *H. parviporum* resistance in response to *H. parviporum* inoculation and wounding at seven dpi proximally (0–5 mm from the inoculation site) and distally (10–15 mm from the inoculation site) in the clones S21K0220126 and S21K0220184 (Chaudhary submitted MS). Asterisks indicate significant different expression levels between the inoculation treatment and the wounding control in *Cuffdiff*. The bar to the left indicates the FPKM values associated with the gene model

5 | DISCUSSION

5.1 | Twelve distinct QTLs for resistance to *H. parviporum* identified by GWAS

In this study, the GWAS identified 11 significant associations across the two traits for *H. parviporum* resistance. QTLs for LL and SWG traits detected in the GWAS explained similar fractions of the observed phenotypic variation, as in the IM-based QTL study by Lind et al. (2014). However, the narrow-sense heritability of the phenotypic traits was considerably higher among the 466 Norway spruce half-sib families than in the single family used in the IM-based QTL study, 0.42 compared to 0.11 for SWG (Arnerup et al., 2010; Chen et al., 2018; Lind et al., 2014). The fact that the Norway spruce genome v 1.0 assembly was highly fragmented comprising >10 million scaffolds over 500 bp (Bernhardsson et al., 2019; Nystedt et al., 2013) made it difficult to evaluate how the QTLs identified by GWAS relate to the previously identified QTLs (Lind et al., 2014), or to each other. However, the newly published ultra-dense genetic map (Bernhardsson et al., 2019) showed that five of the QTLs were independent from the other QTL regions as they were found in different linkage groups. Only one of the QTL regions that was identified in the linkage map

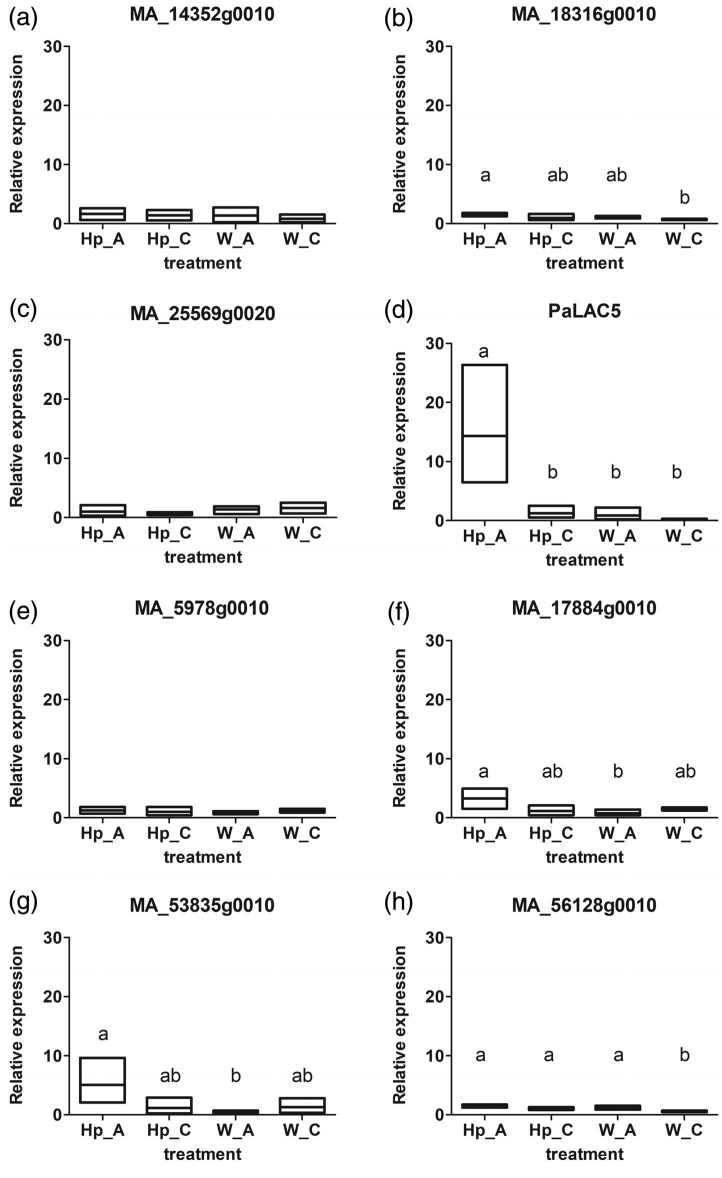

FIGURE 3 Expression profile of candidate genes for *H. parviporum* resistance in response to *H. parviporum* inoculation (Hp) and wounding (W) at seven dpi proximally (0–5 mm from the inoculation site, indicated by the letter "A" in, e.g., the treatment "Hp_A") and distally (10–15 mm from the inoculation site, indicated by the letter "C") in the Norway spruce clone S21K7820222 as detected by qPCR. Candidates a–d are associated with the trait LL and candidate genes e–g with trait the SWG. The floating bars in the graphs indicate min and max values, the line indicates mean, and different letters over the bars in the graph indicate significant differences in the statistical analyses ($N = 4$)

may possibly coincide with a previously identified resistance QTLs (Lind et al., 2014). The SNP MA_53835_9763 is positioned within 4 cM from a probe in the confidence region for the trait infection prevention (IP) on LG 11 (Lind et al., 2014; Chaudhary et al., submitted manuscript). Thus, the possibility that these markers target the same genomic region cannot be excluded, although it is not very likely given the short LD. Overall, the GWAS returned 11 new potential markers for resistance to *H. parviporum* in Norway spruce that could be used to aid selection in breeding programmes.

5.2 | Candidate genes have orthologues whose genetic variation is associated with the control of the responses to multiple stresses

Three of the candidate genes identified in the GWAS, MA_17884g0010, MA_5978g0020 and MA_18316g0010, have *Arabidopsis* orthologues AT3G20000.1, AT5G17640.1 and AT1G34220.2, respectively. These orthologues hold QTLs for responses to multiple stresses (Kawa et al., 2016; Thoen et al., 2017).

The candidate gene MA_18316g0010 was associated with control of lesion length in the inner bark and it was upregulated in response to *H. parviporum* inoculation compared to wounding alone, both proximally and distally. The *Arabidopsis* orthologue AT1G34220.2 encodes IST1-LIKE1 (ISTL1), a protein predicted to be the *Arabidopsis* homologue of yeast IST (Buono et al., 2016). ISTL1 is a regulator of the multivesicular bodies (MVB) pathway in which ubiquitinated and endocytically internalized membrane proteins are degraded (C. Gao, Zhuang, Shen, & Jiang, 2017). ISTL1, in interaction with LIP5 (LYST INTERACTING PROTEIN 5, AT4G26750), is essential for normal plant growth and repression of spontaneous cell death (Buono et al., 2016). The fungus *H. parviporum* is a necrotrophic pathogen and upon infection or inoculation in trees, it will create necrotic lesions in the phloem to gain access to the sapwood (Johansson & Stenlid, 1985; Lindberg & Johansson, 1991). It is, therefore, tempting to propose that the MA_18316g0010 protein fulfils the same role in the control of the cell death process as the ISTL1/LIP5 complex, MA_18316g0010 was upregulated in response to *H. parviporum* inoculation to repress cell death, a mechanism that must be integral to the LL trait. It would be interesting to test if the variation at MA_18316_3156 leads to differential accumulation of the transcript in response to *H. parviporum*.

The *Arabidopsis* orthologue to MA_17884g0010, AT3G20000.1, encodes translocase of the outer mitochondrial membrane 40, TOM40. *AtTOM40* is in LD with a QTL (Ch3:6968031) identified in a multi-trait QTL mixed models GWAS using the responses to a set of 30 biotic and abiotic stresses in 196 accessions of *Arabidopsis* (Thoen et al., 2017). TOM40 protein is the central channel forming units of the TOM complex (Hill et al., 1998). The TOM complex and the mitochondrial outer membrane play a central role in the interaction between the mitochondrion and the cytosol. It mediates the import of preproteins, the passage of small molecules and the transduction of signals between cellular compartments (Duncan, van der Merwe, Daley, & Whelan, 2013). Consequently, it is perhaps not unexpected that genetic variation associated with MA_17884g0010 and *TOM40* may influence plants responses to stress, or that MA_17884g0010 shows a ubiquitous expression in the surveyed Norway spruce tissues, with a slight upregulation in metabolically very active tissues (eg the cambium) and in response to *H. parviporum* inoculation.

5.3 | Candidate genes linked to SWG QTLs are more commonly expressed in wood

Despite the economic and ecological importance of conifers, we know surprisingly little about the genetic basis of resistance to decay pathogens compared to canker-forming pathogens in conifers (Kinloch, Sniezko, & Dupper, 2003; Liu et al., 2017; Sniezko, Smith, Liu, & Hamelin, 2014). Examining the regions under selection in response to given pathogens or stressors, identifying and testing candidate genes, can lead to better understanding of the interaction between the host and the pathogen (Liu et al., 2017; Martin, Rönnberg-Wästljung, Stenlid, & Samils, 2016; Nemesio-Gorriz et al., 2016; Thoen et al., 2017). Under the expectation that candidate genes linked to the control of SWG are involved in processes shaping the cell wall or in production of, for example, specialized metabolites in wood (Oliva et al., 2015; Popoff, Theander, & Johansson, 1975; Stenlid & Johansson, 1987), we predicted that the expression of the candidate genes linked to SWG QTLs should be more commonly detected in the wood-forming tissues than the genes linked to the LL QTLs. A trend for this was observed in the NorWood database (Jokipii-Lukkari et al., 2017), although a larger number of QTLs and candidate genes for both traits studied would probably have been needed to gain conclusive evidence. It is, however, important to point out that none of the QTLs identified for SWG, or LL, coincide with the 52 QTLs for important wood quality traits in Norway spruce reported by Baison et al. (2019). An observation that is fully in agreement with the absence of significant correlations between wood quality, or growth, traits and resistance to *H. parviporum* in this material (Chen et al., 2018), suggesting that the detected SWG QTLs may be associated to distinct defence-related processes. Several of the expressed candidate genes showed their highest transcriptional activity in the cambium and expanding early wood libraries. The candidate gene MA_25569g0020, associated with LL, showed increased transcriptional activity during visual appearance of dead early wood cells in the sapwood. The transcript is also specifically expressed in the phloem in the autumn/winter (Jokipii-Lukkari et al., 2018), but it was not induced by *H. parviporum* inoculation. This points to that the role of MA_25569g0020 in resistance may be associated to the constitutive defence.

5.4 | The Norway spruce laccase *PaLAC5* responds specifically to *H. parviporum* inoculation

Two candidate genes associated with the LL trait in bark, MA_53835g0010 and *PaLAC5*, are likely to be members of the induced defence to *H. parviporum*. The Norway spruce *laccase* gene *PaLAC5* (MA_97119g0010 and MA_97119g0020) was originally isolated from lignin-producing Norway spruce suspension cultures (Koutaniemi, Malmberg, Simola, Teeri, & Kärkönen, 2015), and transcriptome analyses of these lignin-producing Norway spruce suspension cultures under different conditions suggest that *Pa*LAC5 is associated with the activation of stress associated lignin production (Laitinen et al., 2017). *PaLAC5* has a very specific spatial expression pattern in response to *H. parviporum* inoculation. It is strongly, and specifically, upregulated proximally to the *H. parviporum* inoculation site but not regulated 10 mm away from the developing necrotic lesion or in response to the wounding control. In contrast to the induction of *PaLAC5* in stress associated lignin production conditions in vitro, the transcriptional activity of *PaLAC5* is very low in sapwood (Blokhina et al., 2019; Jokipii-Lukkari et al., 2017; Laitinen et al., 2017). Therefore, *Pa*LAC5 is not likely to be associated with lignifying tracheids or ray parenchyma cells indicating that the induction of *PaLAC5* expression under lignin-forming conditions in the cell cultures is stress-associated and not directly connected to lignification processes in wood (Blokhina et al., 2019; Jokipii-Lukkari et al., 2017;

Laitinen et al., 2017). However, if *PaLAC5* would be responding to stress in general, it would likely have had an expression pattern similar to many other studied defense genes, which often show upregulation in proximal to both mechanical wounding sites and to inoculation points (Arnerup et al., 2011; Danielsson et al., 2011; Ralph et al., 2006). Instead, it showed a distinct expression pattern. Thus, it is probable that *PaLAC5* expression is associated with specific cell types or processes such as the formation of the LSZ in the bark adjacent to the inoculation site. The LSZ is characterized by deposition of phenolics and suberin, and an early development of a discernible LSZ is crucial in stopping fungal invasions (Bodles et al., 2007; Lindberg & Johansson, 1991; Solla, Tomlinson, & Woodward, 2002; Woodward et al., 2007). Recently, it was suggested that specific isoforms of peroxidase and laccases may be involved in cross-linking aromatics to form lignin-like polyphenolics in the suberin in bark (Rains, Molina, & Gardiyehewa de Silva, 2017). The expression pattern of *PaLAC5* responding to *H. parviporum* and lignin-forming conditions (Laitinen et al., 2017) clearly makes it an interesting candidate for such a role. It remains to be seen if *PaLAC5*, indeed, is involved in the LSZ formation and if genetic variation associated with *PaLAC5* influences the formation of the LSZ.

6 | CONCLUSIONS

Our large sample sizes and a relatively high number of markers allowed us to link traits to SNPs with GWAS and to identify candidate genes associated with the QTLs. These candidate genes present new insights into the interaction between Norway spruce and *H. parviporum*, such as a putative involvement of the secretory and endosomal trafficking pathways and the *laccase PaLAC5*, in the control of lesion extension in the inner bark or the potential role of mitochondrial protein import and biogenesis in controlling *H. parviporum* spread in the sapwood.

ACKNOWLEDGMENTS

Financial support was received from the Swedish Foundation for Strategic Research (SSF), grant number RBP14-0040 and The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS). We also acknowledge the Swedish Research Council (VR) and the Swedish Governmental Agency for Innovation Systems (VINNOVA). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors thank Anna Kärkönen for discussions on Norway spruce laccases, Louis Mielke for linguistic help and Maria Jonsson and the field staff at the Swedish Forest research institute for assistance with phenotyping.

The R scripts used for the GWAS are publicly available at https://github.com/RosarioGarciaLab. Genotypic data and SNP position files are available upon contacting Rosario Garcia-Gil (m.rosario.garcia@slu.se). The Norway spruce genome assemblies and resources are available from http://congenie.org/pabiesgenome.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceived the study: H.W., J.S., B.K., M.R.G.G. and M.E.

Planned the study: B.K., M.R.G.G., M.E., I.V.

Performed experiments: K.L., I.V., M.E., H.C., M.S.Å., R.C., Å.O.

Analysed data: L.Z., J.B., Z.-Q.C., M.E., K.L., R.C.

Drafted the MS: M.E. and J.B.

Commented on MS: J.B., Z.-Q.C., K.L., B.K., M.E., H.C., M.S.Å., R.C., J.S., Å.O. and M.R.G.G.

Wrote the final MS: M.E., all authors read and approved the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Elfstrand M, Baison J, Lundén K, et al. Association genetics identifies a specifically regulated Norway spruce laccase gene, *PaLAC5*, linked to *Heterobasidion parviporum* resistance. *Plant Cell Environ*. 2020;43:1779–1791. https://doi.org/10.1111/pce.13768

Supplementary table S1. QPCR primers used in the study

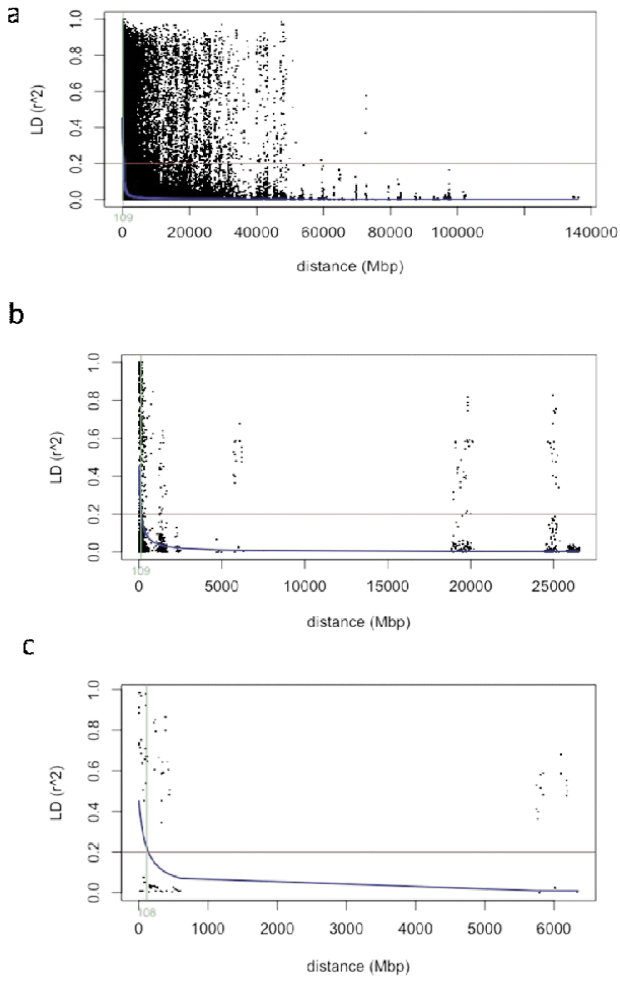
Primer	Sequence	Reference
PaLAC5F	CCCATTCTTCCAGCCTACAA	
PaLAC5R	GAGTCCAAGTCCGATGGTGT	
PhosphoglucomutaseF	AATGCAGTTGAAGCCATTCC	Vestman et al 2011
PhosphoglucomutaseR	CCAGTGCCGAAACTCTCTTC	Vestman et al 2011
ELF1αF	TAGTCCCTCACAGCAAAACGA	Arnerup et al 2011
ELF1αR	TTAAGAATGGAGATGCCGGGTTTGT	Arnerup et al 2011
MA_14352g0010F	TCTGTCGGTTCAGGTGTTCA	
MA_14352g0010R	CACAACTTCAAGCCCACCTT	
MA_17884g0010F	ATGCTTGTGGGACGGATACT	
MA_17884g0010R	TTGAACATCCCCTGAGAGAAA	
MA_18316g0010F	CTTCACAAGCTGTGCCAGAA	
MA_18316g0010R	TGAGAGAGGGCGGTAGAGAA	
MA_19645g0010F	AGAGGCTGAGTGGGATTTCA	
MA_19645g0010R	TGGGGGAGGAATAACAACAA	
MA_25569g0010F	CATCGCAATCAACCAAAAGA	
MA_25569g0010R	CATCGAAGCAAGCATCAAAA	
MA_25569g0020F	ATCGTCTCGATGTCGCTCTT	
MA_25569g0020R	TGTTTTCAAGGGATGCAACA	
MA_53072g0010F	GATTTGCATCTCGTTGTGGA	
MA_53072g0010R	TCCATTGTTGTGATGCTCGT	
MA_53835g0010F	GGTGCATTATTCCTGCCATT	
MA_53835g0010R	GGAGGTCGTAGAATCCGTGA	
MA_56128g0010F	CCATCCTCATGGAAAGGAAA	
MA_56128g0010R	GAACACTGAGCATCCAAGCA	
MA_5978g0010F	TCAAGAGGTTTTTGCCGTCT	
MA_5978g0010R	CCTGCGAATTTTTGCTTTGT	
MA_5978g0020F	TTAGGGAGTAGCGAGCCTGA	
MA_5978g0020R	TGAAAATTGGTTGCCTCACA	
MA_84091g0010F	CATCTGGTCCCTTGCTCACT	
MA_84091g0010R	AGAACTTCGCTTGCCTTTGA	

Supplementary table S2.I. Position of QTLs in the Norway spruce genome and LD estimation

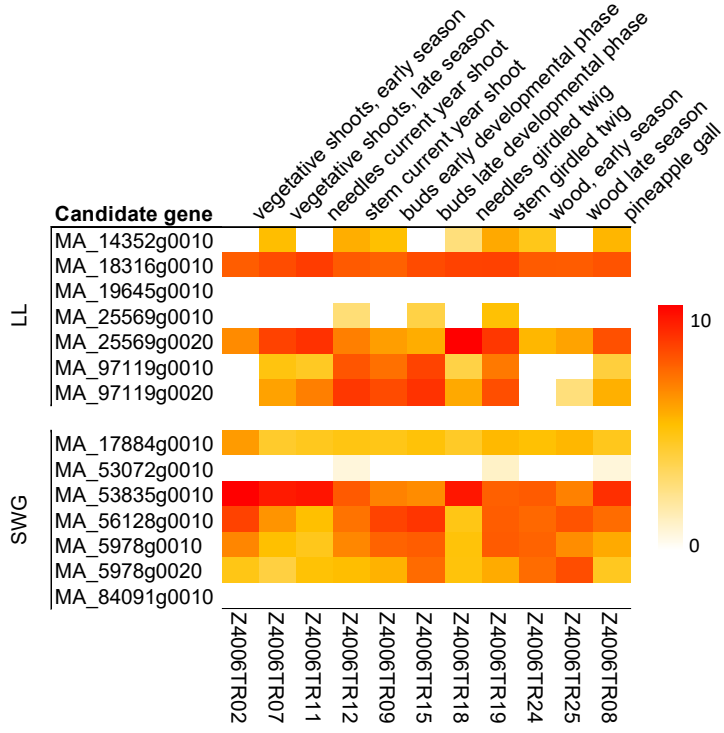
Phenotype[a]	QTL	SNP[b]	LG[c]	cM[d]	IM QTL[e]
	8675	MA_5978_21011	4	270.6	
	26756	MA_17884_58584	5	282.6	
SWG	54184	MA_53072_3732	8	186.0	
	54695	MA_53835_9763	10	214.7	MA_14663 (IM QTL) at 218.8 cM
	56105	MA_56128_7752	N/A		
	71928	MA_84091_11329	N/A		
	21105	MA_14352_27165	N/A		
	27795	MA_18316_3165	5	81.9	
LL	31060	MA_19645_22184	N/A		
	37057	MA_25569_28091	9	92.2	
	81488	MA_97119_12277	N/A		

[a]Phenotype, specify the trait upon which the marker [b]SNP: The SNP name was composed of the contig (MA_number) and SNP position on the contig. For detailed explanation see table 2; [c] LG group in which SNPs from the contigs probe(s) are positioned in the genetic map (Bernhardsson et al 2019) feature allelic variation associated with the SNP; [d] position of the marker in the linkage group in centiMorgans (cM) , and [e] position of the IM QLT in the linkage group in centiMorgans (cM).

Supplementary Figure S2.II. Decay of linkage disequilibrium (LD) across all the tagged genomic sequences (a), the majority being exonic regions. The squared correlation coefficient between loci (r^2) is plotted against distance in base pairs, separating loci; (b) decay of LD with distance across 13 with significant associations to resistance traits and, (c) decay of LD across contig MA_17884, that has a significant association for SWG_tot, on which two probes were captured.



Supplementary Figure S3. Expression of the candidate genes in various tissues in the Norway spruce.



Expression of the candidate genes associated with SWG and LL QTLs in various tissues in the Norway spruce (clone Z4006, data collected from the *P. abies* exATLAS v 1.0) The bar to the left indicate the expression values associated with the gene model

Supplementary

Supplementary Table S4. Expression in sapwood measured by qPCR of *H. parviporum* resistance candidate genes in response to *H. parviporum* inoculation and wounding at seven dpi adjacent to the inoculation site in the Norway spruce clone S21K7820222. None of the candidate genes were differentially expressed between treatments.

Trait	Candidate gene	Wounding[a]	H. parviporum[a]
SWG	MA_5978g0010	1.0 (+/- 0.3)	1.0 (+/- 0.2)
	MA_17884g0010	1.0 (+/- 0.1)	1.0 (+/- 0.3)
	MA_56128g0010	0.9 (+/- 0.1)	1.4 (+/- 1.2)
	MA_53835g0010	1.2 (+/- 0.3)	0.9 (+/- 0.4)
LL	*PaLAC5*	0.9 (+/- 0.9)	1.8 (+/-1.3)

[a] mean relative expression levels with standard deviation in brackets (N = 2-3).

Methods

The inoculation experiment in S21K7820222 is described in the main text. At seven days post-inoculation (dpi), when the surrounding bark were collected the sapwood 0-5 mm away from the inoculation site were also collected and frozen separately in liquid nitrogen and stored at −80°C until further use. RNA extraction cDNA synthesized and qPCR were carried out as described in the main text except that three biological and two technical replicates were used for qPCR.

II

Received: 12 February 2021 | Revised: 22 June 2021 | Accepted: 29 June 2021

DOI: 10.1111/mec.16058

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Killing two enemies with one stone? Genomics of resistance to two sympatric pathogens in Norway spruce

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Funding information
Stiftelsen för Strategisk Forskning, Grant/Award Number: RBP14-0040; Svenska Forskningsrådet Formas, Grant/Award Number: 2017-00402; VINNOVA, Grant/Award Number: 2016-00504

Abstract

Trees must cope with the attack of multiple pathogens, often simultaneously during their long lifespan. Ironically, the genetic and molecular mechanisms controlling this process are poorly understood. The objective of this study was to compare the genetic component of resistance in Norway spruce to *Heterobasidion annosum s.s.* and its sympatric congener *Heterobasidion parviporum*. Heterobasidion root- and stem-rot is a major disease of Norway spruce caused by members of the *Heterobasidion annosum* species complex. Resistance to both pathogens was measured using artificial inoculations in half-sib families of Norway spruce trees originating from central to northern Europe. The genetic component of resistance was analysed using 63,760 genome-wide exome-capture sequenced SNPs and multitrait genome-wide associations. No correlation was found for resistance to the two pathogens; however, associations were found between genomic variants and resistance traits with synergic or antagonist pleiotropic effects to both pathogens. Additionally, a latitudinal cline in resistance in the bark to *H. annosum s.s.* was found; trees from southern latitudes, with a later bud-set and thicker stem diameter, allowed longer lesions, but this was not the case for *H. parviporum*. In summary, this study detects genomic variants with pleiotropic effects which explain multiple disease resistance from a genic level and could be useful for selection of resistant trees to both pathogens. Furthermore, it highlights the need for additional research to understand the evolution of resistance traits to multiple pathogens in trees.

KEYWORDS
cline, disease resistance, genome-wide association study, *Picea abies*, pleiotropy, root-rot

1 | INTRODUCTION

Trees are long lived organisms that withstand the attack of a wide range of pathogens that often occur simultaneously (Tobias & Guest, 2014). Therefore, these organisms have evolved a layered and tuneable defence strategy, which includes pre-formed physical barriers, pathogen and damage recognition, signal transduction, production of metabolites and compartmentalization of damaged areas (Bonello

et al., 2006; Ennos, 2015; Franceschi et al., 2005; Kovalchuk et al., 2013; Nemesio-Gorriz et al., 2016; Oliva et al., 2015; Solla et al., 2002). Although the understanding of major genes conferring disease resistance to single diseases in plants has advanced, the genetic and molecular mechanisms controlling quantitative disease resistance traits and its effectiveness against multiple attackers remains scarce, particularly in trees (Abdullah et al., 2017; Chen et al., 2018; Corwin & Kliebenstein, 2017; Ismael et al., 2020; Weiss et al., 2020).

Quantitative resistance traits have a continuous distribution of phenotypes from susceptible to resistant and are controlled by quantitative trait loci (QTL) - multiple loci with small to moderate effects (Corwin & Kliebenstein, 2017; Nelson et al., 2018). Quantitative disease resistance is assumed to be nonstrain specific and therefore durable (Ismael et al., 2020; Nelson et al., 2018; Wiesner-hanks & Nelson, 2016), however it is not always effective against different pathogens (Corwin & Kliebenstein, 2017). The nature of disease resistance to multiple pathogens could theoretically be explained from an organism level to a single gene level (Wiesner-hanks & Nelson, 2016). At the organism level, individuals can be resistant to multiple diseases because different unlinked QTLs present in an organism's genome are effective against different diseases independently (Risterucci et al., 2003; Wiesner-hanks & Nelson, 2016). At the genic level, multiple disease resistance could arise through the linkage of clusters of loci effective against single diseases (Schweizer & Stein, 2011) or by individual pleiotropic genes, where the same gene confers resistance to multiple diseases (Nelson et al., 2018; Wiesner-hanks & Nelson, 2016; Wisser et al., 2011).

The mapping and identification of QTLs is typically done through linkage mapping studies or genome-wide associations studies (GWAS) (Nelson et al., 2018). To guarantee the success of these experiments, they must be performed with high precision and comparable infection systems between pathogens, which is particularly challenging in forest systems (Ismael et al., 2020; Quesada et al., 2010). In recent years the knowledge of conifer genomics has improved vastly, which has allowed for more detailed studies on the genomic architecture of disease resistance traits (Elfstrand et al. 2020; Lind et al., 2014; Weiss et al., 2020). Within conifers, a well-studied pathosystem that allows for precise phenotyping is stem- and root-rot caused by members of the *Heterobasidion annosum s.l* species complex (Bodles et al., 2007; Chen et al., 2018; Dalman et al., 2013; Lind et al., 2014; Mukrimin et al., 2018; Skrøppa et al., 2015; Steffenrem et al., 2016).

Speciation in the *Heterobasidion annosum s.l* species complex began with a split between the ancestor of the pine-infecting species *H. annosum* s.s. and *H. irregulare*, and the ancestor of the nonpine-infecting species *H. parviporum*, *H. abietinum*, and *H. occidentale* (Chen et al., 2015; Dalman et al., 2010). Species in the complex generally display sexual and somatic incompatibility and have different host ranges (Garbelotto & Gonthier, 2013). *H. parviporum* and *H. annosum s.s.*, however, readily infect Norway spruce and share much of the Norway spruce distribution on the European continent (Figure S1; Chen et al., 2015; Dalman et al., 2010; Garbelotto & Gonthier, 2013; Niemela & Korhonen, 1998).

Norway spruce (*Picea abies* L. Karst) is a dominant conifer in boreal forests in Europe with a vast current population size (Wang et al., 2020). The sequencing of the Norway spruce genome and subsequent work has allowed the description of the species' evolutionary history and population structure (Chen et al., 2019; Nystedt et al., 2013; Wang et al., 2020). Norway spruce is divided into three main domains, probably as a result of refugia through glaciation periods: a northern (Fennoscandian) domain ranging from Norway in the west to central Russia and two other domains in the Alps and Carpathians, with signs of main domain admixture—probably linked to recent expansion following the last glaciation period (Chen et al., 2019; Li, 2020; Tsuda et al. 2016). Recent studies have described the genetics of wood properties, growth, phenology traits (Baison et al., 2019; Milesi et al., 2019) and resistance to *H. parviporum* (Chen et al., 2018; Elfstrand et al. 2020).

Resistance to *H. parviporum* in Norway spruce is heritable (Chen et al., 2018; Lind et al., 2014; Steffenrem et al., 2016) and is characterized by many genes with relatively small effects on resistance (Elfstrand et al. 2020). One QTL in *PaLAR3*, a gene involved in the synthesis of catechin and linked to *H. parviporum* resistance in Norway spruce, is known to respond to other stressors such as *H. annosum s.s.*, the blue-stain fungus *Endoconiophora polonica*, and mechanical wounding (Danielsson et al., 2011; Hammerbacher et al., 2014; Nemesio-Gorriz et al., 2016). Therefore, we hypothesised that quantitative resistance to *H. parviporum* could provide multiple-disease resistance to other members of the *H. annosum s.l.* species complex. In this study we measured disease resistance traits to *H. annosum* s.s. and *H. parviporum* in a well-characterized Norway spruce population part of the Swedish Norway spruce breeding programme (Baison et al., 2019; Chen et al., 2018, 2019; Lind et al., 2014; Milesi et al., 2019). The programme is a result of phenotypic selection of trees across Europe based on growth, survival, stem quality and vitality, resulting in the inclusion of seven recognized Norway spruce genetic clusters in the current breeding population (Chen et al., 2019; Haappanen et al. 2015; Milesi et al., 2019). We formulated the specific hypotheses that (i) Norway spruce has variation in its resistance traits to *H. annosum s.s.*, (ii) resistance to *H. annosum s.s.* is correlated to resistance to *H. parviporum*, and (iii) QTLs could explain multiple-disease resistance in Norway spruce. To test these hypotheses, we studied resistance traits in 400 Norway spruce half-sib families following inoculation with *H. annosum s.s.* using quantitative genetics and genome-wide association methods (GWAS). Furthermore, we compared additive genetic resistance in half-sib families phenotyped for both *H. annosum s.s* and *H. parviporum* and identified potential multiple disease resistance QTLs with pleiotropic effects using multitrait GWAS.

2 | MATERIALS AND METHODS

2.1 | Plant material

A total of 400 open pollinated half-sib families from members of the founder population of the Swedish Norway spruce breeding programme were sown in 2016 (18 seedlings/family). After the first growth season, seedlings were randomised into a complete block design with three replications (Figure 1a), where each family was

planted in 4-tree row-plots in plastic trays consisting of 24 separate 0.124 L plastic pots. The seedlings were grown for another season in Skogforsk's experimental nursery at Ekebo, Sweden (55°56′53.1″N 13°6′52.2″E) and subjected to standard watering and fertilisation. No fungicides were used during cultivation.

2.2 | Artificial inoculations and phenotyping

Artificial inoculations were performed as described in Chen et al. (2018) with *H. annosum* s.s. Sä 16-4. The fungus was grown on Hagem's media plates for three weeks prior the experiment together with 5 mm *P. abies* wood plugs. Immediately prior to inoculation, bark was removed with a 6-mm diameter cork borer at 10 cm from the base of the seedling. A wooden plug colonised by *H. annosum s.s.* was then placed at the wound and covered with Parafilm (Chen et al., 2018). Ambient light and temperature conditions were maintained for 21 days, after which plants were harvested (from 20 August 2018 onwards).

Upon harvest, the diameter at the point of inoculation (D) was recorded and the lesion length (LL) above and below the edge of the inoculation point on the inner side of the bark was measured. Sapwood growth of the fungus (SWG) was measured according to Arnerup and collaborators (2010): The inoculated stem was cut up into 5-mm discs and placed on moist filter paper in 9 cm Petri dishes together with the original colonised wooden plug. To avoid contamination, the stem was cut from the tip to, and the base to the point of inoculation, respectively. After seven days incubation under humid conditions, the presence of *H. annosum s.s.* on the discs was determined by observation of characteristic conidiophores under a stereomicroscope (Arnerup et al., 2010; Swedjemark et al., 1997).

Time of bud-set of seedlings following the first growing season, from mid-October to mid-November 2017, was recorded twice per week, with "1" and "0" representing the presence and absence of a visible bud, respectively.

Out of the 400 half-sib families phenotyped for *H. annosum* s.s., 269 were previously phenotyped for the same resistance traits to *H. parviporum* and reported by Chen et al. (2018).

2.3 | Statistical analyses

Measured traits were checked for recording errors and normality. From a total of 5,924 observations, those with SWG =0 and no

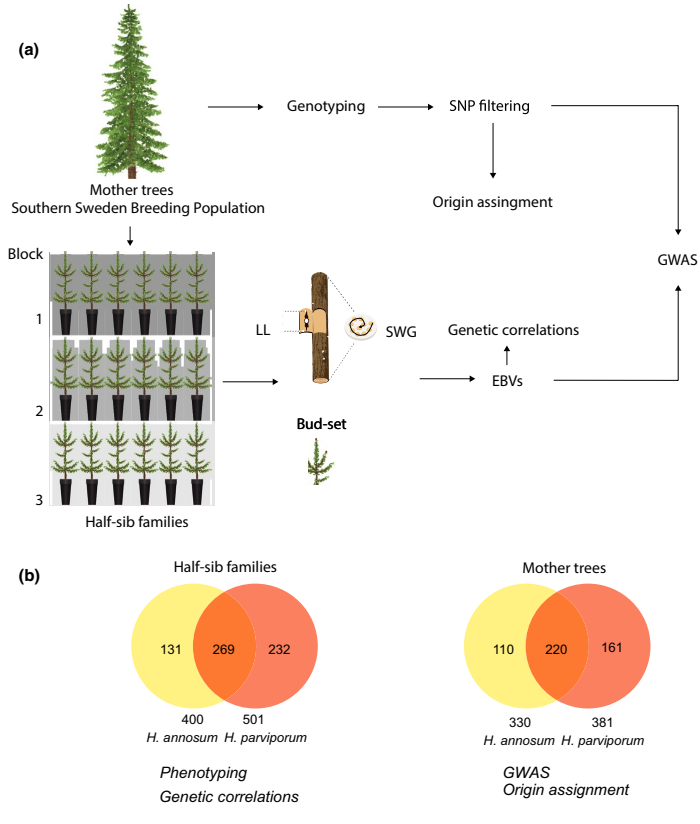

FIGURE 1 Experiment set up. (a) Genotyping and phenotyping. Mother trees were genotyped and SNPs were filtered. Thereafter tree origin prediction and GWAS was performed. Half-sib families from the genotyped mother trees where phenotyped in three different blocks. These values were used to calculate EBVs, which in turn were used to calculate genetic correlations and the GWAS. (b) The half-sib families were phenotyped for resistance traits against *H. annosum s.s.* ($N = 400$) and *H. parviporum* ($N = 501$). The families phenotyped for both pathogens ($N = 269$) were used to calculate genetic correlations. Due to genotype filtering based in SNPs missingness, only a subset of mother trees met the cutoff and was used for origin assignment and GWAS

conidiophores observed at either the point of inoculation or the inoculation plug were excluded from analyses ($N = 235$). Due to experimental errors progenies from the first block, with more than 75% of the seedlings scoring SWG =0, were also excluded ($N = 69$ observations). Resistance traits to *H. parviporum* phenotyped by Chen et al. (2018) were reanalysed in accordance with our criteria to remove bias. As LL showed a significant deviation from a normal distribution, the data was log-transformed, and a 0.5 constant was added to each value. Variance and covariance components for each trait were estimated using ASReml-R 4 (Butler et al., 2007) and the following linear mixed model was fitted for each trait individually:

$$y_{ijkl} = \mu + B_i + D_{ijkl} + F_k + e_{ijkl}$$

Where y_{ijkl} is each observation on the lth seedling from the kth family in the jth block, μ is the general mean and B_i is the fixed effect of the jth block. The variable F_k is the random effect of the kth family, e_{ijkl} is the random residual effect and D_{ijkl} is a covariate for diameter at inoculation point. Wald tests were used to estimate the significance of fixed factors. Estimated breeding values (EBVs) for each family were defined as the coefficients of the random effect. Genetic correlation between traits was assessed by testing the association between EBVs using Pearson's product moment correlation in R.

The individual-tree narrow-sense heritability for each trait was estimated using the equation:

$$\hat{h}_i^2 = \frac{\hat{\sigma}_a^2}{\hat{\sigma}_p^2} = \frac{4 \times \hat{\sigma}_f^2}{\hat{\sigma}_f^2 + \hat{\sigma}_e^2}$$

where h_i^2,$\hat{\sigma}_a^2$, $\hat{\sigma}_f^2$,$\hat{\sigma}_e^2$, and $\hat{\sigma}_p^2$ are narrow-sense heritability, additive genetic effect, family, residual, and phenotypic variance components, respectively.

Time of bud-set was fitted in a nested logistic mixed model as follows:

$$\text{logit}\left(y_{ijklm}\right) = \mu + B_i + F_k + G_{mk} + e_{ijklm}$$

Where y_{ijklm} is each observation on the lth seedling, at the mth week, from the kth family in the jth block where "1" corresponds to presence and "0" to absence of buds in the seedling, μ is the general mean and $B_i$is the fixed effect of the jth block. The variable F_{km} is the random effect of the repeated measurements for the kth family and G_{mk} is the random effect of the mth week within the kth family, with a first order auto regressive variance assumption and e_{ijklm} is the random residual effect. EBVs for each family were defined as the coefficients of the random effect of F_k.

2.4 | SNP identification

Mother trees to the half-sib families were genotyped using 40,018 probes to cover intragenic regions in 26,219 *P. abies* gene models (Vidalis et al., 2018). DNA extraction, sequencing, and initial variant calling is described elsewhere (Baison et al., 2019; Bernhardsson et al., 2020).

Variants were filtered according to Bernhardsson et al. (2020) with minor modifications. Briefly, only biallelic SNPs within the extended probe regions were included with QualbyDepth > 2.0, FisherStrand < 60.0, RMSMappingQuality (MQ) > 40, MappingQualityRankSumTest (MQRankSum) > −12.5, ReadPosRankSumTest (ReadPosRankSum) > −8.0, StrandOddsRatio (SOR) < 3.0 using VCFTOOLS (Danecek et al., 2011). SNPs with depth 6–40, GQ < 15, mean depth between 10–30, 20% missing data, minor allele count 1, and a p-value = >1e−10 for excess of heterozygosity were retained to avoid collapsed reads. Individuals with more than 30% missing variants after filtering were excluded from analysis. Missing variants in the remaining individuals were imputed with BEAGLE 4.1 (Browning & Browning, 2007).

2.5 | Mother trees origin assignment

The ancestral origin of mother trees was assessed following Chen et al. (2019) based on genotype similarity to individuals with known origin collected across *P. abies* natural range. Coordinates of the first five principle components of *P. abies* trees, from a sample population of 2572 (Li, 2020), with documented geographic origins and representative of the seven main genetic clusters were used as a training set in a "Random Forest" regression model ("randomForest" v4.6-14 package [Liaw & Wiener, 2002], R software v.3.3.1). The coordinates of the first five components of unknown individuals were then used to assign each mother tree to a given genetic cluster. The procedure was repeated 200 times with 8,000 iterations to estimate the accuracy of each assignment. Assignment of mother trees to a genetic cluster was determined to be true when the same allocation was repeated on more than 98% of occasions.

2.6 | SNP phenotype associations

Genome wide associations using different data sets were performed. For *H. annosum s.s.*, 330 mother trees were included after filtering for genotyping quality and relatedness (see above; Figure 1b). In order to perform multitrait GWAS between resistance traits to both *H. annosum s.s.* and *H. parviporum* we used the 220 overlapping mother trees between the population phenotyped for *H. annosum s.s.* resistance in this study and the population used in Chen et al. (2018) and Elfstrand et al. (2020). Associations were tested with GEMMA (Zhou & Stephens, 2012, 2014). EBVs calculated with ASreml R-4 (Butler et al., 2007) were used as the phenotype for each trait and kinship was accounted for with a standardized kinship matrix calculated in GEMMA (Zhou & Stephens, 2012, 2014). Principal component analysis (PCA) was computed with PLINK 1.9 (Chang et al., 2015) and used to identify and remove mother trees that were either too different or had very close family relationships with one another. Additionally, to account for population structure, three to four principal components

TABLE 1 Variance and heritability for lesion length (LL), sapwood growth (SWG) and Bud-set

		df	$\hat{\sigma}^2_a$	$\hat{\sigma}^2_e$	$\hat{\sigma}^2_p$	h^2	P(D)
H. annosum s.s.	LL	4994	0.09	0.17	0.19	0.49	<2.20 e−16
	SWG	4994	100.93	135.21	160.45	0.63	4.69 e−09
Budset		31330	16.04	3.28	19.33	0.83	–
H. parviporum	LL	4536	0.16	0.51	0.55	0.28	2.01 e−11
	SWG	4536	102.73	208.31	233.99	0.44	<2.20 e−16

Notes: df: Degrees of freedom; $\hat{\sigma}^2_a$: additive genetic variance; $\hat{\sigma}^2_e$: environmental variance; $\hat{\sigma}^2_p$: phenotypic variance; h^2: narrow sense heritability; P(D): Wald test p-value for diameter in the mixed model.

TABLE 2 Genetic correlations for lesion length (LL) and sapwood growth (SWG) between *H. annosum* s.s. and *H. parviporum* in the 269 families analysed in interaction with both pathogens

Trait combination	Genetic correlation	df	t	*p*-value
H. annosum s.s. LL × *H. annosum* s.s. SWG	0.40	392	8.75	<2.2e−16
H. parviporum LL × *H. parviporum* SWG	0.49	453	12.09	<2.2e−16
H. annosum s.s. LL × *H. parviporum* LL	0.06	262	0.99	.32
H. annosum s.s. SWG × *H. parviporum* SWG	0.08	262	1.32	.18

were used as covariates depending on the subset of samples. Only SNPs with a minor allele frequency (MAF) > 0.05 were used for the associations (63,760 SNPs *for H. annosum s.s.*, 63,372 for *H. parviporum* and 63,606 for the overlap). The tested model was:

$$y = W\alpha + x\beta + u + \epsilon$$

Where y is a matrix of $n \times d$ traits, W a matrix of $c \times d$ covariates (fixed effects), α is a matrix of the corresponding coefficients, x is an n -vector of the SNP genotypes, β is a d vector of effet sizes for the d phenotypes, U is an $n \times d$ matrix of the random effects and ϵ is an $n \times d$ matrix of errors (Zhou & Stephens, 2012, 2014). Wald association tests were performed for each analysis testing the alternate hypothesis $\beta \neq 0$. In order to correct for multiple comparisons, False discovery rate (FDR) and Bonferroni, corrections were calculated with R. Since very few markers were significant following multiple comparisons correction, a suggestive significance threshold of $1x10^{-5}$ (equivalent to the to 99.9 percentile) was used to identify candidate genes. The proportion of phenotypic variance explained by the SNP (PVE) was calculated according to (Shim et al., 2015).

The multitrait combinations were selected based on hypothesized relationships between traits, namely LL and SWG, within the experiment (different traits for the same pathogen) and between pathogens (same trait for different pathogens).

2.7 | Gene model identification

snpEff 4 (default parameters, Cingolani et al., 2012) was used to assess the putative function of the candidate SNPs. Ensembl general feature format (GFF, gene sets) information was utilised to build the *P. abies* snpEff database. Gene annotations were obtained from the *P. abies* v1.0 genome hosted at ConGenIE (http://congenie.org/). The position of the variants in Norway spruce genome was retrieved from the latest genetic map (Bernhardsson et al., 2019).

3 | RESULTS

3.1 | Resistance to *H. annosum s.s.* is not correlated to resistance to *H. parviporum* in Norway spruce

Resistance to *H. annosum s.s.* was variable in Norway spruce half-sib families with individual plant values for LL (lesion length in inner bark) ranging from 0 to 21 mm with a mean of 3.5 mm, and values of SWG (sapwood growth) ranging from 0 to 80 mm with a mean of 15.4 m ($N = 5924$). The block effect was significant in the mixed model for both traits, as well as the diameter at inoculation point, which had a significant positive effect on both LL and SWG (Table 1). Narrow sense heritability estimates (h^2) were 0.49 for LL and 0.69 for SWG (Table 1) and a positive correlation between traits was observed (Table 2).

Out of the 400 half-sib families phenotyped with *H. annosum* s.s., 226 were previously scored for the same resistance traits to *H. parviporum* and reported in Chen et al. (2018) (Figure 1b). Traits measured by Chen et al. (2018) with *H. parviporum*, and reanalysed here show generally larger individual plant values for LL and SWG than those for *H. annosum s.s.* (LL ranged between 0 and 104 mm and SWG between 0 and 85 mm with means of 7.6 and 32.6, respectively). Heritability values however, were lower: 0.28 for LL and 0.44 for SWG for all the half-sib families phenotyped by Chen et al. (2018). Block and diameter at inoculation point were significant in the mixed model (Table 1). Correlation of the resistance traits in response to *H. annosum* s.s. and *H. parviporum* inoculations was low and nonsignificant (0.06 for LL and 0.08 for SWG; Table 2).

To test if there was a geographic effect on resistance, the ancestral origin of mother trees (i.e., before they were introduced in the Swedish breeding programme) was inferred based on genotype similarity to trees of known origin. One tree was assigned to the Carpathian domain, 156 to the Alpine domain, 55 to central Europe, 27 to north Poland, 21 to Russian-Baltic region, 63 to Central and

Southern Sweden genetic cluster and one to the Fennoscandian domain; six trees were unassigned to a specific cluster due to their highly mixed genetic background. The two trees belonging to Fennoscandian and Romanian clusters were removed prior to making comparisons. Breeding values for LL in trees infected with *H. annosum s.s.* were significantly different between the southernmost and northernmost clusters following a latitudinal cline (Figure 2), but that was not the case for SWG or any other phenotypes in *H. parviporum* (Figure S2). Likewise, breeding values for timing of bud-set were significantly correlated with those for LL after infection with *H. annosum s.s.* ($r = 0.154$, $t = -3.34$, $df = 396$, $p = .008$), both following a latitudinal gradient.

3.2 | QTLs associated to *H. annosum s.s.* are novel and different from QTLs associated to *H. parviporum*

Genome-wide associations were performed using 63,760 SNPs from mothers of half-sib families and EBVs for LL and SWG calculated in half-sib families in response to artificial inoculations with *H. annosum s.s.* The distribution of the significance level of associations between the SNPs and EBVs (Figure S3) together with the PVE (Table 3) show that the resistance traits are probably polygenic, with several significant variants having small effects on the traits. After correction for multiple comparisons, no SNPs were significantly associated with either trait. Nonetheless, a suggestive threshold of $p < 1 \times 10^{-5}$ was used to identify the most significant variants associated with LL and SWG individually and together in a multitrait model. 13 SNPs associated with *H. annosum s.s.* resistance traits were found when analysed individually (eight for LL and six to SWG, Table 3) and 12 SNPs when the LL and SWG traits were analysed together in a multitrait model, from which 4 SNPs where exclusively found in the multitrait analysis (Table 3). Only eight markers could be placed in the linkage map and these were distributed in seven different linkage groups (Figure S4). Interestingly, two of the SNPs detected specifically in the multitrait model appear to be involved in plant hormone signalling. MA_27152:21720 is positioned in a putative orthologue of *AtRAE1*, a negative regulator of abscisic acid (ABA) in Arabidopsis (Li et al., 2018) and MA_64875:14168 in an orthologue of an enzyme involved in the last step of T-zeatin biosynthesis (Kiba et al., 2013). Furthermore, one SNP (MA_99821:7939) was found within a gene annotated as an "ethylene responsive transcription factor" (Table 3). A closer inspection of the gene model MA_99821g0010 shows that the gene indeed is a more likely orthologue of *Cytokinin response factor 2* (*AtCRF2*) in Arabidopsis. Several SNPs in *Pentatricopeptide repeat protein*- and *Tetraspanin* genes were also detected (Table 3). No QTLs were found to be associated with resistance to *H. parviporum* in this, or previous studies (Elfstrand et al. 2020; Mukrimin et al., 2018).

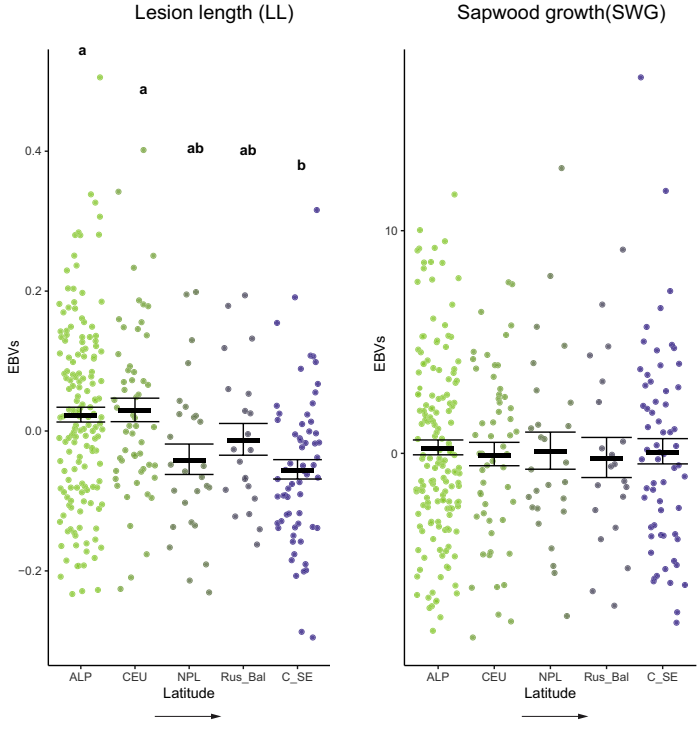

FIGURE 2 Effect of tree origin on estimated breeding values (EBVs) for resistance traits against *H. annosum s.s.* Horizontal bars represent mean and standard error. Half-sib families are grouped according to the predicted origin of their mother, sorted from southern latitudes (green, right-most) to northern latitudes (purple, left-most). ALP, Alpine; CEE, Central Europe; NPL, North Poland; Rus_Bal, Russian Baltic; C_SE, Central and South Sweden. EBVs for LL are in logarithmic scale. Letters represent significant differences according to a pairwise *t* test ($p < .005$)

TABLE 3 SNPs associated with lesion length (LL) sapwood growth (SWG) and both phenotypes together (LL_SWG) after infection with H.annosum (Ha)

Trait	Position	Substitution	Allele frequency	*p*-value[a]	PVE[b]	Variant	Description
Ha LL	MA_10426244_14899	A/G	0.064	9.83E−05	0.045	Downstream gene variant	Soluble inorganic pyrophosphatase chloroplastic-like
Ha LL	MA_10433173_9796	A/C	0.067	3.48E−05	0.05	Nonsynonymous variant	Pentatricopeptide repeat-containing chloroplastic
Ha LL Ha LL_SWG	MA_18641_10534	C/T	0.356	6.56E−05	0.047	Synonymous variant	Unknown
Ha LL Ha LL_SWG	MA_38687_10189	T/C	0.362	7.74E−06	0.051	Nonsynonymous variant	Pentatricopeptide repeat-containing mitochondrial-like
Ha LL Ha LL_SWG	MA_38687_8846	G/A	0.364	9.57E−06	0.057	Synonymous variant	Pentatricopeptide repeat-containing mitochondrial-like
Ha LL Ha LL_SWG	MA_38687_8852	C/G	0.364	9.57E−06	0.057	Synonymous variant	Pentatricopeptide repeat-containing mitochondrial-like
Ha LL Ha LL_SWG	MA_38687_8951	C/T	0.365	6.07E−06	0.060	Synonymous variant	Pentatricopeptide repeat-containing mitochondrial-like
Ha LL Ha LL_SWG	MA_10426146_6141	G/C	0.224	3.15E−05	0.051	Downstream gene variant	Tetraspanin−18-like isoform X2
Ha SWG	MA_100805_9561	A/G	0.086	5.64E−05	0.048	Synonymous variant	Subtilisin-like protease
Ha SWG	MA_10436386_12609	C/T	0.423	5.52E−05	0.048	Upstream gene variant	Villin−3 isoform X1
Ha SWG	MA_99821_7939	A/G	0.148	3.83E−05	0.05	Synonymous variant	Ethylene-responsive transcription factor CRF2
Ha SWG Ha LL_SWG	MA_10293670_1990	C/G	0.064	3.74E−06	0.062	Upstream gene variant	Unknown
Ha SWG Ha LL SWG	MA_10426146_6062	C/T	0.058	2.14E−05	0.053	Synonymous variant	Tetraspanin−18-like isoform X2
Ha SWG Ha LL SWG	MA_10432243_9511	T/C	0.13	5.44E−06	0.06	Upstream gene variant	Splicing factor SF3a60 homologue
Ha LL SWG	MA_10428968_12845	T/C	0.114	9.06E−05	-	Upstream gene variant	Phosphoenolpyruvate phosphate translocator chloroplastic
Ha LL SWG	MA_172610_8287	G/T	0.05	9.37E−05	-	Upstream gene variant	Expansin A10
Ha LL SWG	MA_27152_21720	A/G	0.071	3.23E−05	-	Upstream gene variant	RAE1
Ha LL SWG	MA_64875_14168	G/T	0.147	9.25E−05	-	Upstream gene variant	Cytokinin hydroxylase-like

[a]*p*-value based on Wald tests.

[b]Proportion of variance explained by the variant. Only calculated for unitrait GWAS.

3.3 | Multitrait GWAS identifies loci with pleiotropic effects on resistance in Norway spruce

In order to test if loci have pleiotropic effects on the same trait for resistance to both pathogens, a multitrait GWAS was performed in 220 half-sib families. Considering the same significance threshold as above ($p < 1.10^{-5}$), 12 SNPs were found to be associated with LL and 7 with SWG (Figure 3; Table 4). We then investigated correlations in allele effect sizes by plotting the effect sizes of all SNPs for resistance to *H. parviporum* as a function of their respective effect sizes in resistance to *H. annosum s.s.* (Figure 3). The SNPs were classified as belonging to two main categories (i) those with the same effect size direction for both pathogens (synergistic pleiotropy) (Figure 3, upper-left and lower-right; Table 4); and (ii) those with opposite effect sizes, (antagonist pleiotropy) (Figure 3 lower-left and upper-right). For instance, MA_97119:12145 in the *PaLAC5* gene has synergistic pleiotropic effect for LL to both pathogens (Figure 3b; Table 4). Two loci with SNP variants positively associated with SWG after inoculation *H. annosum s.s.* but negatively associated after inoculation with *H. parviporum* are SNPs in an LRR-kinase receptor (MA_404302:2414) and a secoisolariciresinol dehydrogenase-like gene (MA_57399:6360) (Figure 3a). Additionally, MA_10427923:1055 (FATTY ACID EXPORT chloroplastic-like isoform x2) (Figure 3a, lower left quadrant) has a positive pleiotropic effect and is co-located within 10 centimorgans (cM) from two different SNPs found to be significant in individual GWAS for SWG for both pathogens (Figure S4; Table S2).

4 | DISCUSSION

4.1 | Resistance to *H. annosum s.s.* is under genetic control, but not correlated to resistance to *H. parviporum* in Norway spruce

Resistance traits to *H. annosum s.s.* were found to be quantitative, heritable, and under strong genetic control with high narrow-sense heritability estimates (0.49 for LL and 0.69 for SWG). The narrow-sense heritability values obtained in this study are high and in line with previous studies for resistance against *H. parviporum* (Arnerup et al., 2010; Chen et al., 2018; Karlsson & Swedjemark, 2006; Skrøppa et al., 2015; Swedjemark & Karlsson, 2004). Contrary to our expectations, resistance traits to *H. annosum s.s.* were not significantly correlated to the same resistance traits to *H. parviporum* based on the 269 half-sib families phenotyped after artificial inoculations with both pathogens (Table 2). This could be explained by differences in the pathogens' life strategy (Garbelotto & Gonthier, 2013; Hu et al., 2020; Oliva et al., 2011, 2013), and by the ability of *H. annosum s.s.* to infect *Pinus*, using mechanisms that could also be effective when infecting *Picea* (Dalman et al., 2013), but which are absent in *H. parviporum*. Alternatively, different environmental variables during the years the two experiments were conducted could have introduced variation that we cannot account for in our experimental design. For example, in *Quercus robur* resistance traits to *Erysiphe alphitoides* measured over different years were poorly correlated as well (Bartholomé et al., 2020). Consequently, the observed quantitative resistance to *H. annosum s.s.* and *H. parviporum* is likely to be dependent on both the environment in which infections take place and the genetic variation in resistance, which may have evolved independently to both *Heterobasidion* species.

The LL in response to *H. annosum s.s.* inoculation was significantly different in different genetic clusters of Norway spruce and followed a latitudinal cline; with mother trees from the Alpine domain having the longest lesions and trees from Southern and central Sweden being the most resistant in response to *H. annosum s.s.* (Figure 1), but not to *H. parviporum* (Figure S2). This is, to the best of our knowledge, the first time that a difference between tree origins has been observed in the interaction between a conifer and *H. annosum s.l.* (Bodles et al., 2007), although provenance effects on disease resistance have been reported for other forest pathogens (Hamilton et al., 2013; Perry et al., 2016). Moreira et al. (2014) observed that the level of constitutive defence in pines increases in species from higher latitudes and colder environments and is negatively correlated with early plant growth (Moreira et al., 2014). In Norway spruce quantitative traits such as growth and spring phenology follow environmental gradients in Europe (Milesi et al., 2019) and the LL in response to *H. annosum s.s.* was positively correlated to the timing of bud-set and negatively correlated with diameter at the inoculation point, indicating that trees with later bud-set enabled the growth of longer lesions than trees which terminated their growth early and had thinner stems. Thus, it is possible that growth rhythm displayed by plants from higher latitudes with an earlier termination of growth allows for a better defence response in the bark to *H. annosum s.s.* than the faster growing plants from southern origins. It is worth noting that resistance traits in Norway spruce to *H. parviporum* are also correlated to the diameter at the inoculation point (Chen et al., 2018), but no significant difference between Norway spruce genetic clusters was observed in this interaction (Figure S2). This is possibly influenced by the fungi´s respective tissue preferences, as *H. annosum s.s.* grows preferentially in the cambium and phloem tissues, while *H. parviporum* is concentrated in the sapwood and heartwood tissues (Hu et al., 2020; Oliva et al., 2011). An interaction located in the cambium and phloem tissues would be more susceptible to seasonal changes in fluxes, as shown previously in Norway spruce (Krokene et al., 2012).

4.2 | Novel gene models associated with resistance traits against *H. annosum s.s.*

Novel QTLs associated with resistance traits to *H. annosum s.s.* were found, four of which were exclusively found using multitrait associations (Figure 3; Table 3). Recent use of multitrait GWAS in plant systems have proved useful in increasing the discovery power and understand the genetic make-up of complex traits such as response to stressors or leaf morphology (Chhetri et al., 2019; Thoen et al., 2017). One advantage of this method is that the analysis of different traits

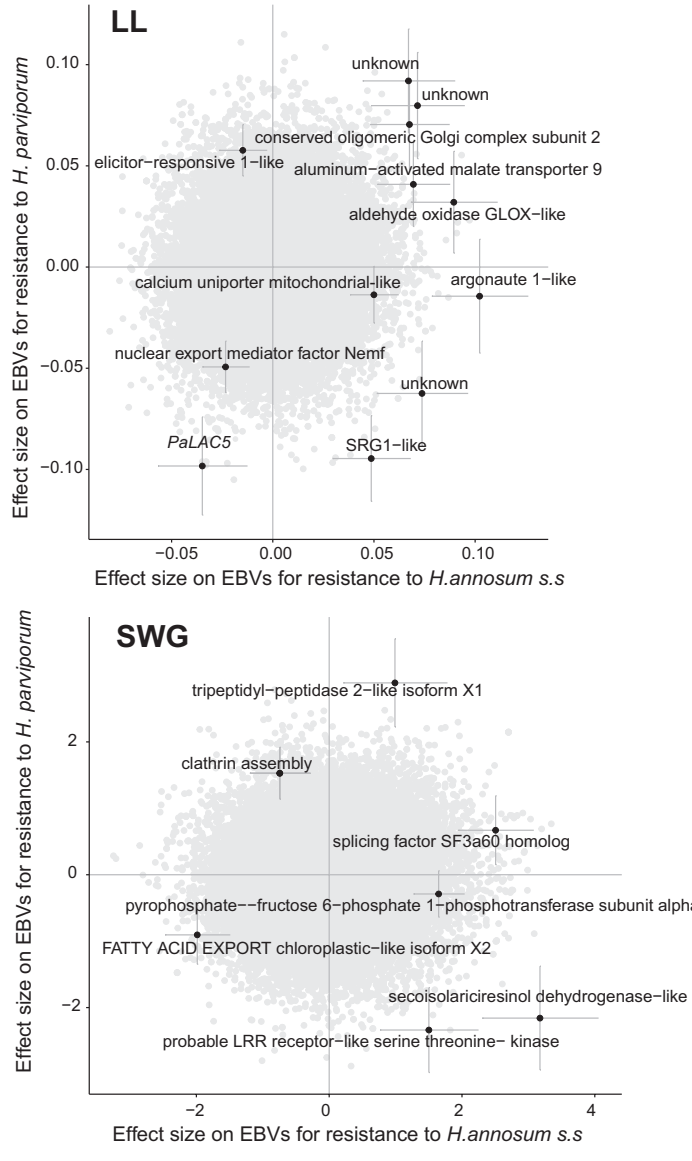

FIGURE 3 Effect size of significant SNPs in the multitrait GWAS for estimated breeding values (EBVs) for resistance traits (LL, lesion length, SWG, sapwood growth) to *H. annosum* s.s. and *H. parviporum*. Dark points represent SNPs significant after the suggested threshold and the bars behind the standard error. EBVs for LL are in logarithmic scale

together can lead to the identification of gene models that have a common effect on traits, and therefore play a central role in their regulation. Indeed, this was observed in *Arabidopsis*, where QTLs associated with multiple stressors were often involved in hormone signalling processes (Thoen et al., 2017). The GWAS of *H. annosum s.s.* resistance traits identified three Norway spruce orthologues of genes in angiosperm ABA and cytokinin hormone signalling pathways: *AtRAE1*, a negative regulator ABA in Arabidopsis (Li et al., 2018); a cytochrome P450 involved in the last step of the T- zeatin biosynthesis (Kiba et al., 2013), and *AtCRF2* (Cutcliffe et al., 2011). Most transcriptomic studies in response to *Heterobasidion* in Norway spruce have suggested that jasmonate is the main hormonal pathway activated (Arnerup et al., 2011, 2013; Lundén et al., 2015), but recently the role of ABA has been highlighted (Kovalchuk et al., 2019). Because of the quantitative and potentially polygenic nature of the resistance traits in Norway spruce, it is likely that hormonal cross-talking takes place in the tissues in order to deploy a successful defence response.

Interestingly, other groups of SNPs in gene models associated with *H. annosum s.s.* point to a possible small RNA-mediated defence strategy in Norway spruce. Previously, it has been shown that a large

TABLE 4 SNPs associated to the same traits (lesion length (LL) and sapwood growth (SWG)) in both *H.annosum s.s.* and *H. parviporum*

Trait	Position	Substitution	Allele frequency	Variant	Description
LL	MA_10243484_2131	T/G	0.106	Upstream gene variant	Aluminum-activated malate transporter 9
LL	MA_10428147_25653	C/T	0.086	Upstream gene variant	Conserved oligomeric Golgi complex subunit 2
LL	MA_10432585_12933	T/C	0.333	Upstream gene variant	Elicitor-responsive 1-like
LL	MA_10435193_11103	G/A	0.063	Missense variant	Unknown
LL	MA_10435979_27030	C/T	0.423	Missense variant	Calcium uniporter mitochondrial-like
LL	MA_18424_36662	A/G	0.063	Missense variant	Unknown
LL	MA_18424_37546	G/T	0.061	Missense variant	Unknown
LL	MA_18547_38950	A/G	0.07	Synonymous variant	Argonaute 1-like
LL	MA_2971_22606	G/A	0.063	Synonymous variant	aldehyde oxidase GLOX-like
LL	MA_922824_4364	T/C	0.113	Upstream gene variant	SRG1-like
LL	MA_97119_12145	C/A	0.077	Upstream gene variant	*PaLAC5*
LL	MA_9987602_612	A/G	0.375	Downstream gene variant	Nuclear export mediator factor Nemf
SWG	MA_10427923_1055	C/T	0.196	Missense variant	Fatty acid export chloroplastic-like isoform X2
SWG	MA_10432243_9511	T/C	0.13	Upstream gene variant	Splicing factor SF3a60 homologue
SWG	MA_138196_4550	A/T	0.077	Downstream gene variant	Tripeptidyl-peptidase 2-like isoform X1
SWG	MA_404302_2414	A/C	0.066	Upstream gene variant	Probable LRR receptor-like serine threonine-kinase At1g56140
SWG	MA_57399_6360	T/C	0.056	Missense variant	Secoisolariciresinol dehydrogenase-like
SWG	MA_736502_3531	A/C	0.457	Upstream gene variant	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha
SWG	MA_8778565_5315	A/G	0.255	Synonymous variant	Clathrin assembly
SWG	MA_8778565_5321	T/C	0.255	Synonymous variant	Clathrin assembly

number of small interfering RNAs in Norway spruce are related to nucleotide-binding site-leucine-rich repeat-type resistance genes (Källman et al., 2013). Here, we found a SNP in an *argonaute*1-like gene model associated to LL in both pathogens (Figure 3). This gene model is the orthologue of *Argonaute*1 in *Arabidopsis*, which is known to modulate defence responses against bacterial and fungal pathogens by utilising endogenous small RNAs (Ellendorff et al., 2009; Katiyar-Agarwal et al., 2006). Interestingly this regulatory pathway is also utilised by pathogens like *Botrytis cinerea*, which use their own small RNAs via *Argonaute1* to silence specific pathways in the host to establish successful infections (Weiberg et al., 2013). Given that pentatricopeptide repeat proteins and tetraspanins are also involved in RNA-mediated defence in Arabidopsis (Cai et al., 2018; Katiyar-Agarwal et al., 2006; Park et al., 2014) it is possible that candidate genes belonging to these groups, which were highlighted in this study, are involved in RNA mediated defence in Norway spruce against *H. annosum s.s.*

4.3 | Multitrait GWAS identifies pleiotropic QTLs associated with *H. annosum s.s.* and *H. parviporum*

Given that the resistance traits to *H. annosum* and *H. parviporum* had no correlation, it is not surprising that the SNPs associated with either pathogen in the univariate analysis were different (Table S1). It is worth mentioning that the exonic probes used cover only ~39% of the predicted gene models in the spruce genome (Vidalis et al., 2018) and that assembly of the genome is highly fragmented (Bernhardsson et al., 2019; Nystedt et al., 2013). There could therefore, be significant variation associated to loci that are not observed in this study. Nonetheless, multitrait GWAS was used here to identify SNPs associated with resistance traits to both pathogens. A number of SNPs had effects that contributed to resistance traits to both *H. annosum* and *H. parviporum*, resulting in a synergistic pleiotropic effect (Figure 3). Interesting examples are the three different SNPs located within 10 cM in linkage group 3 (Figure S4; Table S2). Two of these SNPs were found independently in the univariate GWAS for SWG for both pathogens and one other in the multitrait model for SWG (Figure 3, "FATTY ACID EXPORT chloroplastic-like isoform X2"). It is possible that genetic variation in the region linked to this QTL drives the positive pleiotropic effect we observe and could therefore be an example of multiple disease resistance conferred by individual genes clustered in the genome. Similarly, a SNP in *PaLAC5* with a synergistic pleiotropic effect on LL to both pathogens (Figure 3, lower-left quadrant), encodes a stress induced laccase (Koutaniemi et al., 2015; Laitinen et al., 2017) which is associated with resistance to *H. parviporum* (Elfstrand et al. 2020). This gene

is specifically and differentially expressed in tissues after infection by *H. parviporum*, and is likely to be involved in the formation of the ligno-suberized boundary zone (Elfstrand et al. 2020). Ligno-suberized boundary zone formation is a common feature of angiosperm and gymnosperm trees in response to a wide range of pathogens (Pearce, 1996; Woodward, 1992), which is in line with the synergistic pleiotropic effect observed in *PaLAC5*. Therefore, these results indicate that disease resistance to these two pathogens exists at genic level.

Another group of SNPs had the opposite effect for the same trait to the two pathogens (antagonist pleiotropy). Among the gene models harbouring such variants are an LRR-kinase receptor (MA_404302_2414) which is positively associated with resistance to *H. annosum* but negatively associated with resistance to *H. parviporum* (Figure 3, lower-right quadrant). LRR receptors with kinase functions are important components of both innate immunity and effector-triggered immunity in plants (Nürnberger & Kemmerling, 2006; Zhao et al., 2009). This particular LRR receptor harbours a conserved Malectin domain which is known to determine nonhost resistance in barley to powdery mildew strains adapted to wheat (Rajaraman et al., 2016). It is therefore possible that this LRR receptor recognises specific molecular patterns in only one of the pathogens leading to a successful defence response. Likewise, a secoisolariciresinol dehydrogenase-like gene (Figure 3, lower-right quadrant), which encodes for an enzyme involved in the production of matairesinol (Suzuki & Umezawa, 2007) had a negative pleiotropic effect. Lignans, such as matairesinol, have been shown to inhibit the activity of extracellular enzymes produced by a *H. annosum* s.l. isolate in vitro (Johansson et al., 1976; Popoff et al., 1975). In summary, SNPs associated to resistance traits to both pathogens can also have antagonistic pleiotropic effects on the infection outcome.

4.4 | Implications for disease resistance breeding in Norway spruce to *Heterobasidion* root- and stem-rot

Understanding the genetic architecture of tree resistance traits is an important task, as it will facilitate the development of resistance breeding strategies and ultimately ensure the success of reforestation programmes in the future (Buggs, 2020; Hall et al., 2016; Sniezko & Koch, 2017). *H. annosum* s.l. remains as one of the most devastating forest pathogens in the northern hemisphere and improved resistance to this species complex would be a desirable trait in breeding programmes (Garbelotto & Gonthier, 2013). Our results show that in areas where *H. parviporum* and *H. annosum* s.s. exist in sympatry, resistance to both species must be considered in prospective breeding programmes. Interestingly, we were able to show that some SNPs have a synergic pleiotropic effect, and selection based on these markers could be a useful strategy in breeding for resistance to both pathogens simultaneously. Furthermore, the significant variation in resistance to *H. annosum* s.s. with the predicted geographical origin of the mother trees indicates that disease resistance should be further studied in the ongoing assisted migration of Norway spruce trees.

5 | CONCLUSIONS

Here, we have used quantitative genetics together with exome-capture genomic data to understand the genetics behind resistance in Norway spruce to two closely related forest pathogens. The results show that resistance to *H. annosum s.s.* is quantitative, under strong genetic control and associated with variation in genes with known involvement in defence responses. Interestingly, we demonstrate that resistance traits in Norway spruce against *H. annosum s.s.* and *H. parviporum* have no correlation and are most probably the result of different underlying genetic mechanisms of resistance and/or genotype-environment interactions. Additionally, we show that resistance in bark is significantly affected by the geographic origin of the trees following a latitudinal cline in *H. annosum s.s.*, but not in *H. parviporum*.

Furthermore, we found that these uncorrelated traits are associated with genomic variation in gene models with antagonist and synergic pleiotropic effects which are potentially involved in disease resistance, such as an *PaLAC5*, an *LRR-kinase receptor* and a *secoisolariciresinol dehydrogenase*. The QTLs with a synergic pleiotropic effect are an example of multiple disease resistance at the genic level and are of special interest as they could be utilised to select for trees with higher resistance to multiple pathogens. On the other hand, markers with an antagonistic pleiotropic effect could explain why these pathogens have evolved to inhabit different niches when infecting conifers. Finally, the results of this study highlight the need for further research to understand the plasticity of resistance traits in response to different pathogens under different environments – a key aspect in the success of reforestation programmes.

ACKNOWLEDGEMENTS

We would like to thank all the seasonal workers at the Skogforsk research station in Ekebo for taking care of the plant material and phenotyping resistance traits. Authors also thank Linghua Zhou and John Baison for their advice on data analysis and Katharine King for English language editing. This study was supported with grants from the Swedish Governmental Agency for Innovation Systems (VINNOVA) (UPSC Centre for Forest Biotechnology, reference number 2016-00504), The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) (2017-00402) and the Swedish Foundation for Strategic Research (SSF), grant number RBP14-0040. The computations were performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under Project SNIC 2017/7-296 and uppstore2017112.

AUTHOR CONTRIBUTIONS

M.E., J.S., B.K. conceived the study. M.E., J.S., B.K., H.D.C.-B., I.V., and K.L. planned the study. H.D.C.-B., I.V., M.E., and K.L. performed

the experiments and phenotyped progenies. H.D.C.-B., C.B., P.K.I., and M.E. were responsible for data curation and SNP filtering. H.D.C.-B., P.K.I., M.E., and P.M. provided data analysis. M.E., J.S., P.M., and P.K.I. interpreted the results. H.D.C.-B. visualized and drafted the manuscript. M.E., J.S., B.K., H.D.C.-B., I.V., C.B., P.M., K.L., and H.X.W. commented on the manuscript. H.D.C.-B. and M.E. wrote the final MS. All authors read and approved the final version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study have been made openly available in Zenodo at https://doi.org/10.5281/zenodo.4088142.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Capador-Barreto, H. D., Bernhardsson, C., Milesi, P., Vos, I., Lundén, K., Wu, H. X., Karlsson, B., Ingvarsson, P. K., Stenlid, J., & Elfstrand, M. (2021). Killing two enemies with one stone? Genomics of resistance to two sympatric pathogens in Norway spruce. *Molecular Ecology*, 00, 1–15. https://doi.org/10.1111/mec.16058

MOLECULAR ECOLOGY

Supplemental Information for:

Killing two enemies with one stone?: Genomics of resistance to two sympatric pathogens in Norway spruce

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Table of Contents:

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Table S1 SNPs associated to every trait individually and together in the multi-trait model.

Trait	Position	Allele	Allele frequency	P value	SNP feature	Gene description
Ha bivariate	MA_10428968_12845	T/C	0.114	9.06E-05	upstream gene variant	phosphoenolpyruvate phosphate translocator chloroplastic
Ha bivariate	MA_172610_8287	G/T	0.05	9.37E-05	upstream gene variant	expansin A10
Ha bivariate	MA_27152_21720	A/G	0.071	3.23E-05	upstream gene variant	RAE1
Ha bivariate	MA_64875_14168	G/T	0.147	9.25E-05	upstream gene variant	cytokinin hydroxylase-like
Ha LL	MA_10426244_14899	A/G	0.064	9.83E-05	downstream gene variant	soluble inorganic pyrophosphatase chloroplastic-like
Ha LL	MA_10433173_9796	A/C	0.067	3.48E-05	non-synonymous variant	pentatricopeptide repeat-containing chloroplastic
Ha LL	MA_18641_10534	C/T	0.356	6.56E-05	synonymous variant	unknown
Ha LL, Ha bivariate	MA_38687_10189	T/C	0.362	7.74E-06	non-synonymous variant	pentatricopeptide repeat-containing mitochondrial-like
Ha LL, Ha bivariate	MA_38687_8846	G/A	0.364	9.57E-06	synonymous variant	pentatricopeptide repeat-containing mitochondrial-like
Ha LL, Ha bivariate	MA_38687_8852	C/G	0.364	9.57E-06	synonymous variant	pentatricopeptide repeat-containing mitochondrial-like
Ha LL, Ha bivariate	MA_38687_8951	C/T	0.365	6.07E-06	synonymous variant	pentatricopeptide repeat-containing mitochondrial-like
Ha LL,Ha bivariate	MA_10426146_6141	G/C	0.224	3.15E-05	downstream gene variant	tetraspanin-18-like isoform X2
Ha SWG	MA_100805_9561	A/G	0.086	5.64E-05	synonymous variant	subtilisin-like protease
Ha SWG	MA_10436386_12609	C/T	0.423	5.52E-05	upstream gene	villin-3 isoform X1

					variant	
Ha SWG	MA_99821_7939	A/G	0.148	3.83E-05	synonymous variant	ethylene-responsive transcription factor CRF2
Ha SWG, Ha bivariate	MA_10293670_1990	C/G	0.064	3.74E-06	upstream gene variant	unknown
Ha SWG, Ha bivariate	MA_10426146_6062	C/T	0.058	2.14E-05	synonymous variant	tetraspanin-18-like isoform X2
Ha SWG, Ha bivariate, SWG Bivariate	MA_10432243_9511	T/C	0.13	5.44E-06	upstream gene variant	splicing factor SF3a60 homolog
Hp bivariate	MA_10434138_17036	T/A	0.476	5.80E-05	synonymous variant	Drug metabolite transporter
Hp bivariate	MA_190532_5339	A/G	0.203	5.54E-05	synonymous variant	unknown
Hp bivariate	MA_9987602_529	T/C	0.387	4.07E-05	downstream gene variant	nuclear export mediator factor Nemf
Hp bivariate	MA_9987602_581	C/T	0.43	4.35E-05	downstream gene variant	nuclear export mediator factor Nemf
Hp bivariate, LL bivariate	MA_9987602_612	A/G	0.375	3.68E-05	downstream gene variant	nuclear export mediator factor Nemf
Hp bivariate, SWG Bivariate	MA_404302_2414	A/C	0.066	5.56E-05	upstream gene variant	probable LRR receptor-like serine threonine-kinase At1g56140
Hp LL	MA_10434825_2251	C/A	0.117	3.73E-05	upstream gene variant	peroxidase 25
Hp LL	MA_10434825_2326	G/A	0.121	7.54E-05	upstream gene variant	peroxidase 25
Hp LL	MA_10436325_5007	T/G	0.151	8.72E-05	upstream gene variant	nucleobase-ascorbate transporter 12
Hp LL	MA_9106_21377	A/T	0.26	4.64E-05	non-synonymous variant	polyamine oxidase 1
Hp LL	MA_9989575_4597	T/C	0.058	4.98E-05	synonymous variant	unknown
Hp LL, Hp	MA_14333_31191	T/C	0.126	2.95E	synonymo	NRT1 PTR FAMILY -like

bivariate				-05	us variant	
Hp LL, Hp bivariate	MA_14333_31287	T/C	0.126	2.95E-05	synonymous variant	NRT1 PTR FAMILY -like
Hp LL, Hp bivariate	MA_497147_4409	G/A	0.454	2.13E-05	non-synonymous variant	Blue copper oxidase
Hp LL, Hp bivariate	MA_8829511_1045	C/T	0.052	9.45E-05	synonymous variant	U-box domain-containing 33-like isoform X1
Hp SWG	MA_13984_30658	A/G	0.331	4.89E-05	upstream gene variant	uncharacterized protein LOC109792139
Hp SWG	MA_209838_1878	G/A	0.202	4.75E-05	non-synonymous variant	unknown
Hp SWG	MA_9483804_3085	C/A	0.118	8.01E-05	stop lost	transcription factor MYB34-like
Hp SWG, Hp bivariate	MA_3214_3952	G/A	0.264	3.20E-05	upstream gene variant	unknown
Hp SWG, Hp bivariate	MA_427213_6299	T/C	0.063	1.09E-05	non-synonymous variant	2-methylene-furan-3-one reductase-like
Hp SWG, Hp bivariate	MA_5480022_1363	T/C	0.064	6.12E-05	non-synonymous variant	glutathione S-transferase
Hp SWG, Hp bivariate	MA_84091_11168	G/T	0.463	8.25E-05	upstream gene variant	unknown
Hp SWG, Hp bivariate	MA_84091_11215	G/C	0.471	9.72E-05	upstream gene variant	unknown
LL bivariate	MA_10243484_2131	T/G	0.106	8.18E-05	upstream gene variant	aluminum-activated malate transporter 9
LL bivariate	MA_10435193_11103	G/A	0.063	9.55E-05	non-synonymous variant	unknown
LL bivariate	MA_18424_36662	A/G	0.063	2.32E-05	non-synonymous variant	unknown
LL bivariate	MA_18424_37546	G/T	0.061	8.92E-05	non-synonymous variant	unknown
LL bivariate	MA_18547_38950	A/G	0.07	5.91E	synonymo	argonaute 1-like

				-05	us variant	
LL bivariate	MA_2971_22606	G/A	0.063	7.36E-05	synonymous variant	aldehyde oxidase GLOX-like
LL bivariate	MA_97119_12145	C/A	0.077	9.41E-05	upstream gene variant	laccase
LL bivariate, Multivariate	MA_10428147_25653	C/T	0.086	1.83E-05	upstream gene variant	conserved oligomeric Golgi complex subunit 2
LL bivariate, Multivariate	MA_10432585_12933	T/C	0.333	8.20E-06	upstream gene variant	elicitor-responsive 1-like
LL bivariate, Multivariate	MA_10435979_27030	C/T	0.423	5.08E-05	non-synonymous variant	calcium uniporter mitochondrial-like
LL bivariate, Multivariate	MA_922824_4364	T/C	0.113	3.85E-07	upstream gene variant	SRG1-like
Multivariate	MA_10427963_43939	A/G	0.162	7.81E-05	upstream gene variant	ATP synthase subunit mitochondrial-like
Multivariate	MA_10428951_639	A/T	0.227	6.08E-05	synonymous variant	cytochrome DM13 and DOMON domain-containing At5g54830
Multivariate	MA_10428951_789	C/A	0.227	6.08E-05	non-synonymous variant	cytochrome DM13 and DOMON domain-containing At5g54830
Multivariate	MA_10430342_2416	C/T	0.133	4.38E-05	upstream gene variant	unknown
Multivariate	MA_126037_5976	T/G	0.259	5.66E-05	synonymous variant	pentatricopeptide repeat-containing mitochondrial
Multivariate	MA_9424008_2490	G/A	0.306	4.54E-05	non-synonymous variant	unknown
SWG Bivariate	MA_138196_4550	A/T	0.077	3.51E-05	downstream gene variant	tripeptidyl-peptidase 2-like isoform X1
SWG Bivariate	MA_8778565_5315	A/G	0.255	7.63E-05	synonymous variant	clathrin assembly
SWG Bivariate	MA_8778565_5321	T/C	0.255	7.63E-05	synonymous variant	clathrin assembly
SWG	MA_10427923_105	C/T	0.196	2.28E	non-	FATTY ACID EXPORT

Bivariate, Multivariate	5			-05	synonymous variant	chloroplastic-like isoform X2
SWG Bivariate, Multivariate	MA_57399_6360	T/C	0.056	1.27E-05	non-synonymous variant	secoisolariciresinol dehydrogenase-like
SWG Bivariate, Multivariate	MA_736502_3531	A/C	0.457	4.29E-05	upstream gene variant	pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha

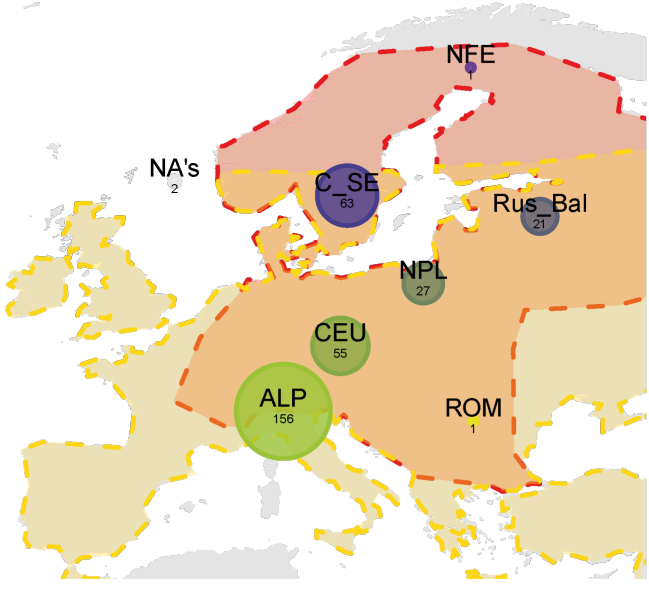
MOLECULAR ECOLOGY

Table S2 Location of scaffolds bearing significant SNP's in the linkage map for all traits.

LG	Position (cM)	Scaffold	Trait
1	317.5	MA_64875	Ha_LL_SWG
2	255.4	MA_922824	bi_LL, multi
3	153.86	MA_99821	Ha_SWG
3	156	MA_3214	Hp_SWG, Hp_LL_SWG
3	163.84	MA_10427923	biSWG
4	4.75	MA_10432585	bi_LL, multi
4	159.19	MA_10435193	bi_LL
4	178.48	MA_9483804	Hp_SWG
5	2.64	MA_427213	Hp_SWG, Hp_LL_SWG
5	191.45	MA_10435979	bi_LL, multi
6	2.88	MA_10436325	Hp_LL
6	56.83	MA_13984	Hp_SWG
6	158.5	MA_10427963	multi
6	161.7	MA_5480022	Hp_SWG, Hp_LL_SWG
6	233.21	MA_10432243	Ha_SWG, Ha_LL_SWG, bi_SWG
7	88.02	MA_10426244	Ha_LL
7	113.66	MA_10428951	multi
7	153.91	MA_8778565	biSWG
7	230.41	MA_172610	Ha_LL_SWG
8	259.04	MA_10428968	HaLL_SWG
8	261.15	MA_9987602	bi_LL, HP_LL_SWG
9	3.79	MA_57399	biSWG
9	126.62	MA_8829511	Hp_LL, Hp_LL_SWG
11	35.74	MA_10428147	bi_LL
11	171.73	MA_18641	Ha_LL, Ha_LL_SWG
12	151.07	MA_100805	Ha_SWG

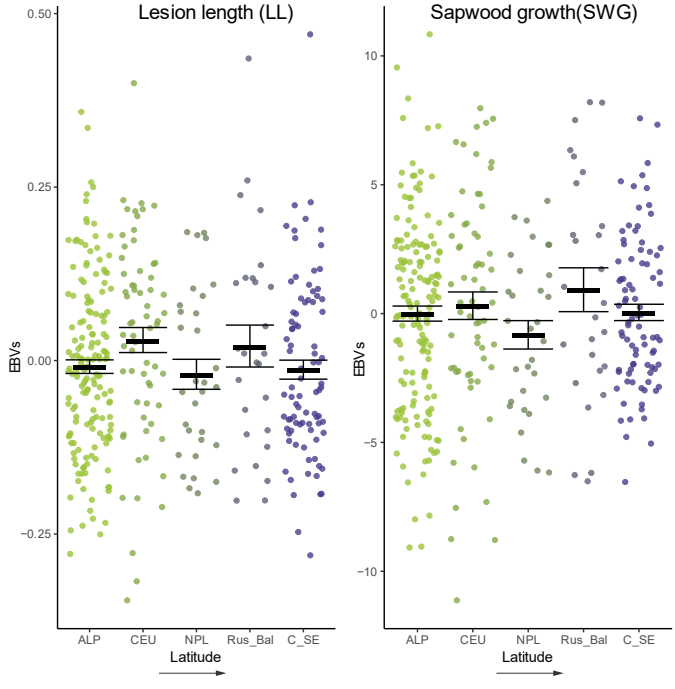
MOLECULAR ECOLOGY

Fig. S1 Pathogen distribution and tree origin assignment. Yellow area corresponds to *H. annosum s.s.,* red to *Heterobasidion parviporum*, and orange to the overlap between them. Circles represent the number of trees assigned to each cluster: ROM (Romanian), ALP (Alpine), CEE (Central Europe), NPL (North Poland), Rus_Bal (Russian Baltic), C_SE (Central and South Sweden), NFE (Fennoescandian).



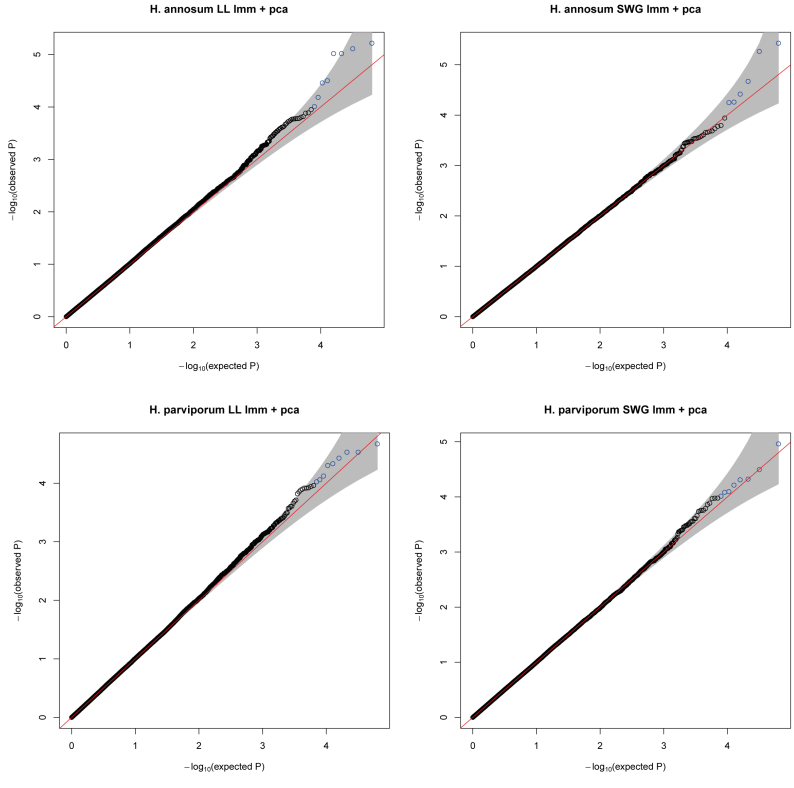
MOLECULAR ECOLOGY

Fig. S2 Effect of tree origin on estimated breeding values (EBVs) for resistance traits against *H. parviporum* . Horizontal bars represent mean and standard error. Half-sib families are grouped according to the predicted origin of their mother, sorted from southern latitudes (green, right-most) to northern latitudes (purple, left-most): ALP (Alpine), CEE (Central Europe), NPL (North Poland), Rus_Bal (Russian Baltic), C_SE (Central and South Sweden). EBVs for LL are in logarithmic scale.



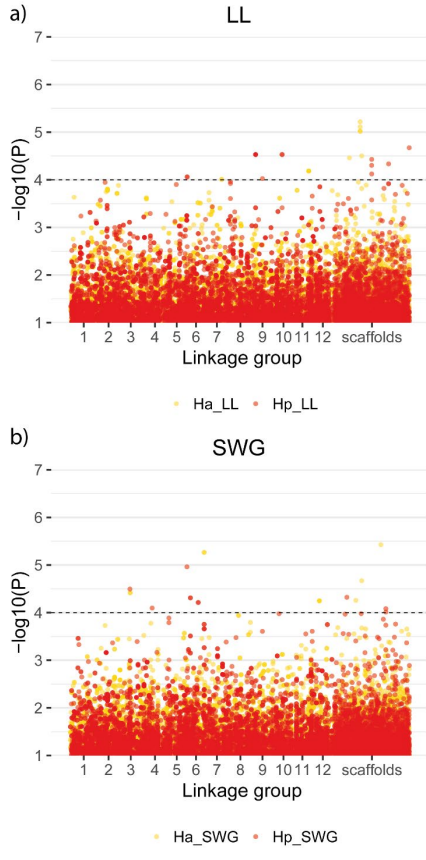
MOLECULAR ECOLOGY

Fig. S3 QQ-plots for the univariate GWAs for LL (Lesion length) and SWG (Sapwood growth) for both *H. annosum s.s.* (upper row) and *H. parviporum* (lower row). The red line is the one to one quantile, line and the grey area is the 95% confidence intervals around it. Blue points represent the significant SNPs after the suggested significance threshold.



MOLECULAR ECOLOGY

Fig. S4 Manhattan plots for univariate GWAs according to the latest linkage map (Bernhardsson et al., 2019). a) LL (Lesion length) and b) SWG (Sapwood growth) for both H. annosum s.s. (upper row) (yellow dots) and H. parviporum (lower row) (red dots). SNPs that have not been mapped to the latest map are shown in the "scaffolds" field randomly. Horizontal dashed line represents the suggested significance threshold



Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2022:3

The aim of this thesis was to study the genetic variation in disease resistance in Norway spruce. First, the genomic basis of resistance was studied with genome-wide association studies (GWAS). Also, signatures of balancing selection in *PaLAR3* were studied. Finally, transcriptional regulation was studied in different Norway spruce genotypes infected with pathogen isolates varying in virulence. This thesis advances the knowledge on disease resistance in Norway spruce and its results will support the Swedish Norway spruce breeding program.

Hernán Dario Capador-Barreto received his PhD at the Department of Forest Mycology and Plant Pathology at SLU, Uppsala. He obtained a M.Sc. in Plant Biology also at SLU, and his B.Sc. in Biology and Industrial Microbiology at the Pontificia Universidad Javeriana in Bogotá, Colombia.

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Online publication of thesis summary: http://pub.epsilon.slu.se/

ISSN 1652-6880
ISBN (print version) 978-91-7760-881-3
ISBN (electronic version) 978-91-7760-882-0