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

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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

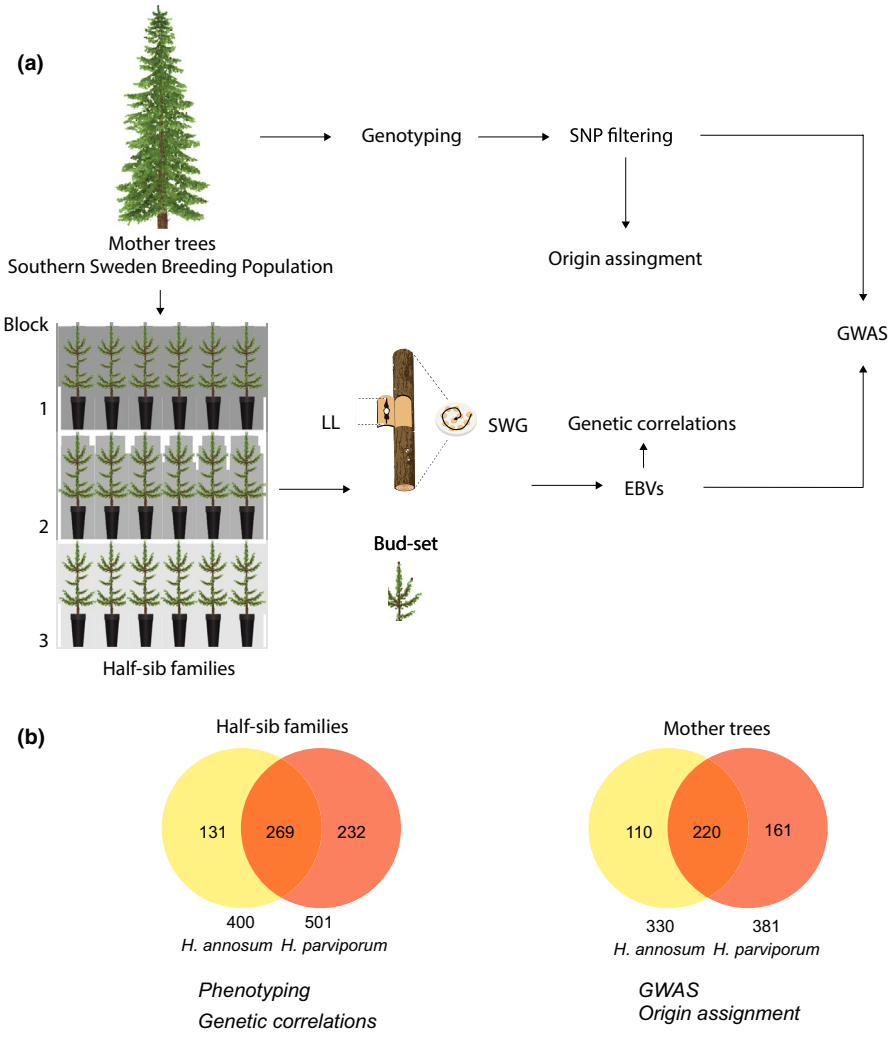

FIGURE 1 Experiment set up. (a) Genotyping and phenotyping. Mother trees were genotyped and SNPs were filtered. Thereafter tree origin prediction and GWAS was performed. Half-sib families from the genotyped mother trees where phenotyped in three different blocks. These values were used to calculate EBVs, 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	$\hat{\sigma}_a^2$	$\hat{\sigma}_e^2$	$\hat{\sigma}_p^2$	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; $\hat{\sigma}_a^2$: additive genetic variance; $\hat{\sigma}_e^2$: environmental variance; $\hat{\sigma}_p^2$: 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).

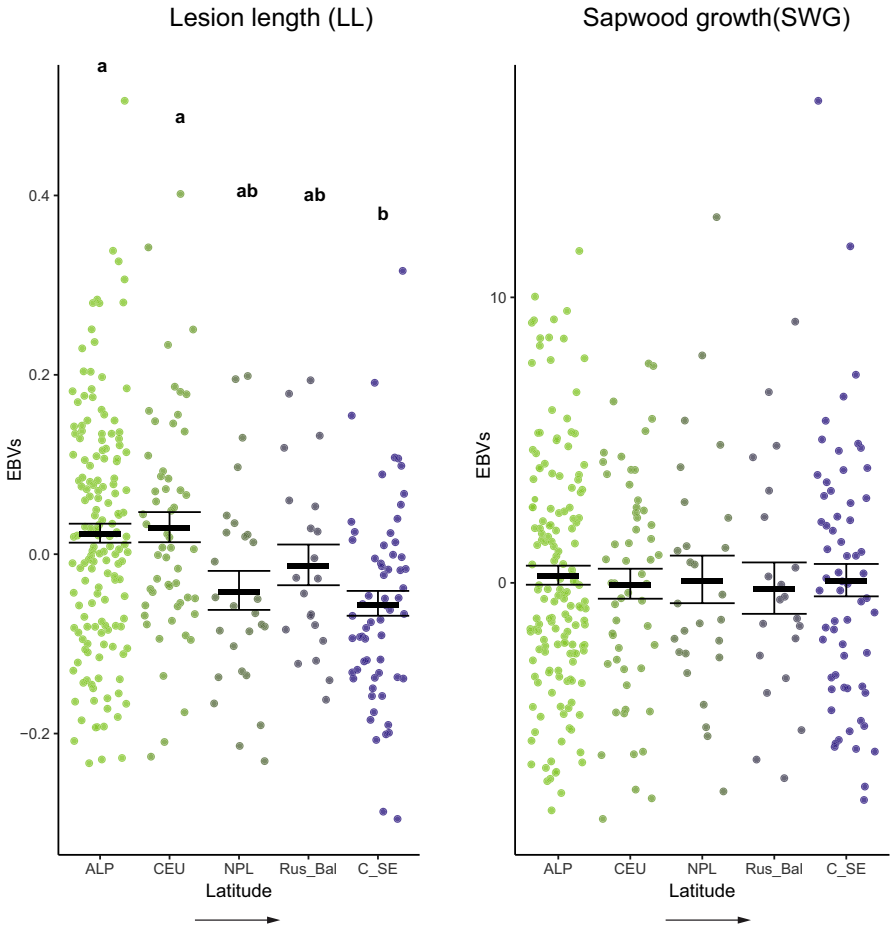

FIGURE 2 Effect of tree origin on estimated breeding values (EBVs) for resistance traits against *H. annosum s.s.* Horizontal bars represent mean and standard error. Half-sib families are grouped according to the predicted origin of their mother, sorted from southern latitudes (green, right-most) to 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

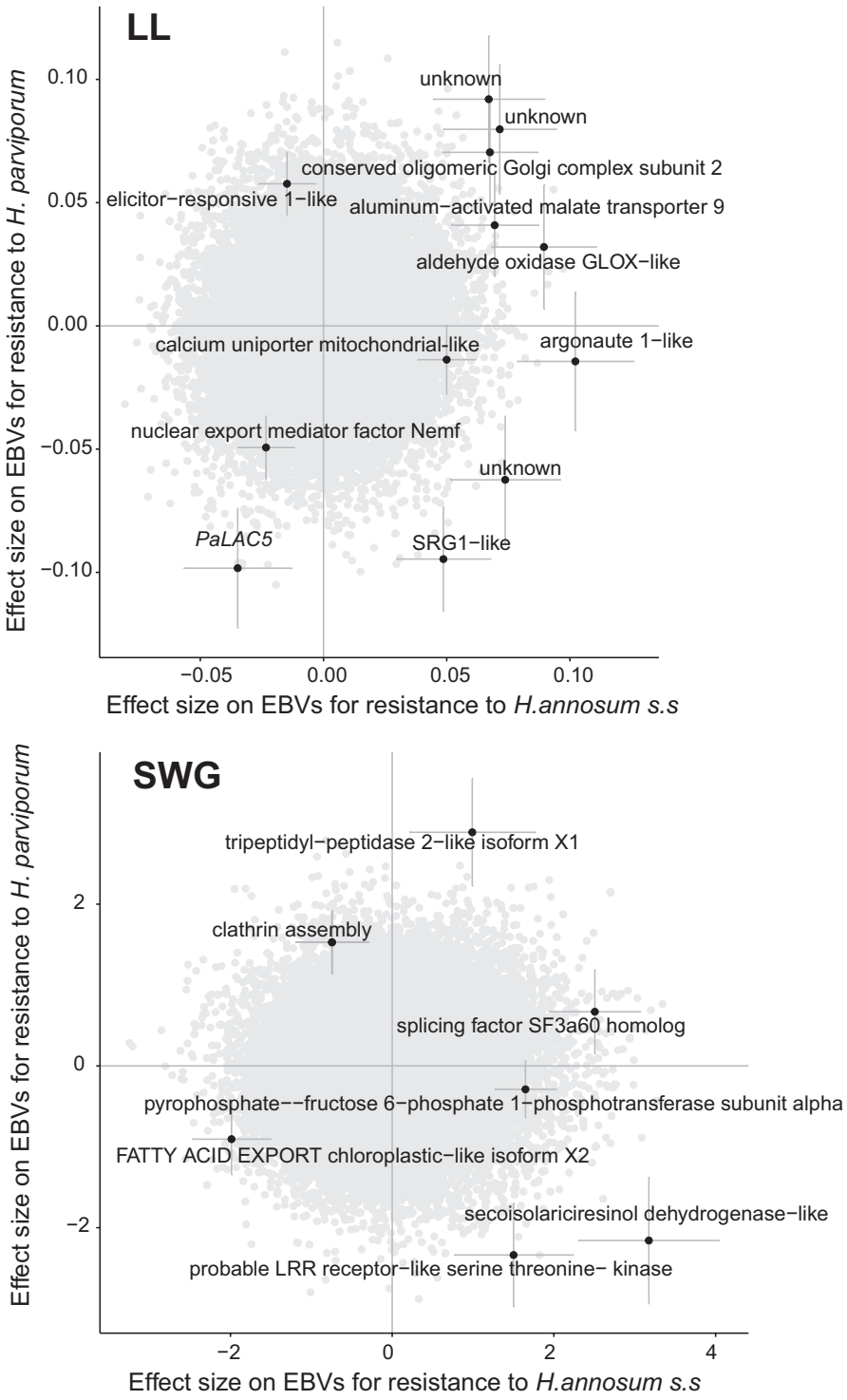

FIGURE 3 Effect size of significant SNPs in the multitrait GWAS for estimated breeding values (EBVs) for resistance traits (LL, lesion length, SWG, sapwood growth) to *H. annosum* s.s. and *H. parviporum*. Dark points represent SNPs significant after the suggested threshold and the bars behind the standard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

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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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