Genetic architecture underlying response to the fungal pathogen *Dothistroma septosporum* in lodgepole pine, jack pine, and their hybrids

Abstract

In recent decades, *Dothistroma* needle blight (DNB), a pine tree disease caused by the fungal pathogen *Dothistroma septosporum,* has severely damaged lodgepole pine (*Pinus contorta* Dougl. ex. Loud.) in British Columbia, Canada. The pathogen has already shown signs of host shift eastward to lodgepole × jack hybrid pine populations, and possibly into pure jack pine (*Pinus banksiana* Lamb.). However, we have little knowledge about mechanisms of resistance to *D. septosporum*, especially the underlying genetic basis in pines. In this study, we conducted controlled inoculations to induce infection by *D. septosporum* and performed a genome-wide case-control association study with pooled sequencing (pool-seq) data to dissect the genetic architecture underlying response in lodgepole pine, jack pine, and their hybrids. We identified candidate genes associated with *D. septosporum* response in lodgepole pine and in lodgepole × jack hybrid pine samples. We also assessed genetic structure in hybrid populations and inferred how introgression may affect the distribution of genetic variation involved in *D. septosporum* response in the studied samples. These results can be used to develop genomic tools to evaluate DNB risk, guide forest management strategies, and potentially select for resistant genotypes.

KEY WORDS

Conifer, fungal pathogen resistance, F_{ST}, hybrid, introgression, pool-GWAS

1 | INTRODUCTION

Pests and pathogens constantly threaten forest trees, and their impacts are changing as climates are altered (Seidl et al., 2017; Simler-Williamson et al., 2019). Knowledge about the genetic architecture of pathogen resistance and the identified candidate resistance (R) genes provides the basis for breeding practices to improve tree resilience to forest pathogens. Genes and alleles that confer disease resistance or tolerance can eventually be used to develop genomic tools for rapidly selecting resistant genotypes (Isabel et al., 2019). Current breeding practices for forest trees involve recurrent selection, which includes repeated cycles of selection, breeding, and testing, to search for genotypes with better disease resistance, and then deployment of these selected tree genotypes (White, 2004). This takes years of effort to design and implement. Increasingly, genome editing is used to develop crops resistant to disease (van Esse et al., 2020). CRISPR/Cas9 system has been applied to enhance the disease resistance in citrus (Jia et al., 2017; Wang et al., 2019), apple (Zhou et al., 2020), and timberland species (Min et al., 2022). Given the long generation time in conifers and the frequent threats from diseases and insects, identifying R genes that can be targeted for selection or editing is a useful step in developing resistant genotypes for reforestation.

Dothistroma needle blight (DNB), which is caused by the fungal pathogen *Dothistroma septosporum,* has caused increased damage to lodgepole pine (*Pinus contorta* Dougl. ex. Loud.) in recent decades in Western Canada (Dale et al., 2011; Woods, 2003). DNB is a foliar disease of a wide range of pine trees (Gibson, 1972), with infected trees showing reddish-brown bands in needles, defoliation, and growth reduction. Historically, DNB has had only minor impacts on native forest trees in western North America (Woods et al., 2005). Recently, however, DNB is causing increased outbreak incidence and extent, which is correlated with climate change (Welsh et al., 2009). While *D. septosporum* tends to be more common in moist western forests closer to the Pacific Ocean, *Dothistroma*-like symptoms were observed in drier lodgepole and lodgepole × jack hybrid pines in northern Alberta in 2012 and 2013 with further tests confirming *D. septosporum* was the cause of the disease observed (Ramsfield et al., 2021). Feau et al. (2021) performed a controlled inoculation experiment and demonstrated that lodgepole pine, jack pine (*Pinus banksiana* Lamb.), and their hybrids are susceptible to *D. septosporum*, with jack pine showing a higher disease severity than lodgepole or hybrid pine. Although there is no report showing DNB infection in natural jack pine forests, their higher susceptibility to *D. septosporum* raises serious concern.

The history of host-pathogen coevolution might explain the stronger resistance to DNB infection in lodgepole than in jack pine. A previous study has identified candidate resistance genes by RNA-seq analysis on experimentally infected lodgepole pine seedlings (Lu et al., 2021). These candidate genes include 43 genes that are present in the plant-pathogen interaction pathway, as well as nine R genes that contained sites under positive selection. It seems that both constitutive (baseline) and induced (defenses activated after being attacked) defenses have been developed in lodgepole pine. Moreover, the host-pathogen coevolution can promote a broad-spectrum resistance (Hulse et al., 2023). It is likely that the induced and increased general defense have contributed to the lodgepole pine's resistance to DNB infection, however, little is known about resistance in jack pine. Previous studies have compared the molecular responses between lodgepole and jack pine when subjected to other biotic agents. Lodgepole and jack pine showed unique monoterpene profiles in response to mountain pine beetle (Hall et al., 2013) and significant differences in chitinase gene expression in response to the fungal pathogens *Grosmannia clavigera* and *Cronartium harknessii* (Peery et al., 2021). These previous studies used particular gene families to examine the species-specific pathogen responses in a focused set of candidates, whereas a genome-wide gene survey might give us a broad perspective.

To dissect the genetic architecture of pathogen resistance and to identify candidate genes, researchers have employed genetic markers, mainly SNPs, and methods like quantitative trait locus (QTL) mapping and genome-wide association analysis (GWAS). Polygenic disease responses, comprising numerous loci of small effect, appear to be the rule (Stocks et al., 2019). Large numbers of SNPs were associated with the pitch canker resistance in *Pinus taeda* L. (De La Torre et al., 2019; Lu et al., 2017; Quesada et al., 2010), with the white pine blister rust resistance in *Pinus lambertiana* Dougl. (Weiss et al., 2020), and with the *Heterobasidion* root rot resistance in *Picea abies* (L.) Karst. (Capador-Barreto et al., 2021). To identify the candidate R genes, most GWAS studies on forest trees employ individual-based methods, with both phenotypic and genotypic data collected on each sampled individual. Case-control methods have been developed to study disease where individuals are pooled into healthy vs. infected groups, but such approaches typically still use individual-based sequencing to call genotypes before

pooling (Wu et al., 2010). When multiple replicates are available, it is possible to combine pool-seq and the case-control design: Endler et al. (2016) analyzed the genetic differences underlying abdominal pigmentation variation among *Drosophila* populations, while Stocks et al. (2019) identified SNPs associated with low versus high ash dieback damage in *Fraxinus excelsior* L. using this approach. Recently, Singh et al. (2024) used a case-control pool-seq approach similar to that deployed here to search for signatures of resistance to Swiss needle cast in *Pseudotsuga menziesii* (Mirb.) Franco. Pool-seq is a cost-effective alternative approach to sequencing of individuals, but the weakness and limitations are also evident, such as unequal representation of individuals and suboptimal allele frequency estimate when the pool size is small, as well as misaligned mapping and sequencing errors (Schlötterer et al., 2014). Nonetheless, in a study on conifer genotyping, Lind et al. (2022) found that allele frequencies estimated from pooled DNA sequencing samples were highly correlated with frequencies estimated from individual sequencing.

An important factor in understanding the genetics of resistance in these species is whether there is any interplay between any alleles conferring resistance and introgression between the species. Lodgepole pine mainly inhabits montane regions of western North America (Figure 1), throughout the interior of British Columbia and the Rocky Mountains of Alberta (Lotan & Critchfield, 1990), whereas jack pine is widely distributed across boreal forests in eastern North America, extending in Canada from Northwest Territories to Nova Scotia (Rudolph & Laidly, 1990). Lodgepole and jack pine evolved in allopatry, with an estimated divergence time of ~8 million years before present (Hao et al., 2015), but hybridized in areas of contact, primarily in central and northwestern Alberta (Burns et al., 2019; Rudolph & Yeatman, 1982). Following secondary contact of these two species, additional patchy regions of introgression have been developed in central Alberta north through to the Northwest Territories and northeastern British Columbia to the Alberta-Saskatchewan border (Burns et al., 2019). Natural hybrids display a wide range of phenotypes that are intermediate or more typical of one parent, depending on the level of introgression (Wood et al., 2009; Yeatman & Teich, 1969). The hybrid zone between these species has been very well studied by Cullingham et al. (2012), who found that the lodgepole × jack pine hybrid zone presents a mosaic zone with variable introgression and patchy distributions of hybrids (Burns et al., 2019). While it is clear that these species share a broad hybrid zone, it is unclear whether any alleles for disease resistance will be more or less introgressed than the average region in the genome.

In this study, our aim is to identify the genetic basis of DNB resistance in the studied pine species and to explore spatial patterning in any identified loci. The specific objectives of our study were to: 1) develop a GWAS case-control approach using pool-seq samples to identify loci with consistent associations with *D. septosporum* response across replicates; and 2) compare the genetic architecture underlying DNB resistance within the studied pine trees and infer how introgression may affect the genetic basis of *D. septosporum* responses. This study provides an important step towards identifying candidate genes to develop tools for screening trees resistant to DNB infection.

2 | METHODS

2.1 | Plant materials

Seeds were obtained from 40 natural seedlots across Western Canada (Figure 1, seedlot numbers and locations can be found in Table S1, seed contributors http://adaptree.forestry.ubc.ca/seed-contributors/), including 25 lodgepole pine seedlots from British Columbia (BC_pl) and three from Alberta (AB_pl), seven lodgepole × jack hybrid pine seedlots from Alberta (AB_px), and five jack pine seedlots from Alberta (AB_pj). The range maps were downloaded from https://sites.ualberta.ca/~ahamann/data/rangemaps.html (Hamann et al., 2005). When the seeds were collected in the wild, they were assigned to pure lodgepole pine, pure jack pine, or lodgepole × jack hybrid pine, based on their location and morphological traits such as cone and branch characteristics, microfibril angle, and cell area (Wheeler & Guries, 1987; Wood et al., 2009; Yeatman & Teich, 1969). The ancestry of the collected seeds were genotyped using 11 microsatellite loci by Cullingham et al. (2012). Briefly, seeds were germinated to obtain seedlings, then DNA was isolated from the seedlings. DNA was used to amplify 11 microsatellite loci and allele sizes were determined for genotyping as described by Cullingham et al. (2011).

For the present study, seeds from the 40 seedlots were used to grow seedlings in a greenhouse at University of British Columbia, Vancouver, BC, for their first year (a flow chart of experimental procedure is shown in Figure S1). Resistance was phenotyped by inoculating 100 individuals per seedlot with one of two *D. septosporum* isolates (D1 & D2, 50 individuals of each isolate). D1 was isolated from needles of an infected lodgepole × jack hybrid pine seedlot in Alberta, Canada, while D2 was isolated from an infected lodgepole pine seedlot in the Kispiox Valley region close to Smithers in Northwestern British Columbia, Canada (Feau et al., 2021; Ramsfield et al., 2021). The seedlots were identified as lodgepole or lodgepole × jack hybrid pine as aforementioned (Cullingham et al., 2012). The controlled inoculation experiment was performed as described in Feau et al. (2021). Briefly, the one-year-old seedlings were placed in a completely randomized experimental design in a growth chamber with the condition of 16 hours daylight at 20 °C, 8 hours a night at 12 °C, and minimum relative humidity of 80 %. *D. septosporum* conidia were harvested from colonies grown for 10~16 days on *Dothistroma* sporulation medium plates and then suspended in sterile distilled water. Each seedling was sprayed twice at 10-day interval with a standard inoculum of approximately 3 mL of 1.6×10^6 conidia mL^{-1}, using a household trigger action atomizer. Once the needles were dry (~45 minutes), the seedlings were wrapped in transparent plastic bags and kept in the growth chamber. After 48h the plastic bags were removed and the seedlings were kept in the growth chamber for 15 weeks till phenotyping. A mister spraying tap water was activated hourly for 3 minutes during the trial to maintain needle wetness. Control seedlings, at least one for each seedlot, were inoculated with sterile water.

After 15 weeks since the beginning of the experiment, seedlings were rated for proportion of necrotic needles with red bands and/or fruiting bodies according to a disease severity scale of 1 to 5 (1 = least, 5 = most disease severity). Following disease rating, the ten most- and ten least-infected individuals per seedlot (five individuals for each *D. septosporum* isolate) were identified and retained for DNA extraction. Genomic DNA of each of these individuals was extracted as described in Lind et al. (2022), using the Nucleospin 96 Plant II Core kit (Macherey–Nagel GmbH & Co. KG, Germany) on an Eppendorf epMotion 5075 liquid-handling platform. The DNA of five most-infected and five least-infected individuals by each of the two *D. septosporum* isolates was combined in equimolar amounts to compose four pooled libraries per population,

two susceptible (D1-S, D2-S) and two resistant libraries (D1-R, D2-R). Hence,160 pooled DNA libraries were generated.

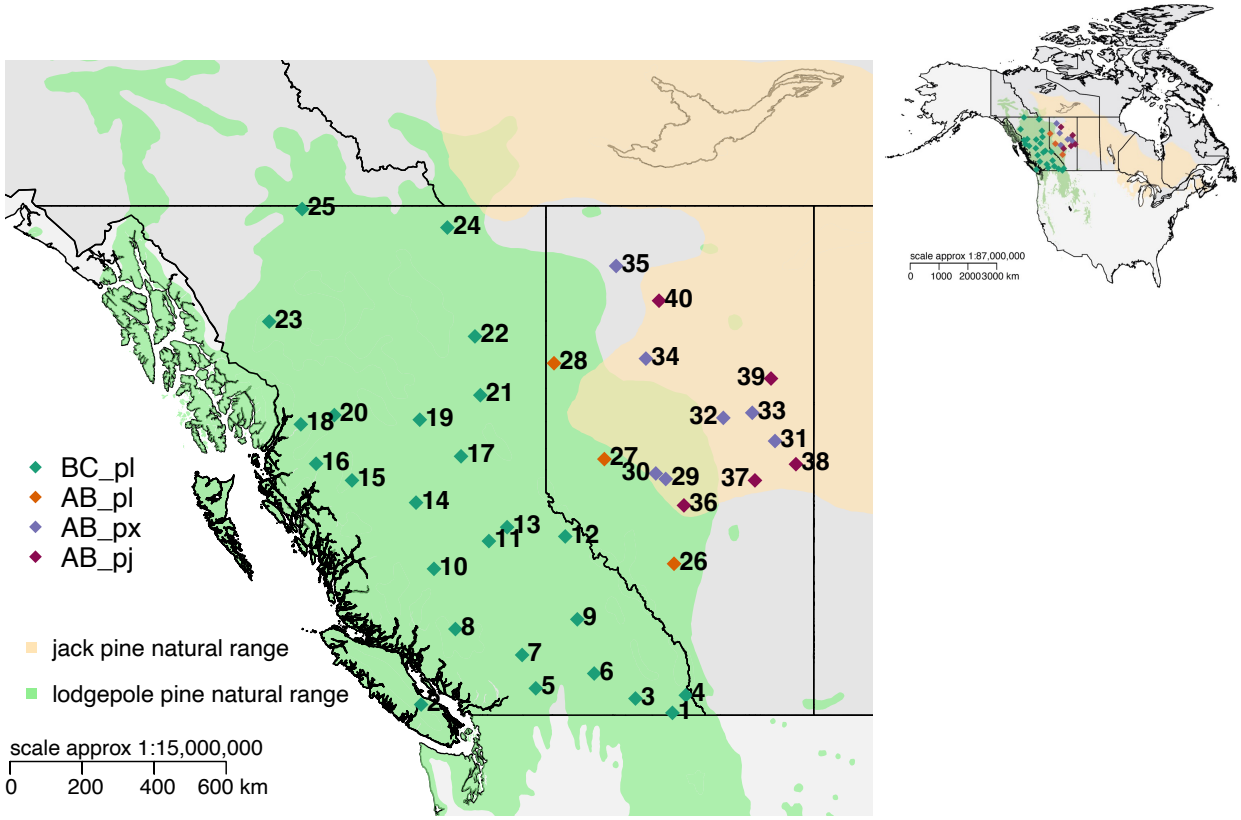


FIGURE 1 Sampling locations (Table S1) of the 40 pine seedlots analyzed in this study, plotted within the natural range of lodgepole and jack pine in Canada and USA. The topright map shows the context of the sampling locations. The range maps were downloaded from https://sites.ualberta.ca/~ahamann/data/rangemaps.html. BC_pl (green diamonds) — lodgepole pine seedlots from British Columbia (N=25); AB_pl (orange diamonds) — lodgepole pine seedlots from Alberta (N=3); AB_px (purple diamonds) — lodgepole × jack hybrid pine seedlots from Alberta (N=7); AB_pj (brown diamonds) — jack pine seedlots from Alberta (N=5).

2.2 | Sequence capture and pool-seq genotyping

The capture probes were designed based on an existing lodgepole pine sequence capture array (Suren et al., 2016) by removing the probes that did not yield successful genotyping in Yeaman et al. (2016) and adding new probes derived from newly assembled *D. septosporum*-induced genes. Probe sequences of the existing capture array were aligned to the reference genome using

204 GMAP v2019-03-15 (Wu & Watanabe, 2005). Probes that covered the genomic regions with
205 called SNPs in the dataset from Yeaman et al. (2016) were retained, otherwise the probes were
206 discarded. Since there is no available lodgepole pine reference genome, a masked loblolly pine
207 reference genome Pita.2_01.masked3k2.fa
208 (https://treegenesdb.org/FTP/Genomes/Pita/v2.01/genome/) (Neale et al., 2014) was used
209 instead. Loblolly pine is a closely related species to lodgepole pine (Jin et al., 2021). New probes
210 targeting *D. septosporum*-induced genes were designed based on a lodgepole pine reference
211 transcriptome assembled using the RNA-seq data of *D. septosporum* infected lodgepole pine
212 samples (Lu et al., 2021). To avoid duplicates, only those *D. septosporum*-induced genes with
213 low homology to the retained probe sequences were used to design the new probes. To do so, the
214 retained probe sequences were aligned to this transcriptome using blastn v2.9.0 with an E-value
215 of 1e-10. A total of 8,778 *D. septosporum*-induced genes did not have any aligned probe
216 sequences. These non-duplicate *D. septosporum*-induced genes were subsequently aligned to the
217 reference genome to predict the exon-intron boundaries using GMAP v2019-03-15. Exon
218 sequences from these induced genes with a length of at least 100 bp were combined with the
219 previously designed working probe sequences, and this combined set of sequences was
220 submitted to Roche NimbleGen (Roche Sequencing Solutions, Inc., CA USA) for Custom
221 SeqCap EZ probe design (design name: 180321_lodgepole_v2_EZ). This updated lodgepole pine
222 sequence capture array has a capture space of 44 Mbp, containing roughly 35,467 assembled
223 genes. Most lodgepole pine genes responsive to environment stress and fungal pathogen attack
224 were included in the current capture probe design, though genes expressed in different
225 development periods might be missed, genes that have evidence of substantial expression have
226 been covered in this capture probe design.
227
228 The capture libraries for each of the 160 pools were constructed following NimbleGen SeqCap
229 EZ Library SR User's Guide and as described in Lind et al. (2022). Then the R (resistant) and S
230 (susceptible) libraries (two libraries per capture, R1+S1 or R2+S2, indexed with different
231 barcodes) per population and per isolate were combined for sequence capture and enrichment.
232 Sequencing was performed using the Illumina NovaSeq 6000 S4 PE 150 platform in Centre
233 d'expertise et de services Génome Québec. Our in-house pool-seq pipeline (Lind, 2021) was
234 employed to align the reads to the reference genome and call SNPs. For raw SNPs, only bi-allelic
235 loci in regions without annotated repetitive elements or potentially paralogous genes were
236 retained. The annotated repetitive elements were acquired from the loblolly pine genome
237 annotation (Wegrzyn et al., 2014). The potentially paralogous genes were identified as described
238 in Lind et al. (2022) using haploid megagametophyte sequences, for the heterozygous SNP calls
239 for haploid sequences are likely to represent misalignments of paralogs. Afterwards, the SNP
240 loci with depth (DP) < 10, DP > 400, global minor allele frequency < 0.05, or > 25% missing
241 data were also removed.
242
243 **2.3 | Genetic structure analyses**
244
245 The genetic structure among the 160 pooled samples, which represented four pooled libraries
246 (D1-R, D1-S, D2-R, D2-S) for each of the 40 populations, was detected by using principal
247 component analysis (PCA), reconstructing a phylogenetic tree, and by assessing correlation of
248 allele frequencies and population differentiation (F_{ST}) between samples using unlinked SNPs. To
249 reduce the impact of linkage disequilibrium on estimation, the SNP set was thinned using

vcftools (Danecek et al., 2011), so that no two sites were within 100,000 bp. PCA and genetic distance were calculated using the major allele depth and the R package "adegenet" (Jombart, 2008). An unrooted phylogenetic tree was constructed using the distance output and the Neighbour-Joining algorithm, which was implemented by the R package "ape" (Paradis & Schliep, 2019). A bootstrap analysis was performed using 5000 bootstrap replicates. Correlation coefficients of major allele frequencies between all pairs within the 160 samples were calculated and plotted using the R package "corrplot" (Wei & Simko, 2017). F_{ST} was estimated between all pairs within the 160 samples using the R package "poolfstat" (Hivert et al., 2018).

2.4 | Dissection of genetic architecture underlying *D. septosporum* response

The alleles and candidate genes associated with *D. septosporum* response were detected using the pool-GWAS method. A GWAS case-control approach was developed using pool-seq samples to identify allele frequency differences between susceptible and resistant pines inoculated with *D. septosporum*. This method was based on Cochran-Mantel-Haenszel (CMH) method and implemented in our in-house pipeline (Lind, 2021). While sequencing read depth was commonly used as an estimation of allele count in such applications of test (Futschik & Schlötterer, 2010; Schlötterer et al., 2014), reliable results would be only achieved when the haploid size of the sample is much greater than the depth of coverage (so that most reads arise from uniquely sampled haplotypes). As the pool size used in the present study (diploid size of 2N=10) is often smaller than the sequencing depth of coverage, read counts were converted into allele frequencies and then multiplied by the ploidy to yield an approximate count for each pool that represents the real replication level. A simulation was conducted to compare the false positive rate from the CMH test using uncorrected or corrected allele counts across a range of number of demes, individuals, and depths. Results showed that the false positive rate of the CMH method is high, especially when the sequencing depth is much higher than the pool size (Figure S2a). Using corrected allele counts, the allele frequencies multiplied by the ploidy (N=10) tends to lower the false positive rate (Figure S2b). Thus, the corrected allele counts were used for CMH test.

As the genetic structure analysis showed that lodgepole × jack hybrid pine and jack pine samples tend to cluster, jack pine samples and lodgepole × jack hybrid pine samples were combined as jack-hybrid samples for downstream analyses. Pool-GWAS was performed separately within lodgepole and jack-hybrid pine samples. Analysis of R and S samples was conducted by combining individuals inoculated with D1 or D2 isolates to maximize statistical power. To identify candidate genes, SNPs in the top 1% of $-\log_{10}(p)$ values from a pool-GWAS test were classified as outliers. As linkage disequilibrium can amplify signatures of selection, candidate resistance genes were identified as those with a large number of outlier SNPs relative to the average genome-wide expectation, as represented by an index based on the binomial distribution as per Yeaman et al. (2016). The identified loci on the enriched genes were realigned to the 12 lodgepole pine linkage groups (MacLachlan et al., 2021) and Manhattan plots were drawn. The lodgepole pine transcriptome (Lu et al., 2021) was used to annotate the reference genome. The aligned transcripts to the reference genome were identified as those with a minimum alignment identity of 90 % and a minimum alignment coverage of 85 % using GMAP v2019-03-15.

296 **2.5 | Linkage disequilibrium (LD) between loci**
297
298 A non-linear model (Hill & Weir, 1988) was used to estimate the decay of LD with physical
299 distance. Pairwise correlation (r^2) of allele frequencies between loci on the same scaffold were
300 calculated. The r^2 values and the physical distances between loci on all scaffolds (genome-wide)
301 or on scaffolds containing the identified top candidate genes (significant) were used to fit the
302 non-linear model as described by Marroni et al. (2011). The LD decay graph was plotted using R
303 (R Core Team, 2024).
304
305 **2.6 | Introgression**
306
307 To study patterns of introgression affecting different regions of the genome and among different
308 samples, the mean pairwise F_{ST} was calculated for each locus using the R package "poolfstat".
309 Loci with high F_{ST} values have high genetic differentiation, which may represent species barriers
310 or genomic regions under divergent selection, while loci with low F_{ST} values imply
311 introgression. To estimate the genetic similarity of studied jack pine and lodgepole pine
312 populations, the five jack pine samples (p36 to p40) and six pure lodgepole samples (the six
313 westmost lodgepole pine samples, p15, p16, p18, p20, p23, p25) were used to calculate F_{ST}
314 values, which were then averaged. To evaluate whether regions associated with *D. septosporum*
315 response had atypical patterns of introgression, the F_{ST} values of the top 1 % *D. septosporum*
316 response outliers identified from lodgepole pine (N = 3,358) and those from jack-hybrid pine (N
317 = 3,041) were compared, with F_{ST} values of randomly drawn SNPs (N = 3,400).
318
319 Similarly, F_{ST} between the jack-hybrid samples (p29 to p40) and six pure lodgepole samples
320 (p15, p16, p18, p20, p23, p25) were averaged to estimate the genetic similarity of studied jack-
321 hybrid and lodgepole pine. Different genomic regions were represented by different SNP sets,
322 including *D. septosporum* response outliers identified from lodgepole pine (N = 3,358) and
323 outliers identified from jack-hybrid pine (N = 3,041), as well as the unlinked SNP set (N =
324 31,716), which was used for detecting genetic structure and representing random genomic
325 region. The genetic similarity (F_{ST}) of jack-hybrid and lodgepole pine was regressed to the
326 longitude of these jack-hybrid samples. The regression slopes for the three SNPs sets were
327 compared using analysis of covariance method implemented by the R package "lsmeans" (Lenth,
328 2016; R Core Team, 2024).
329
330
331 **3 | RESULTS**
332
333 **3.1 | Patterns of genetic structure**
334 After filtering, a total of 364,691 SNP loci were retained for downstream analyses, and most loci
335 had a minor allele frequency between 0.05 and 0.1 (Figure S3). A thinned set of 31,716 unlinked
336 SNPs was used for studying genetic structure among the 160 pooled samples (Figure 2). The
337 most prominent patterns of structure were associated with species (Figure 2a & b, Figure S4),
338 with lodgepole pine samples readily distinguishable from jack and hybrid pine samples. Hybrid
339 samples clustered more closely with jack than lodgepole pine. Compared to those sampled from
340 the eastern hybrid zone, western hybrid pines tend to be more similar to lodgepole pine (samples
341 were arranged according to longitude from left to right in Figure 2 c & d). Given this clear

pattern of genetic structure, we conducted GWAS in lodgepole pine and jack-hybrid pine samples separately.

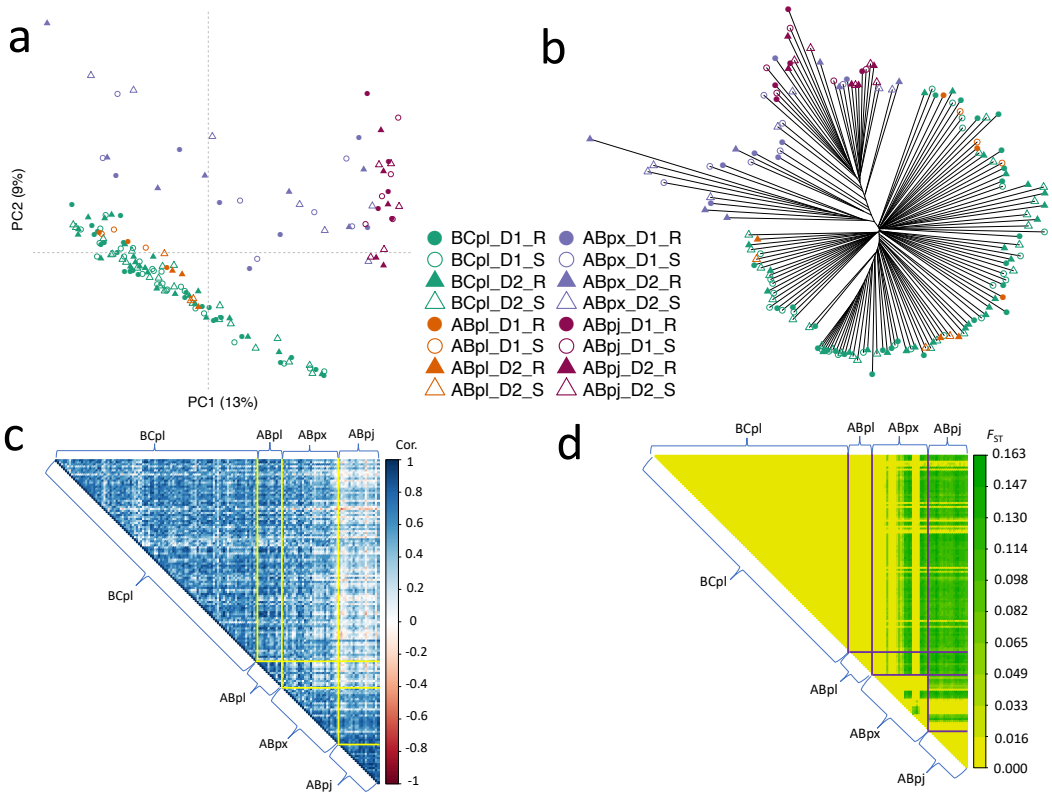
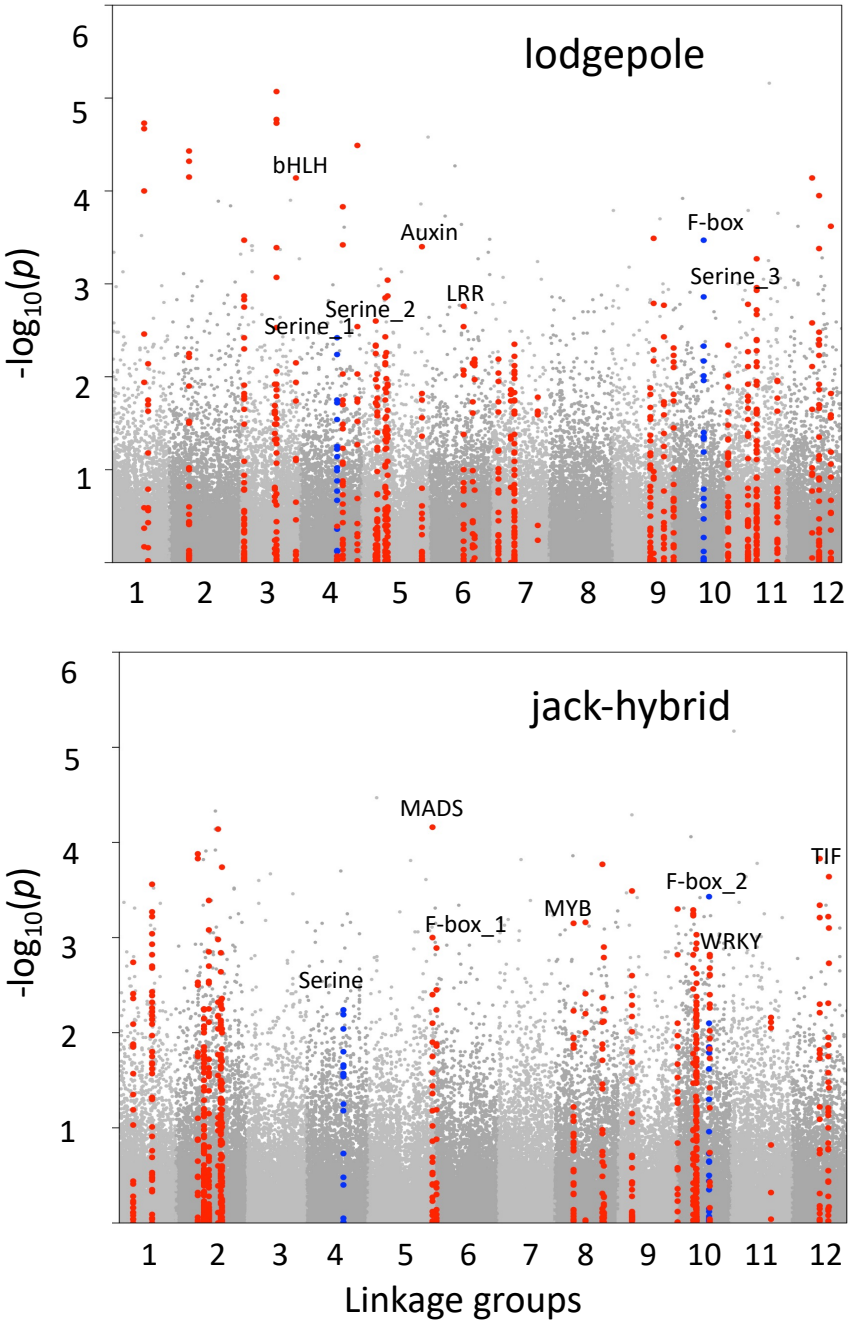


FIGURE 2 Genetic structure among the 160 pooled samples. a) Principal component analysis. b) Unrooted phylogenetic tree. The bootstrap values for each branch were shown in Figure S4. c) Correlation plot of the major allele frequencies between samples. d) Pairwise F_{ST} values between pools. BC_pl — lodgepole pine samples from British Columbia; AB_pl — lodgepole pine samples from Alberta; AB_px — lodgepole × jack hybrid pine samples from Alberta; AB_pj — jack pine samples from Alberta. D1 — *D. septosporum* isolate 1; D2 — *D. septosporum* isolate 2. R — resistant tree; S — susceptible tree. In c) and d), each column represents a pooled sample. The samples were arranged from left to right according to the longitude of sampling location, and four pooled libraries per seedlot (D1-R, D1-S, D2-R, D2-S) were arranged together.

3.2 | Signatures of association to infection response

Out of the 100 top-ranked candidate genes in each test (Table S2), three were identified within both the lodgepole and jack-hybrid pine samples (Figure 3). This is significantly more than the hypergeometric expectation, based on the probability of overlap for two draws of 100 genes from 20,026 genes. A previous study anchored 10,093 scaffolds on the lodgepole pine linkage map (MacLachlan et al., 2021), so we plotted SNPs and labeled the enriched plant-pathogen interaction candidate genes on the 12 linkage groups (Figure 3). The three genes identified in both species encode ABC transporter, F-box Kelch-repeat protein, and serine/threonine protein

367 kinase (F-box protein and serine protein kinase were highlighted in Figure 3, ABC transporter
368 genes were not included in the linkage groups, so they are not included in the figure). We also
369 checked the enrichment of differentially expressed genes (DEGs) in the top 100 ranked genes.
370 Out of 631 DEGs identified in the previous study (Lu et al., 2021), four were also candidate
371 genes in the present study. Fisher's Exact test showed there is no significant enrichment of DEGs
372 in the candidate genes.
373



374
375
376 FIGURE 3 Manhattan plot showing SNPs associated with *D. septosporum* response within
377 lodgepole and jack-hybrid pine samples. The x axis represents 12 linkage groups. The y axis

represents significance of associations identified from pool-GWAS. Candidate genes were identified using a binomial test for enrichment of outlier SNPs per gene. Candidate genes and their surrounding 50 kbp regions are highlighted in red. Of the three top-ranked candidate genes identified within both the lodgepole and jack-hybrid pine samples, one was not present in the linkage map, while the other two are highlighted in blue ("Serine" on linkage group 4 and "F-box" on linkage group 10). Labels in the graphs represent candidate genes encoding plant-pathogen interaction proteins: bHLH — bHLH transcription factor; Serine — serine/threonine-protein kinase; Auxin — auxin response factor; LRR — proteins with leucine-rich repeat; F-box — F-box Kelch-repeat protein; MADS — MADS transcription factor; MYB — MYB transcription factor; WRKY — WRKY transcription factor; TIF — translation initiation factor. The candidate gene IDs (in Table S2) are: In lodgepole pine, bHLH — Scaffold_2015-465162-569336; Serine_1 — Scaffold_3728-494595-736720; Serine_2 — Scaffold_3141-1-105566; Serine_3 — Scaffold_1072-1299306-1374553; Auxin — Scaffold_3823-1571412-1689102; LRR — Scaffold_1361-487806-570406; F-box — Scaffold_3698-733755-839782; in jack-hybrid pine, Serine — Scaffold_3728-494595-736720; MADS — Scaffold_427-2324701-2717580; F-box_1 — Scaffold_3698-733755-839782; MYB — Scaffold_4478-441537-627389; F-box_2 — Scaffold_342-1965787-2253515; WRKY — Scaffold_830-1136302-1239031; TIF — Scaffold_207-7583953-7695067.

Loci on scaffolds with outliers (the SNPs with top 1 % of -$\log_{10}(p)$ values from pool-GWAS test) tend to exhibit a more gradual decay in LD compared with the genome-wide loci on all scaffolds (Figure 4), with jack-hybrid pine outliers having a much slower decay rate than lodgepole pine. In lodgepole pine, the distance at which LD decays to half of its maximum value is 96 bp for genome-wide loci, and 174 bp for outlier loci. In jack-hybrid pine, the half LD decay distance is 1,357 bp for genome-wide loci, and 68,215 bp for outlier loci. The slower LD decay pattern for the outliers may indicate selection on these identified outlier loci.

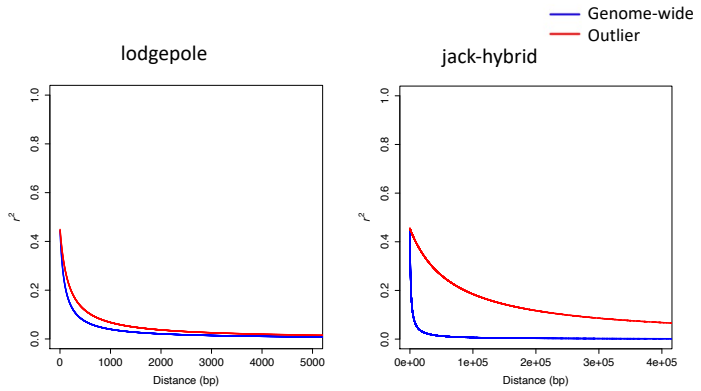


FIGURE 4 Linkage disequilibrium (LD) decay for genome-wide loci and outlier loci identified from lodgepole and jack-hybrid pine samples. The r^2 values (pairwise correlation of allele frequencies between loci) and the physical distances between loci were used to fit the non-linear model as described by Marroni et al. (2011).

3.3 | Introgression

By calculating average F_{ST} between pairs of jack pine and lodgepole pine samples, we found that the *D. septosporum* response outliers, which were identified separately in lodgepole or jack-hybrid samples, have higher F_{ST} values than randomly drawn SNPs (Wilcoxon test p-value < 0.01, Figure 5a & 5b), with outliers identified in jack-hybrid samples showing higher F_{ST} values than those identified in lodgepole samples (Wilcoxon test p-value < 0.01). These results suggest that the outliers associated with disease response constitute or are linked to genomic regions for divergent selection or species barriers, rather than facilitating introgression. F_{ST} estimates between jack-hybrid samples and a set of pure lodgepole pine samples showed a pattern of increased differentiation with distance eastwards of the jack-hybrid samples (Figure 5c), which was higher for the outliers than randomly chosen SNPs. Given the small number of samples, significance of linear model fitting is borderline, with p-value = 0.06 for outliers identified in lodgepole, p-value = 0.05 for outliers identified in jack-hybrid, and p-value = 0.06 for randomly chosen SNPs (df = 10). The differences between regression slopes on different gene sets (lodgepole outliers, jack-hybrid outliers, and unlinked SNPs) were not significant (p-values of pairwise comparisons > 0.3). Given the limited sample size, it is not possible to test whether a linear vs. discontinuous model would fit better to these data, but these results suggest that isolation-by-distance is occurring here.

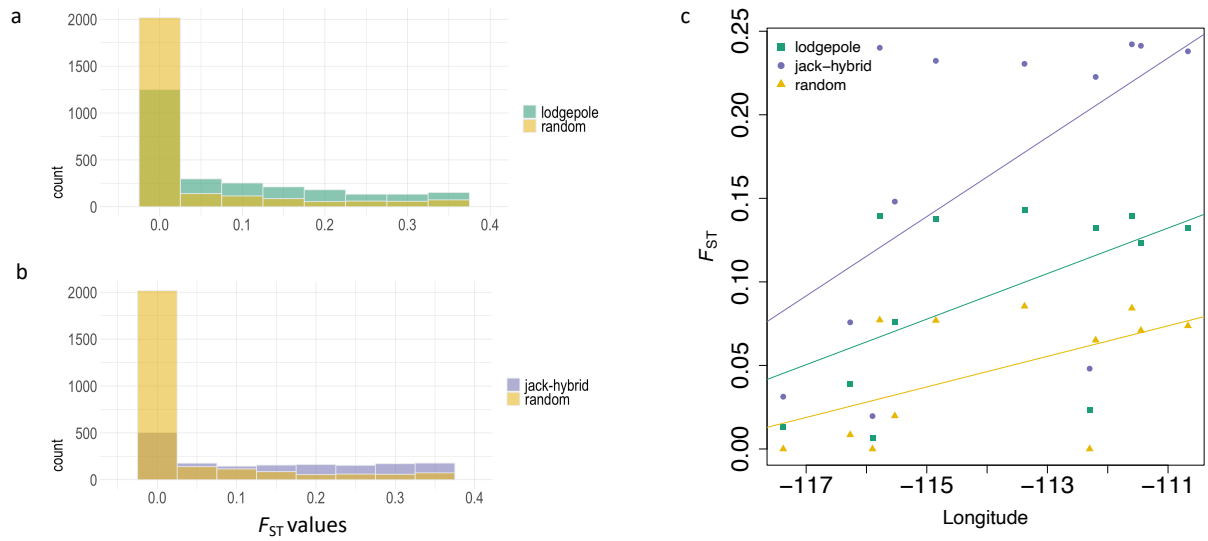


FIGURE 5 F_{ST} values of *D. septosporum* response outliers (a & b) and regression patterns of F_{ST} values with longitude. For a & b, the F_{ST} values were calculated between the jack pine samples (p36 to p40) and six pure lodgepole samples (p15, p16, p18, p20, p23, p25). F_{ST} values of *D. septosporum* response outliers were identified from lodgepole (N=3,358) and jack-hybrid (N=3,041) samples, and randomly drawn SNPs (N=3,400). Outliers identified from both species have higher F_{ST} values than randomly drawn SNPs (p-value < 0.01). For c, the F_{ST} values were calculated between jack-hybrid samples (p29 to p40) and six pure lodgepole samples (p15, p16, p18, p20, p23, p25) and then averaged for each jack-hybrid sample. Different genomic regions were represented by different SNP sets, including lodgepole outliers (N=3,358), jack-hybrid

445 outliers (N=3,041), and unlinked SNPs (N=31,716). The F_{ST} values represented by different SNP
446 sets were regressed to the longitude of these jack-hybrid samples. Differences between slopes are
447 not significant (ANCOVA, *p*-value > 0.3).
448
449 **4 | DISCUSSION**
450
451 In recent decades, the fungal pathogen *D. septosporum* has become an increasing threat to
452 lodgepole pine in British Columbia (Feau et al., 2021; Welsh et al., 2009). Although
453 *Dothistroma* needle blight (DNB) has not been observed in natural stands of jack pine in Alberta,
454 Ramsfield et al. (2021) confirmed jack pine's susceptibility to infection by *D. septosporum* using
455 a trial under natural conditions. The potential of climate change to allow this pathogen to spread
456 across the wide natural distribution of jack pine raises serious concerns. The hybrid zone of
457 contact between the sister species lodgepole and jack pine may act as a bridge, allowing this
458 fungal pathogen host shift (Taylor et al., 2015). The genetic basis of pathogen resistance in each
459 species is therefore an important factor affecting the potential for host shift. To study the genetics
460 of resistance to DNB, we analyzed the genetic architecture underlying *D. septosporum* response
461 using the study samples of lodgepole pine, jack pine, and their hybrids. We identified candidate
462 genes associated with *D. septosporum* response and inferred the impact of introgression on the
463 genetic structure underlying *D. septosporum* response in the studied samples. We detected
464 largely non-overlapping sets of candidate genes in lodgepole and jack-hybrid pine in response to
465 *D. septosporum*, which might be shaped by different evolutionary forces of selection and
466 introgression. Alternatively, the non-overlapping sets of candidate genes might reflect that many
467 non-causal genes exist in the top 1 % GWAS outliers of each species, given that we did not test
468 for significance at the gene-level. In either case, it seems clear that resistance to DNB is a highly
469 polygenic trait with alleles of small effect.
470
471 This study is the first report of genome-wide scanning of *D. septosporum* response genes in
472 lodgepole pine, jack pine, and their hybrids. We identified a few top candidate genes encoding
473 leucine-rich repeats (LRR)-containing protein domain and serine threonine kinases in lodgepole
474 pine samples (Figure 3, Table S2). Plant breeders have long used nucleotide-binding leucine-rich
475 repeat (NB-LRR) receptor resistance genes (R genes) for preventing or reducing diseases in
476 crops. When attacked by infectious pathogens, plant NB-LRR receptors can recognize pathogen
477 effectors by either direct or indirect mechanisms (Dodds & Rathjen, 2010). Such strategy is
478 called effector-triggered immunity (ETI), which occurs after a basal resistance, pathogen-
479 associated-molecular-patterns-triggered immunity (PTI). In a previous study using RNA-seq
480 data, Lu et al. (2021) identified genes involved in PTI and ETI, as well as R genes with positive
481 selection signals in lodgepole pine. These results indicate that the long coevolutionary history
482 between lodgepole pine and *D. septosporum* (Capron et al., 2021; Welsh et al., 2009) has given
483 rise to some tolerance or resistance mechanism against DNB. Since most of the identified
484 associated markers are likely not causal but rather are in LD with the causal loci, the observed
485 fast LD decay rate in lodgepole pine implies promising fine mapping resolutions as the identified
486 markers are likely to be proximal to the causative genes.
487
488 On the contrary, jack pine, as a potential new host, may not have coevolved such a specific
489 defense, and the long LD blocks surrounding jack-hybrid pine candidate genes, which are typical
490 of hybrid zones, limit the resolution of association mapping, so it may be difficult to locate the

causative genes for follow-up studies. Additionally, we caution that the capture probes were designed using lodgepole pine not jack pine genes, ascertainment bias might give rise to the underrepresentation of jack pine alleles. However, we can use these jack-hybrid pine candidate genes to infer how introgression affects the frequencies of disease-resistant alleles. Since we analyzed pool-seq data and lacked the pure jack pine samples out of the hybrid zone, it is not possible to analyze parental origin information, thus we inferred the ancestry using clustering. The clustering patterns in Figure 2 show that most hybrid pine trees in our study had greater jack pine than lodgepole pine ancestry, likely in part due to our limited sampling of jack pines from further Eastwards. Western hybrid pines tend to be more genetically similar to lodgepole pine, while eastern hybrid pines tend to be more genetically dissimilar to lodgepole pine. This is a typical characteristic for ongoing introgression and hybridization in the contact zone with a possible trend of lodgepole pine expanding its range eastward.

In hybrid zones, alleles in some genomic regions are able to introgress across species boundaries, whereas alleles that constitute species barriers or are under divergent selection will remain differentiated (Harrison & Larson, 2014). We found significantly high F_{ST} values between jack and lodgepole pine samples for the candidate genes associated with *D. septosporum* infection response. This can occur if these candidate genes tend to reside in genomic regions with restricted gene flow, driven by linkage with genes for divergent selection or species barriers. Bechsgaard et al. (2017)showed that R genes can adaptively introgress between closely related species. Since lodgepole pine has a long coevolutionary history with *D. septosporum* (Capron et al., 2021; Welsh et al., 2009), the introgression of lodgepole pine genes into hybrids may increase resistance to *D. septosporum*. However, variation in F_{ST} along longitude (Figure 5c) did not differ significantly between background and candidate loci, so we cannot draw strong conclusions about adaptive introgression here.

5 | CONCLUSION

The recent outbreaks of DNB in lodgepole pine and its ability to infect jack pine pose a pressing need for understanding the genetic architecture of resistance to *D. septosporum*. The present study provides an array of candidate genes associated with *D. septosporum* response using pool-GWAS. The identified candidate genes within lodgepole and jack-hybrid pines are largely non-overlapping, and the adaptive introgression is yet to be found. This knowledge can be used to develop genomic tools to minimize DNB risk and guide forest management strategies. Further testing and validation of these associations using a SNP array is currently underway.

DATA AVAILABILITY STATEMENT

The raw pool-seq data were deposited in NCBI SRA (accession number: PRJNA602898; http://www.ncbi.nlm.nih.gov/sra). The assembled transcriptomes and their annotation were deposited in Dryad https://doi.org/10.5061/dryad.prr4xgxtk .The scripts used for analyses were deposited in https://github.com/Mengmeng-Lu/Genetic-architecture-underlying-response-to-the-fungal-pathogen-Dothistroma-septosporum-in-Pinus-con

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