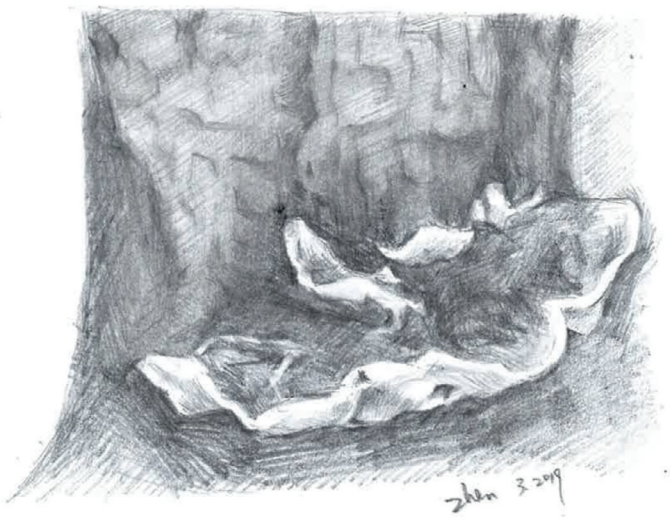
DISSERTATIONES SCHOLA DOCTORALIS SCIENTIAE CIRCUMIECTALIS,
ALIMENTARIAE, BIOLOGICAE. UNIVERSITATIS HELSINKIENSIS

ZHEN ZENG

GENOME, TRANSCRIPTOME, AND METHYLOME IN THE CONIFER PATHOGEN *HETEROBASIDION PARVIPORUM*



DEPARTMENT OF FOREST SCIENCES
FACULTY OF AGRICULTURE AND FORESTRY
DOCTORAL PROGRAMME IN SUSTAINABLE USE OF RENEWABLE NATURAL RESOURCES
UNIVERSITY OF HELSINKI

Department of Forest Sciences

Faculty of Agriculture and Forestry

and

Doctoral Programme in Sustainable Use of Renewable Natural Resources (AGFOREE)

Doctoral School in Environmental, Food and Biological Sciences (YEB)

University of Helsinki

Genome, transcriptome, and methylome in the conifer pathogen *Heterobasidion parviporum*

Zhen Zeng

ACADEMIC DISSERTATION

To be presented for public examination, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, in the Auditorium B2 (Latokartanonkaari 9, B-building) on 14th June 2019 at 12 o'clock.

Helsinki 2019

Supervisor **Professor Fred O. Asiegbu**
Department of Forest Sciences
Faculty of Agriculture and Forestry
University of Helsinki, Finland

Pre-examiners **Professor Louis Bernier**
Department of Wood and Forest Sciences
Laval University, Quebec, Canada

Research Professor Ari Hietala
Division of Biotechnology and Plant Health
Norwegian Institute of Bioeconomy Research, Ås, Norway

Opponent **Associate Professor Li-Jun Ma**
Department of Biochemistry and Molecular Biology
University of Massachusetts Amherst, USA

Custos **Professor Fred O. Asiegbu**
Department of Forest Sciences
Faculty of Agriculture and Forestry
University of Helsinki, Finland

Dissertationes Schola Doctoralis Scientiae Circumiectalis, Alimentariae, Biologicae
ISSN 2342-5423 (Print)
ISSN 2342-5431 (Online)

ISBN 978-951-51-5175-9 (paperback)
ISBN 978-951-51-5176-6 (PDF)
https://ethesis.helsinki.fi

Hansaprint
Helsinki 2019

Cover: Fruiting bodies of the *Heterobasidion* species at the base of a tree, Zhen Zeng, sketch.

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ABBREVIATIONS

AA	Auxiliary activity
AFLP	Amplified fragment length polymorphism
AIC	Akaike information criteria
ANOVA	Analysis of variance
APx	Ascorbate peroxidase
BS-seq	Bisulfite sequencing
BUSCO	Benchmarking universal single-copy ortholog
CAZyme	Carbohydrate-active enzyme
CBM	Carbohydrate-binding module
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
CE	Carbohydrate esterase
CFI	Chlorophyll fluorescence imaging
CGI	CpG island
DMATS	Dimethylallyltryptophan synthase
DMS	Differentially methylated cytosine site
DNA	Deoxyribonucleic acid
DNMT	C5-DNA methyltransferase
Dpi	Days post-inoculation
EF	Elongation factor
ERF	Ethylene response factor
ET	Ethylene
ETI	Effector-triggered immunity
FDR	False discovery rate
gDNA	Genomic DNA
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GH	Glycoside hydrolase
GO	Gene ontology
GT	Glycosyltransferase
GWAS	Genome-wide association study
Hpi	Hours post-infiltration/inoculation
HR	Hypersensitive response
HSB	Hue-saturation-brightness
InDel	Insertion/Deletion
JA	Jasmonic acid
LogFC	Log2-fold-change
LOX	Lipoxygenase
LTR	Long terminal repeat
Mb	Mega base pair
mC	Methylated cytosine site
MEA	Malt extract agar
MFS	Major facilitator superfamily

MP	Mate-paired
NGS	Next-generation sequencing
NPQ	Non-photochemical quenching
NPR1	Non-expressor of pathogenesis-related gene 1
NRPS-like	Nonribosomal peptide synthetase-like
ORF	Open reading frame
PAL	Phenylalanine ammonia lyase
PAMP	Pathogen-associated molecular pattern
PCA	Principal component analysis
PE	Paired-end
PHI-base	Pathogen-host interaction database
PI	Protease inhibitor
PKS	polyketide synthases
PL	Polysaccharide lyase
PSII	Photosystem II
PR	Pathogenesis-related protein
qPCR	Real time quantitative polymerase chain reaction
QTL	Quantitative trait locus
QY max	Maximum quantum yield
RIN	RNA integrity number
RIP	Repeated-induced point mutation
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
SA	Salicylic acid
SNP	Single nucleotide polymorphism
SP	Signal peptide
SSP	Small-secreted protein
SSR	Simple sequence repeat
TC	Terpene cyclases
TE	Transposable element
TF	Transcription factor
TM	Transmembrane
TPM	Transcript per million
Trx	Thioredoxin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I. **Zeng Z**, Sun H, Vainio EJ, Raffaello T, Kovalchuk A, Morin E, Duplessis S, Asiegbu FO. Intraspecific comparative genomics of isolates of the Norway spruce pathogen (*Heterobasidion parviporum*) and identification of its potential virulence factors. BMC Genomics. 2018, 19:220. https://doi.org/10.1186/s12864-018-4610-4

II. Wen ZL, Raffaello T, **Zeng Z**, Pavicic M. Asiegbu FO. Chlorophyll fluorescence imaging for monitoring effects of *Heterobasidion parviporum* small secreted protein induced cell death and *in planta* defense gene expression. Fungal Genetics and Biology. 2019, 126. https://doi.org/10.1016/j.fgb.2019.02.003

III. **Zeng Z**, Wu JY, Kovalchuk A, Raffaello T, Wen ZL, Liu MX, Asiegbu FO. Genome-wide DNA methylation and transcriptomic profiles in the lifestyle strategies and asexual development of the forest fungal pathogen *Heterobasidion parviporum*. Epigenetics. 2019, 14:1. https://doi.org/10.1080/15592294.2018.1564426

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Other articles not included in this thesis:

- **Zeng Z**, Raffaello T, Liu MX, Asiegbu FO. Co-extraction of genomic DNA & total RNA from recalcitrant woody tissues for next-generation sequencing studies. Future Science OA. 2018, FSO306.
- Kovalchuk A, **Zeng Z**, Ghimire RP, Kivimäenpää M, Raffaello T, Liu MX, Mukrimin M, Kasanen R, Sun H, Julkunen-Tiitto R, Holopainen JK, Asiegbu FO. Dual RNA-seq analysis provides new insights into interactions between Norway spruce and necrotrophic pathogen *Heterobasidion annosum* s.l. BMC Plant Biology. 2019, 9:2.
- Kovalchuk A, Mukrimin M, **Zeng Z**, Raffaello T, Liu MX, Kasanen R, Sun H, Asiegbu FO. Mycobiome analysis of asymptomatic and symptomatic Norway spruce trees naturally infected by the conifer pathogens *Heterobasidion* spp. Environmental Microbiology Reports. 2018, May 4.
- Ren F, Kovalchuk A, Mukrimin M, Liu MX, **Zeng Z**, Ghimire RP, Kivimäenpää M, Holopainen JK, Sun H, Asiegbu FO. Tissue microbiome of Norway spruce affected by *Heterobasidion*-induced wood decay. Microbial Ecology. 2018, August 9:1-11.

Author contributions:

I. **ZZ** designed and performed the experiment, conducted the bioinformatic analysis, interpreted the data, and wrote the manuscript; HS conducted the single spore isolation and generated the homokaryotic isolates; EJV genotyped and validated the homokaryotic isolates; AK and TR contributed to data interpretation; SD and EM contributed to data analysis; FOA conceived the study, contributed to the experimental design and writing of the article.

II. **ZZ** identified the small secreted protein in the fungal genome, contributed to data analysis and writing of the article. ZLW contributed to the experimental design, performed the experiment, interpreted the data and wrote the manuscript; MP contributed to the experiment on chlorophyll phenotyping, interpretation of data and writing of the manuscript; FOA and TR conceived the study and contributed to the experimental design and writing of the article.

III. **ZZ** designed and performed the experiment, conducted the bioinformatic analysis, interpreted the data and wrote the manuscript; JW conducted the phylogenetic analysis of DNA methyltransferases; AK contributed to data interpretation; TR contributed to experimental design and lab work; ZLW and MXL helped with the lab work; FOA conceived the study and contributed to the experimental design and writing of the article.

ABSTRACT

The fungus *Heterobasidion parviporum* Niemelä & Korhonen is a member of the species complex *Heterobasidion annosum* (Fr.) Bref. *sensu lato* (s.l.) which is considered as the most economically important and destructive disease agent of conifers. The main host of *H. parviporum* is Norway spruce (*Picea abies*), and the primary infection is mediated by aerial sexual spores (basidiospores) landing on fresh stump surfaces or tree injuries. The secondary infection is mediated by vegetative spread of the fungus via root contacts formed between neighboring trees. *H. parviporum* also reproduces asexually by forming conidiospores, which germinate and grow into mycelia. Like its sibling species, *H. parviporum* features a dual and flexible lifestyle (saprotrophy and necrotrophy). Due to the unavailability of a reference genome, the study of the molecular pathology of *H. parviporum* has heavily relied on parallel studies on its sibling species *H. irregulare*.

The rapid development of next-generation sequencing (NGS) techniques has revolutionized the scalability, reliability, and resolution of sequencing in life sciences. The first aim of this study was to provide a reference genome for *H. parviporum* by application of whole-genome sequencing. By characterization of 15 *H. parviporum* isolates of variable phenotypic traits (vegetative mycelial growth, sporulation, necrotrophic pathogenicity, and saprotrophic wood decay), we selected the most virulent isolate (isolate 96026) as the reference, which presented us a genome assembly of 37.76 Mb, hosting 10,502 protein-coding genes. To identify genomic variations potentially accountable for the higher virulence of the reference isolate, the remaining 14 isolates were also sequenced. Comparative genomic analysis uncovered not only the remarkable intraspecific level of polymorphism (13.9 single nucleotide polymorphisms [SNPs]/Kb) with marked bias in CpG to TpG mutation, but also two genomic regions exclusive to the reference isolate. Annotation of the two regions revealed the presence of encoded proteins such as secreted proteins, cytochrome P450, and major facilitator superfamily (MFS) general substrate transporter, suggestive of their potential implication in the virulence of *H. parviporum* reference isolate. To propose candidate virulence factors for functional characterizations, secreted protein-coding genes under genome-wide selection pressure or possessing featured variants (e.g., nonsynonymous SNPs) were explored, and examples were listed.

Having access to the *H. parviporum* reference genome, we then targeted small-secreted proteins (SSPs) in *H. parviporum* isolate 96026 to provide promising SSP candidate(s) with functional evidence in terms of promoting disease development. The preliminary selection of SSPs was guided by their ability to trigger cell death in *Nicotiana benthamiana.* A particular SSP (HpSSP35.8)

capable of inducing rapid cell death on *N. benthamiana* leaves together with high levels of *in planta* expression during the pre-symptomatic phase of host infection was chosen for further study. Defense-related genes including chitinase *PR4*, transcription factor *WRKY12,* and ethylene response factor *ERF1* were significantly upregulated in both *N. benthamiana* infiltrated by HpSSP35.8 and host seedlings infected by *H. parviporum*. Collectively, all evidence suggests this SSP as a potentially important virulence candidate, presumably, in the early stage of pathogenic interaction with the host.

To further understand the dual lifestyle and asexual development of *H. parviporum* isolate 96026, we collected samples from its saprotrophic (SAP) and necrotrophic growth (NECT) in mature host trees under field conditions as well as from its conidiospores (SPORE) and derived mycelia (MYCEL) in axenic culture. RNA-seq and whole-genome bisulfite sequencing of the collected samples enabled transcriptome and methylome profiling in the four conditions. RNA-seq revealed the enrichment of highly expressed genes encoding carbohydrate-active enzymes in both SAP and NECT stages. Signaling- and transcriptional factor-related genes specially induced in SAP might be potentially associated with lifestyle transition from SAP to NECT. A number of significantly upregulated genes involved in primary cellular activities including energy production were documented throughout asexual developmental stages. Bisulfite sequencing demonstrated the clear preference of DNA cytosine methylation in CpG dinucleotide and in transposable element (TE)-rich regions in *H. parviporum* genome. Together with the negative correlation of TE expression with TE methylation levels, our data substantiated the long-believed function of DNA methylation in fungal genome defense against TEs. A small group of genes with differential methylation and expression levels in SAP and NECT, relative to MYCEL, opened the avenue of DNA methylation as a transcriptional regulatory mechanism for *H. parviporum* different lifestyle strategies.

By taking advantage of NGS, our study provided the reference genome and first methylome of the economically important fungus *H. parviporum*. Candidate virulence factors based on *in silico* and web-lab evidence were highlighted. Follow-up research into the molecular mechanisms of *Heterobasidion* pathogenesis could be greatly facilitated by the availability of the genome, transcriptome, methylome, and secretome data.

1. INTRODUCTION

1.1 Conifer fungal pathogen *Heterobasidion annosum* species complex

The fungal species complex *Heterobasidion annosum* (Fr.) Bref. *sensu lato* (s.l.) (Basidiomycota; Agaricomycotina) is one of the most devastating forest pathogens, causing root and stem rot to conifers in the northern hemisphere (Asiegbu et al., 2005). This fungal species complex comprises three distinct Eurasian species (*H. annosum sensu stricto* [s.s.], *Heterobasidion abietinum* Niemelä & Korhonen, and *Heterobasidion parviporum* Niemelä & Korhonen) and two North American species (*Heterobasidion irregulare* Garbel. & Otrosina and *Heterobasidion occidentale* Otrosina & Garbel.) (Niemelä and Korhonen, 1998, Otrosina and Garbelotto, 2010).

These five species display distinct yet partially overlapping host preferences. The main hosts of *H. annosum* s.s. are different species of pines (*Pinus* spp.) with an affinity for Scots pine (*Pinus sylvestris* L.) in northern Europe. Yet, other gymnosperms, such as *Picea* spp. and broad-leaved trees have also been found colonized (Garbelotto and Gonthier, 2013). The preferred host for *H. parviporum* is Norway spruce (*Picea abies* [L.] Karst.), whereas *H. abietinum* preferentially infects silver fir (*Abies alba* Mill.) and other species of the genus *Abies* (Garbelotto and Gonthier, 2013). The North American species *H. irregulare* is associated with pines, junipers (*Juniperus* spp.), and incense cedar (*Calocedrus decurrens*), whilst *H. occidentale* has a broader host range including species of *Abies, Picea, Tsuga,* and *Pseudotsug*a (Garbelotto and Gonthier, 2013).

H. annosum s.l. produces both asexual and sexual spores known as conidiospores and basidiospores, respectively. Nevertheless, the airborne basidiospores constitute the primary infective source (Redfern and Stenlid, 1998). When the spores land on exposed stump surfaces and wounds on the stems or roots generated during thinning or logging operations, basidiospores germinate and initiate the infection. The established infection could then spread to adjacent stumps and healthy trees by the vegetative growth of mycelia through root-to-root contacts or grafts (secondary infection) (Asiegbu et al., 2005, Stenlid and Redfern, 1998). Fungal mycelia can persist in infected root debris and stumps for decades, readily providing the woody inoculum for intergenerational secondary infection (Asiegbu et al., 2005).

The success of fungal colonization and rate of spread are influenced by abiotic factors such as temperature, substrate pH, and wood moisture content. *H. annosum* s.l. grows optimally at the temperature of 22-28 °C and at pH of 4-5.7 (Korhonen and Stenlid, 1998). Stump sapwood is wetter than heartwood. Thus, the sapwood of spruce stumps is favored for colonization in dry

environments, whereas in regions with high precipitation and poorly drained soils, the heartwood is preferable (Redfern, 1993). The preference of fungal growth in heartwood of Norway spruce trees could be attributed to the absence of metabolic active cells to activate host defense response in heartwood, contrary to living sapwood (Garbelotto and Gonthier, 2013). In addition, wood density could be another influential factor in fungal growth. In a 19-year-old Norway spruce infected by *H. parviporum,* wood density negatively correlated to weight loss (Rodriguez et al., 2013). The vitality of host species and other competing wood-inhabiting fungi, such as *Phlebiopsis gigantea* and *Armillaria* spp. could also affect the survival and spread of *H. annosum* s.l. (Redfern and Stenlid, 1998).

1.2 Lifestyles in *Heterobasidion* species complex

H. annosum s.l. lives on woody substrates with limited capability to grow through soil (Korhonen and Stenlid, 1998). It grows saprotrophically in the dead cells of the wood but swiftly adopts necrotrophic lifestyle upon encountering living cells of stems and roots of host trees (Garbelotto and Gonthier, 2013). This flexible dual lifestyles makes *H. annosum* s.l. a valuable fungal species to be used as a model for studying the role of genomics, transcriptomics, and even DNA methylation in determining lifestyles changes relevant to tree pathosystems.

1.2.1 Necrotrophic interactions with host trees

When interacting with living host trees, *H. annosum* s.l. is confronted with the needs to penetrate the plant cell wall, to assimilate nutrients, and to modulate plant innate immunity. To access the nutrients from the host, *H. annosum* s.l. secretes a multitude of extracellular enzymes such as cellulases, mannanases, cellobiose dehydrogenases, polygalacturonases, pectin lyases, pectin esterases, and laccases to degrade the host plant polysaccharides and lignified tissues for carbon sources necessary for propagation inside host cells (Asiegbu et al., 1998). Fungal pathogens also deliver a myriad of small-secreted proteins (SSPs) termed as effectors that enable pathogenic invasion by manipulating their host cellular program including plant defense (Girard et al., 2013, McCotter et al., 2016). In *H. irregulare*, one SSP candidate that could cause rapid, strong, and consistent cell death in *Nicotiana benthamiana* and activate *N. benthamiana* defense-related genes was identified recently (Raffaello and Asiegbu, 2017). Another *H. annosum* s.s. SSP, having sequence similarity to cerato-platanin from *Ceratocystis platani,* was also found to cause cell death and upregulation of defense-related genes in *N. tabacum* (Chen et al., 2015). This SSP could additionally retard root growth of Scots pine seedlings (Chen et al., 2015). Nonetheless, the mechanism of the action of these SSPs remains to be addressed.

More proteins and biological processes of relevance to pathogenesis have been revealed by studying the changes in transcriptional profiles during host infection. In *H. irregulare* mycelial infection of Scots pine roots, genes encoding a mitochondrial carrier protein and a NADH-ubiquinone oxidoreductase implicated in mitochondrial energy production, a glutaredoxin-encoding gene putatively involved in DNA replication and cell division, and a gene coding for a cytochrome P450 potentially functional in detoxification of plant-derived compounds were upregulated (Karlsson et al., 2003). The same cytochrome P450 was later found highly induced after 20 days of *H. parviporum* growth in the bark tissue of a 10-year old Norway spruce tree (Karlsson et al., 2008), which further underlined the importance of this gene to *H. annosum* s.l. pathogenicity. In the mature Norway spruce naturally colonized by *H. parviporum*, genes engaged in cell cycle, DNA processing, and detoxification were similarly induced in addition to genes acting in protein synthesis, transport, and signal transduction, and of unknown functions (Yakovlev et al., 2008). The majority of significantly induced genes when the bark tissue of mature Norway spruce tree was inoculated with *H. annosum* s.s. were reported to encode metabolic machinery for sustaining fungal growth, such as the three delta-12 fatty acid desaturases which produce fungal cell membrane components (Lunden et al., 2015). By contrast, the pine bark tissue infected with *H. irregulare* led to a shift in gene expression towards production of pectinolytic enzymes and secondary metabolite, and stress tolerance, with fewer genes involved in lignocellulose degradation and nutrient transportation relative to colonizing on woods (Olson et al., 2012).

Production of spores and development of germ tubes into infective hyphae are prerequisites for the successful establishment of infection. Highly expressed genes during the development of *H. parviporum* conidiospores (germ tube emergence, polarized apical, and lateral growth) fall into most of the aforementioned functional categories with enriched genes of metabolic functions (e.g., phosphoglucomutase for glucose metabolism) and genes associated with protein synthesis (e.g., ribosomal proteins), indicating the increased cellular and metabolic activities required for conidiospore germination and hyphal development (Abu et al., 2004, Li et al., 2006).

Apart from the gene transcriptional profile that provides the snapshot of molecular activities occurring at a particular stage of the fungal infection process, the genome of an organism, *per se,* could readily provide additional information that might explain the observed varied phenotypes. Prior to the release of the complete genome of *H. irregulare* isolate TC-32-1 in 2012 by Olson et al., an AFLP marker-based genetic linkage map was constructed based on 102 progeny isolates derived from the compatible mating between *H. irregulare* isolate TC-32-1 and *H. occidentale* isolate TC-122-12 (Lind et al., 2005). The resulting linkage map enabled the localization of genomic

regions with possible linkage to the variation of several phenotypic traits such as growth rate (Olson, 2006), virulence (Lind et al., 2007), and interaction with the saprotrophic fungus *Phlebiopsis gigantea* (Samils et al., 2008). An improved version of the linkage map based on the alignment of first-generation map to the parental genomes (Lind et al., 2012) greatly facilitated and refined the assembly process of the *H. irregulare* genome (Olson et al., 2012). Through re-mapping of the virulence data from an earlier study (Lind et al., 2007) to the second-generation linkage map, three quantitative trait locus (QTL) regions potentially important for pathogenic interaction with Norway spruce and Scots pine were pinpointed on two chromosomes of the *H. irregulare* genome (Olson et al., 2012). The identified virulence QTL regions were characterized by a higher density of transposable elements (TEs) and of orphan genes (i.e., having no homology to genes from other reported fungal species), which indicated that these regions may undergo higher evolutionary rate. Additionally, microarray gene expression profiles during necrotrophic growth in Scots pine cambium led to the identification of three strong candidate virulence genes that were significantly upregulated and situated within the virulence QTL regions. These genes encode a sugar transporter, a flavin-containing Baeyer-Villiger monooxygenase putatively needed for phytotoxin synthesis, and a protein without homology to known proteins (Olson et al., 2012).

More virulence genes were further proposed in a genome-wide association study (GWAS) by Dalman et al. (2013). By sequencing 23 haploid *H. annosum* s.s. isolates, 12 single nucleotide polymorphism (SNPs) distributed on 7 genomic regions were found significantly associated with fungal growth in Norway spruce and Scots pines. Genes located within those genomic regions included both novel and known candidate virulence genes from other fungal pathogens, such as quinone oxidoreductase similar to genes encoding host-selective toxins in *Pyrenophora tritici-repentis*, flavin-containing monooxygenases metabolizing xenobiotic compounds, calcineurin involved in calcium-dependent signal transduction in eukaryotes, and SWI5 transcription factor affecting virulence in *Candida albicans* (Dalman et al., 2013). Interestingly, two of the identified regions were found close to or overlapping with the previously mentioned virulence QTL regions, implying that the generic virulence factors might be present at least among species of *H. irregulare, H. occidentale*, and *H. annosum* s.s. (Lind et al., 2007, Dalman et al., 2013).

Heterobasidion species were reported capable of producing at least ten different secondary metabolites, such as the phytotoxins fomannosin (Kepler et al., 1967) and fomannoxin (Hirotani et al., 1977). Both compounds have been detected in axenic cultures and in the course of plant colonization (Lind et al., 2014, Olson et al., 2012). The application of fomannosin and fomannoxin to pine seedlings and Sitka spruce seedlings, respectively, caused browning of needles (Bassett et

al., 1967, Heslin et al., 1983). In *H. irregulare* genome, three putative terpene cyclases (TCs) and one dimethylallyltryptophan synthase (DMATS) were identified which might be required for biosynthesis of fomannosin and fomannoxin, respectively (Olson et al., 2012). Notably, *H. irregulare* genome also hosts genes coding for other natural products, including 3 polyketide synthases (PKSs), 13 nonribosomal peptide synthetase-like (NRPS-like) enzymes, and several tailoring enzymes for post-backbone assembly modification, implying the potential of this species for synthesizing a wider range of secondary metabolites (Olson et al., 2012).

In addition to nuclear genetic factors, Olson and Stenlid (2001) reported the possible association of fungal virulence of *H. annosum* s.l. to mitochondrial genome. Using the artificially created interspecific hybrid isolates between *H. occidentale* and *H. irregulare*, they found a significant correlation between the virulence of the hybrids and their acquired mitochondrial type. The hybrid isolates displayed strong resemblance in virulence to their progenitors from which they inherited mitochondria. This suggested that mitochondrial genome (likely the exchangeable parts of the genome such as intronic genes, plasmid-derived genes, and non-conserved open reading frames) or certain nuclear-mitochondrial combinations might contribute to fungal virulence (Olson and Stenlid, 2001, Garbelotto and Gonthier, 2013, Garbelotto et al., 2007, Himmelstrand et al., 2014). This could be further supported by the upregulation of genes related to mitochondrial functions during infection of Scots pine roots (Karlsson et al., 2003).

1.2.2 Saprotrophic wood degradation

H. annosum s.l. is the most common wood decayer of conifers (Garbelotto and Gonthier, 2013). As a white-rot fungus, *Heterobasidion* species are capable of degrading all major constituents of plant cell wall, including the aromatic heteropolymer lignin and the plant polysaccharides cellulose, hemicellulose, and pectin (Lind et al., 2014, Rytioja et al., 2014). Accordantly, *H. irregulare* genome harbors genes encoding a broad range of plant cell wall degrading enzymes including oxidoreductases and carbohydrate-active enzymes (CAZymes) (Olson et al., 2012). CAZymes are classified into families of glycoside hydrolases (GHs), carbohydrate esterases (CEs), polysaccharide lyases (PLs), glycosyltransferases (GTs), carbohydrate-binding modules (CBMs), and enzymes with auxiliary activities (AAs) that enhance the activities of GHs, PLs and CEs (Carbohydrate Active Enzymes database http://www.cazy.org/). *H. irregulare* is equipped with comparable number of GHs, PLs, and CEs as also observed in the model white-rot fungal species *Phanerochaete chrysosporium* (Olson et al., 2012). Detailed inspection of *H. irregulare* CAZyme families revealed the presence of typical cellulose degrading gene families (CBM1, GH5, GH6, GH7, and AA9) characteristic of white-rot fungi (Floudas et al., 2012, Riley et al., 2014). *H. irregulare*

also has enriched gene families targeting hemicellulose (xyloglucan) and its side chains (GH12, GH27, GH29, and GH74), and pectin and its side chains (GH28, GH43, GH78, GH88, GH105, PL1, PL4, CE8, and CE12) (Olson et al., 2012, Rytioja et al., 2014).

In contrast to brown-rot fungi, a distinctive feature of white-rot fungi is the ability to modify and depolymerize lignin, which confers rigidity to the plant cell wall and provides protection of cellulose and hemicellulose from microbial attack and mechanical stress (Floudas et al., 2012, Eastwood et al., 2011). Lignin degradation is mediated with oxidative processes involving a panel of enzymes, such as class II heme-containing peroxidases (lignin peroxidase [LiP], manganese peroxidase [MnP], and versatile peroxidase [VP]), multicopper oxidases (MCO laccases [AA1_1]), glucose-methanol-choline (GMC) oxidoreductases (AA3), and glyoxal oxidase (AA5) (Hildén and Mäkelä, 2018). Compared to typical white rotters, the ligninolytic enzymatic repertoire of *H. irregulare* was predicted to consist of a slightly contracted number of class II peroxidases (6 short type MnP without the presence of LiP), but an expanded number of MCO laccases (16 laccase *sensu stricto*), and a spectrum of other oxidoreductases such as 35 GMC oxidoreductases and 5 glyoxal oxidases (Yakovlev et al., 2013).

Transcriptomic analysis of *H. irregulare* growing on pine sapwood shavings underlined the importance of cellulose degrading gene families (particularly CBM1, GH5, GH6, and AA9) and the preferential utilization of simple sugar sucrose (Olson et al., 2012, Garbelotto and Gonthier, 2013). When *H. annosum* s.s. was cultivated on separate pine wood compartments (bark, sapwood, and heartwood), more genes but less CAZyme-coding genes were specifically induced in sapwood than in heartwood and bark. This was putatively attributed to the higher content of more accessible and utilizable carbon sources in sapwood (Terziev et al., 1997, Raffaello et al., 2014). Correspondingly, sugar transporters or other proteins facilitating transport of nutrients (e.g., members of the major facilitator superfamily) were concomitantly induced (Olson et al., 2012, Raffaello et al., 2014). Members of CAZyme families displayed both general induction in all wood compartments and selective expression in heartwood (particularly AA9), suggesting the fine-tuned regulation of CAZyme families in response to the varied chemical composition of different wood compartments (Raffaello et al., 2014).

Additionally, induction of genes (MnPs, MCOs) relevant for oxidative lignin depolymerization was recorded when *Heterobasidion* species was grown on pine sapwood shavings (Olson et al., 2012). A similar observation was documented with the growth of the fungus on diverse pine wood materials (bark, sapwood, and heartwood) (Raffaello et al., 2014). Both MnPs and MCO were also reported to be highly expressed in reaction zone and in the heartwood of Norway spruce,

indicating their roles in detoxification and delignification (Nagy et al., 2012, Yakovlev et al., 2013). Other oxidases, such as glyoxal oxidases and aryl-alcohol oxidases, generating the extracellular hydrogen peroxide (H_2O_2) required in oxidative lignin breakdown were similarly induced (Olson et al., 2012, Yakovlev et al., 2013).

1.3 Control and management strategies for *Heterobasidion* species complex

Stump Infection via germination of airborne basidiospores (primary infection) and the subsequent spread to surrounding trees by root to root contacts (secondary infection) presented a rationale to control the disease in infested sites by removing the infected stumps. Nonetheless, stump removal needs to be carried out thoroughly to minimize the remnants of fungal inocula on woody debris on the site for successive rotation, thereby, rendering this measure time-consuming and expensive (Korhonen et al., 1998, Garbelotto and Gonthier, 2013).

Other preventative control strategies, based on limiting spore deposition and germination, are currently the most commonly implemented as they are effective and sustainable in practical forestry (Garbelotto and Gonthier, 2013). Thinning and logging operations create fresh stumps and scars, favoring the spread of *Heterobasidion* species. Therefore, thinning and logging in the wintertime when the risk of spore infection is very low are generally recommended (Korhonen et al., 1998).

In addition, stump protection after logging by immediate treatment with chemical or biological control agents on the stump surfaces has proved sufficiently effective and is widely practiced (Garbelotto and Gonthier, 2013). Amongst a large number of chemicals that have been tested as stump protectants over the past decades, urea and borates have demonstrated to be the most consistently effective against *Heterobasidion* infection and economically favorable (Pratt et al., 1998). Urea hydrolysis by ureases from living host tissues results in a substantial elevation of pH and ammonia content. The raised pH (>7) inhibits *Heterobasidion* spore germination and growth, and the ammonium ions might favor the growth of more tolerant competing fungi (Johansson et al., 2002). Borates were found to rapidly penetrate into stump tissues. The fungicidal mechanism of borates could be due to their effects on general fungal metabolism, resulting from the interaction of borate anion with fungal compounds containing polyhydric alcohol, and coenzymes NAD^+ and $NADP^+$ (Lloyd, 1998). However, concerns about the application of both stump-treatment substances have been raised since the consequential damages to ground-vegetation species and changes in soil properties have been reported (Westlund and Nohrstedt, 2000). Biological control agents, on the contrary, are generally biodegradable, free of artificial

pesticides, and hence more preferable over chemical control (Holdenrieder and Greig, 1998). Currently, spore suspensions of the saprotrophic fungus *Phlebiopsis gigantea* (Fr.) Jülich is used as a biocontrol agent against *Heterobasidion* species due to the competitive edge of this fungus in colonization of fresh stumps, decaying wood, and interference of antagonistic hyphae (Holdenrieder and Greig, 1998). Moreover, no apparent negative effect of *P. gigantea* on ground vegetation has been recorded (Westlund and Nohrstedt, 2000).

Lastly, disease control could be achieved by combining the above mentioned strategies with other silvicultural approaches, such as changing tree species composition to incorporate more resistant deciduous trees and to avoid monocultures of susceptible trees, widening spacing among planted trees to eliminate the chances of root contagion, and reducing the rotation time in stands heavily infested by *H. annosum* s.l. (Korhonen et al., 1998, Lygis et al., 2004a, Lygis et al., 2004b).

1.4 Epigenetics in fungi

An epigenetic trait is defined as a stably heritable phenotype resulting from changes in a chromosome without alterations in DNA sequence (Berger et al., 2009). Epigenetic processes include DNA methylation, chromatin remodeling by histone modifications, and non-coding RNA induced DNA silencing (Gomez-Diaz et al., 2012). Epigenetic modifications mediate gene transcriptional potentials and provide versatile, relatively fast and reversible phenotypic variations, which appear to be particularly essential to the host-pathogen interaction for adaptive advantage (Gomez-Diaz et al., 2012, Kasuga and Gijzen, 2013).

The methylation of DNA cytosine base is an important epigenetic modification in eukaryotes, implicated in various vital biological processes such as X chromosome inactivation, genomic imprinting, and silencing of retrovirus and repetitive elements for genomic stability (Jones, 2012). The distribution of methyl marks across eukaryotic genomes is not uniform, with demarcation of genomic regions of heavy methylation and regions that are not methylated. The methylation pattern also varies dramatically in different eukaryotic organisms (Jones, 2012, Baubec and Schubeler, 2014). In vertebrates, DNA methylation occurs throughout the entire genome, with the exception of specific genomic elements such as CpG islands (CGIs), active promoters, and enhancers (Baubec and Schubeler, 2014, Schubeler, 2015, Zhong, 2016). By contrast, most of the studied plants and invertebrates display mosaic but distinct DNA methylation pattern (Schubeler, 2015). Plants have DNA methylation in their TEs and gene bodies, whereas invertebrates show preference for genic methylation (Zemach and Zilberman, 2010, Su et al., 2011, Zhong, 2016). In addition, despite the prevalence of DNA methylation in many species, it is curiously absent in

some model organisms such as *Schizosaccharomyces pombe, Saccharomyces cerevisiae,* and *Caenorhabditis elegans*, implying the more complicated mode of actions and divergent functions that DNA methylation may have in diverse species (Capuano et al., 2014, Schubeler, 2015).

Compared to the higher eukaryotic counterparts, DNA methylation in fungi was originally considered as a genome defense mechanism against deleterious TEs proliferation due to its exclusive occurrence in transcriptionally silent, repetitive DNA sequences (Zemach et al., 2010, Zemach and Zilberman, 2010). Emerging studies in various fungal species have unraveled significantly varying numbers of methylated cytosines, ranging from a barely detectable level in the pathogen *Aspergillus flavus* (Liu et al., 2012) to a relatively high proportion in the black truffle *Tuber melanosporum* (36.9-39.6%) (Montanini et al., 2014). More importantly, DNA methylation has also been uncovered in and around structural genes in some species, thereby proposing the presence of additional functions in fungi. For example, considerable variations in the distribution of methylated genomic cytosines were observed in different developmental stages of the pathogenic species *Magnaporthe oryzae* (mycelia, conidia, and appressoria) (Jeon et al., 2015), *Metarhizium robertsii* (mycelia and conidia) (Li et al., 2017), and *Cordyceps militaris* (mycelia and fruiting body) (Wang et al., 2015). It was suggested that DNA methylation undergoes global reprogramming throughout the life cycles, putatively contributing to fungal development. In the human pathogen *Candida albicans*, DNA methylation was found to primarily target and regulate the transcriptional activities of genes associated with dimorphic transition between yeast and hyphal forms, switching between white and opaque cells, and iron metabolism, which might be cued by nutrition and host interaction (Mishra et al., 2011). In the medicinal fungus *Ganoderma sinense*, 5 transcription factors (TFs) and 36 transporters located in secondary metabolism gene clusters were predominantly methylated and transcriptionally silenced, indicating that DNA methylation may be involved in the modulation and transport of secondary metabolites (Zhu et al., 2015). Moreover, DNA methylation also appeared associated with phenotypic changes in sectorization in the chestnut blight fungus *Cryphonectria parasitica,* and the cell wall integrity signal transduction pathway was demonstrated to be important for maintaining DNA methylation in this fungus (So et al., 2018).

2. OBJECTIVES

H. parviporum, the most severe causal agent of root and stem rot in Norway spruce, greatly benefits from the coupling of saprotrophic and necrotrophic lifestyles. Due to the absence of a reference genome and the lack of an efficient transformation system, the molecular mechanisms underlying the pathogenesis and lifestyle changes of *H. parviporum* remain less understood. Therefore, by employing the latest "Omics" techniques, the objectives of this study are as outlined below:

1. To provide the first *H. parviporum* reference genome (I).

2. To propose several candidate virulence genes, based on comparative genomic analysis and transcriptomic profiles, for further functional characterization (I, III),

3. To functionally analyze a subset of genes encoding small-secreted proteins with cell death inducing effects on *Nicotiana benthamiana* and showing significantly activated expression level during host infection (II).

4. To understand the regulatory roles of DNA methylation in *H. parviporum* fungal genome and in its lifestyle strategies and asexual development, through integration of the corresponding transcriptomic profiles (III).

3. MATERIALS AND METHODS

The materials and methods used in this study are summarized in Table 1 and Table 2. Detailed description can be found in the original publications.

Table 1. Biological materials used in this study.

Organism	Information	Publication
Heterobasidion parviporum	Isolate 96026, homokaryotic	I, II, III
Heterobasidion parviporum	Isolate 03020, homokaryotic	I
Heterobasidion parviporum	Isolate 04121, homokaryotic	I
Heterobasidion parviporum	Isolate 93242, homokaryotic	I
Heterobasidion parviporum	Isolate 04051, homokaryotic	I
Heterobasidion parviporum	Isolate 99055, homokaryotic	I
Heterobasidion parviporum	Isolate 91271, homokaryotic	I
Heterobasidion parviporum	Isolate 99058, homokaryotic	I
Heterobasidion parviporum	Isolate 01039, homokaryotic	I
Heterobasidion parviporum	Isolate 96160, homokaryotic	I
Heterobasidion parviporum	Isolate 92150, homokaryotic	I
Heterobasidion parviporum	Isolate 99067, homokaryotic	I
Heterobasidion parviporum	Isolate 94174, homokaryotic	I
Heterobasidion parviporum	Isolate 98038, homokaryotic	I
Heterobasidion parviporum	Isolate 03014, homokaryotic	I
Agrobacterium tumefaciens	GV3101	II
Escherichia coli	TOP10F	II
Picea abies	Seedlings, 14-17 days old	I, II
Picea abies	Clones, 6 years old	I
Picea abies	Mature, 20-30 years old	III
Nicotiana benthamiana	Two months old	II

Table 2. Methods used in this study.

Method	Publication
Fungal cultivation	I, II, III
Fungal growth rate and sporulation assay	I
Pathogenicity screening and wood decay test	I
Statistical analysis	I, II, III
gDNA extraction	I, III
RNA extraction	I, II, III
Whole genome sequencing with a hybrid strategy (PacBio and Illumina Hiseq)	I
De novo genome assembly, gene prediction, annotation, secretome prediction	I
TEs and SSRs identification	I
Whole genome alignment	I
Variants calling, annotation and nucleotide polymorphism analysis	I
RNA-seq	I, III
De novo transcript assembly	I
Differential gene expression analysis	III
Whole genome bisulfite sequencing	III
Genome-wide bisulfite sequencing data analysis	III
Identification of differentially methylated loci	III
Phylogenetic analysis	III
Field work	III
Primer design, cDNA synthesis, qPCR and data analysis	II
Gene cloning	II
Chlorophyll fluorescence imaging	II
Agrobacterium-mediated transient expression	II

4. RESULTS AND DISCUSSION

4.1 Intraspecific comparative genomics of *H. parviporum* isolates and identification of its virulence factors (I)

In this study, several phenotypic traits (vegetative mycelial growth, sporulation, necrotrophic pathogenicity, and saprotrophic wood decay) important for *Heterobasidion* species fitness were characterized among 15 *H. parviporum* homokaryotic isolates originating from diverse geographic locations across Finland. The correlations of the pathogenic trait with the other traits plus the latitude and longitude of fungal sampling origins were analyzed. Furthermore, the most virulent isolate was deeply sequenced with a hybrid sequencing strategy and presented as the first reference genome of *H. parviporum.* The rest of the 14 isolates were re-sequenced and compared with the reference isolate. Genomic variations putatively associated with pathogenesis were explored, and candidate virulence factors were consequently proposed.

4.1.1 Phenotypic characterizations of *H. parviporum* homokaryotic isolates

The necrotrophic pathogenicity was reflected by the mortality rate of Norway spruce seedlings infected with *H. parviporum* mycelia for 15 and 25 days. Significant variations in the mortality rate of seedlings infected by *H. parviporum* isolates were observed. Among the isolates, S15 and S12 were the most and least virulent, respectively. The drastic difference in virulence between S15 and S12 isolates was further validated by the observed lesion length in xylem and phloem of six-year-old Norway spruce clones **(I, Figure 2c).**

Differences in vegetative mycelial growth rate, sporulation, and saprotrophic wood decay among all isolates were also recorded. With the exception of a moderate correlation ($r = 0.54$, $P < 0.05$) between wood decay capacity and mortality rate of seedlings at 15 days post-inoculation (dpi), no significant pairwise correlations between the assessed traits were identified. The lack of correlation between sporulation/vegetative growth and virulence/wood decay could be possibly due to inherent genetic make-up of each isolate or to their responses to the varied growth conditions. The former two traits were measured on axenic solid agar culture, whereas the latter two traits were assayed in the presence of either living seedlings or wood blocks that do not provide readily available nutrients for the fungal isolates. For example, isolate S15 appeared more aggressive in colonizing seedlings and decomposing woody materials than isolate S12 despite its slower growth on axenic culture **(I, Figure 2ab, Additional file 4: Figure S2b).** As similarly concluded by James (1982), our results caution about the simple reliance on cultural

characteristics, such as asexual sporulation and vegetative growth rate, to adequately estimate the virulence of *H. parviporum* isolates.

All the studied phenotypic traits together with longitude and latitude of fungal sampling sites were analyzed collectively using the mortality rate of 15 dpi and 25 dpi as the response variables. Latitude, wood decay capacity, and an interaction between these two features were shown to be significant parameters in determining the variation in mortality rate caused by the tested fungal isolates. This indicated that latitude-dependent environmental factors (e.g., temperature and soil type) and the flexible dual trophic strategies could jointly influence the virulence of this root pathogen.

4.1.2 Genome of *H. parviporum* isolate 96026

PacBio subreads and Illumina reads of a mate-paired and a pair-ended libraries were assembled *de novo.* The resulting genome of *H. parviporum* 96026 (also known as S15 in this study) was 37.76 Mb, distributed in 287 scaffolds. *H. parviporum* 96026 genome is slightly larger than that of *H. irregulare* (33.6 Mb), and its sequencing reads covered 66.24% of *H. irregulare* genome. TEs occupied 20.29% of the genome assembly, with *Gypsy*-like LTR retrotransposons being the most frequent elements (13.36% genome coverage). This was consistent with what was presented by Muszewska et al. (2011) that fungi generally have *Ty3/Gypsy* LTR retrotransposons as the highest-copy TEs. A notable dominance of tri- and hexanucleotide type simple sequence repeats (SSRs) were found inside the gene coding sequences, indicative of selection against possible lethal frameshift mutations (Metzgar et al., 2000). Trinucleotides were also clearly dominant in the upstream of coding sequences, suggesting the involvement of SSRs in gene transcriptional regulation, for instance by altering the repeat length of SSRs to modulate protein-protein interaction between TFs or to change the number of nuclear protein binding sites (Gemayel et al., 2010, Gonthier et al., 2015, Wagner and Lynch, 2008).

A total of 10,502 protein-coding genes were predicted with putatively 759 genes encoding secreted proteins. The secretome of *H. parviporum* includes 238 CAZymes (e.g., targeting both plant and fungal cell walls), 30 peroxidases (e.g., catalases and thioredoxins for scavenging reactive oxygen species), and 75 peptidase/peptidase inhibitors (e.g., aspartic and serine peptidases). In particular, secreted cytochrome P450, aspartic peptidases (A1A), subtilisin-like serine peptidases (S8A), thioredoxins (Trx), ascorbate peroxidases (APx), class II peroxidases, chitinases (GH18), chitin deacetylase (CE4), and almost all secreted CAZymes involved in cellulose and lignin degradations shared homology to entries annotated as “reduced virulence”, “loss of

pathogenicity", or "effector_(plant_avirulence_determinant)" in the Pathogen-host interaction database (PHI-base), highlighting the potential roles of these proteins in *H. parviporum* virulence and pathogenesis.

4.1.3 Intraspecific comparative genomics of *H. parviporum* homokaryotic isolates

Sequence reads of the 14 re-sequenced *H. parviporum* homokaryotic isolates were *de novo* assembled prior to the whole genome alignment to the reference or directly mapped to the reference genome. More than 91% of the genomic sequences of all isolates (except for isolate S6 with more fragmented assembly) could be aligned to the reference genome with an average of at least 96% nucleotide identity **(I, Table 3)**, demonstrating the overall high genomic similarity of these isolates when compared with the reference isolate **(I, Additional file 14: Figure S5**).

Conversely, an average of 139,488 SNPs and 33,643 insertions/deletions (InDels) per isolate were identified in 639,222 non-redundant polymorphic sites. The SNP density was 3.7/Kb when each isolate was compared to the reference (inter-individual level). This is much lower than that when the SNPs of all isolates were considered collectively (13.9/Kb, intraspecific level), reflecting a substantial level of polymorphisms. Our SNP density was higher than that of an intraspecific comparison study of three *H. irregulare* isolates (4 SNPs/Kb), but lower than that of the interspecific comparison between *H. irregulare* and *H. annosum* s.s isolates (20 SNPs/Kb) (Sillo et al., 2015). The higher level of intraspecific polymorphism in *H. parviporum* could be due to the diverse geographic origins of the isolates in this study. In contrast, *H. irregulare* isolates originated from Castelfusano Pinewood Urban Park, Rome (Sillo et al., 2015).

Furthermore, a biased mutation of CpG to TpG was noted among all transition and transversion mutations. This could be attributed to the methylation of cytosine, which showed a strong preference in CpG dinucleotide context **(see section 4.3.1 in this dissertation)**, followed by spontaneous deamination of methylcytosine, resulting in the CpG-to-TpG mutation (Nabel et al., 2012). Alternatively, elevated C-to-T transition mutation rates were also reported as a consequence of repeated-induced point mutation (RIP) (Faugeron, 2000), which has been experimentally validated in several Ascomycota species such as *Neurospora crassa* (Selker, 1990), *Podospora anserina* (Graia et al., 2001), and *Leptosphaeria maculans* (Idnurm and Howlett, 2003). By introducing mutations on different cytosines of duplicated DNA sequences or TEs, RIP could discourage ectopic recombination and inactivate TEs (Faugeron, 2000). There is variation in the preferred nucleotide context in TEs for RIP C-to-T mutations to occur. Ascomycota species *Botrytis cinerea, Sclerotinia sclerotiorum,* and *Magnaporthe oryzae* were shown to have mutational biases

at two different dinucleotides (CpA and CpT), whereas *L. maculans* exhibited a strong preference for CpA dinucleotide only (Amselem et al., 2015). These four species had at least one gene coding for a protein of the RID family required for RIP (Amselem et al., 2015). In Basidiomycota, *Microbotryum violaceum, Melampsora larici-populina,* and *Puccinia graminis* displayed low (10-20%) to intermediate frequency (~40%) of C-to-T mutation bias only in CpG dinucleotide in their TE copies with the presence of only one gene encoding Masc2. This raises the question on whether RIP operates in Basidiomycota or does so by targeting CpG dinucleotide (Amselem et al., 2015). Apart from the similar mutation bias in CpG dinucleotide, *H. parviporum* was found to contain two orthologs of Masc2 **(see section 4.3.1 in this dissertation)**. Further analysis of C-to-T mutation in TEs of *H. parviporum* could shed more light on the underlying mechanism for this mutational bias.

Additionally, selection of candidate virulence factors could also benefit from the application of comparative genomic and population genetic approaches. Such selection could be based on the observed DNA polymorphism in *H. parviporum* and the realization that fungal virulence candidates often undergo positive selection (Rech et al., 2014). Tajima's *D* statistic is the most commonly used allele frequency spectrum-based method to detect selection footprints in genomic regions at intraspecific level (Tajima, 1989, Vitti et al., 2013). A negative *D* value suggests a surplus of rare alleles relative to expectation, and may be indicative of positive selection (Vitti et al., 2013). Therefore, genome-wide distribution of Tajima's *D* value was assessed in a 5-kb non-overlapping sliding window manner. Genes located within windows of negative *D* value less than 5th percentile were extracted and deemed to be subject to positive selection. The secreted protein coding genes situated therein could be important candidate virulence factors **(I, Table 4).** Admittedly, genomic evidence for natural selection is only suggestive and awaits functional evidence to validate the selection.

As significant phenotypic variations, particularly in terms of virulence, were observed, it is presumed that gene number and associated polymorphisms among isolates could account for the varied virulence. The reads mapping strategy was utilized to evaluate the gene number variations among all isolates. Based on the mapping coverage breadth and depth, genes were classified into core genes shared by all isolates (9619 genes), deleted genes shared by a subset of isolates (863 genes), duplicated genes (208 genes), genes exclusive to the reference isolate (20 genes), and novel genes absent in the reference isolate (116-190 genes in the 14 isolates). Highly conserved (1457 genes) and divergent (1456 genes) core genes were further defined and separated by the number and annotations of detected variants (SNPs and InDels).

The reference-specific genes were mainly (16 out of 20 genes) localized on two genomic regions (7 genes on scaffold38 and 9 genes on scaffold51). The absence of the two regions in other isolates was further confirmed by whole genome alignment of the 14 re-sequenced isolates to the reference genome **(I, Additional file 14: Figure S7)**. Five out of the seven reference-specific genes on scaffold38, such as the genes encoding a cytochrome P450 and a MFS general substrate transporter, had PHI-base database hits with either "reduced virulence" or "loss of pathogenicity" annotations, while the reference-specific region on scaffold51 contained two secreted proteins with one of them considered as likely effector candidate (187 amino acids, 20 cysteines) **(I, Table 7)**. This result suggests the potential contributions of these two genomic regions to the high virulence of the reference isolate.

Numerous gene ontology (GO) terms regarding essential biological processes, such as ribosome biogenesis, fatty acid biosynthesis, and regulation of translation, were significantly overrepresented in the conserved core genes **(I, Additional file 20: Table S11).** By contrast, no significantly enriched GO terms could be identified in the divergent core gene and deleted gene sets, implying the occurrences of genetic variations in genes of miscellaneous functions. Nonetheless, genes associated with oxidation-reduction process and encoding TF-related domains were relatively more abundant in both gene sets. Particularly, GO terms of oxidation-reduction process and heme-binding were enriched in the divergent secreted protein coding genes, a similar result of which was also found in the comparison of *H. annosum* s.s. to *H. irregulare* (Sillo et al., 2015). The divergent secreted oxidoreductases include the ligninolytic enzyme multicopper oxidases (5 out of 12 genes), manganese peroxidase (2 out of 5 genes), and members of cytochrome P450 superfamily (9 out of 30 genes). P450s are heme-containing monooxygenases, implicated in versatile metabolisms and reactions. In fungi, P450s play crucial roles in the biosynthesis of secondary metabolites and biodegradation of xenobiotic compounds (Chen et al., 2014). The ubiquity and diverse functions of P450s were documented mainly arisen from gene duplication events and the subsequent mutations that diverge the redundant genes into new family (Sezutsu et al., 2013, Mgbeahuruike et al., 2017). The wide range of sequence polymorphism of P450s enables their substrate promiscuity in response to changing and increasing metabolic needs (Sezutsu et al., 2013). Gene loss is also a common P450 evolutionary event, and may account for the drastically low number of P450s in yeasts from Saccharomycotina (Chen et al., 2014). As expected, cytochrome P450-coding genes were also present in *H. parviporum* deleted, duplicated, and novel gene sets (lost from the reference isolate). One secreted P450 (evm.scaffold10.169) was absent in the less aggressive isolates (S3, S2, S4, S10 and S12), and one secreted P450 (evm.scaffold6.188) was putatively duplicated in isolates of both

weak (S4) and intermediate virulence (S1, S8). Hence, specific classification and function assignments of P450 are needed to consider their association to *H. parviporum* virulence variation.

Although gene copy number variations (deletion and duplication) were observed in the 15 isolates of *H. parviporum*, except for the reference-specific genes, isolates having more gene copies do not appear to be more virulent (e.g., isolate S6 having the highest number of gene copies being less virulent) and vice versa (e.g., virulent isolate S7 having the most deletions). However, we noticed the remarkable prevalence of genes encoding TF-related domains, such as zinc finger of C2H2 type and NF-X1-type, in all our classified gene sets (divergent, deletion, duplication and novel genes). TFs are key determinants of cellular functionalities by modulating gene expression. Additionally, TFs are highly evolvable, thus, contributing to phenotypic evolution (Shelest, 2008, Wagner and Lynch, 2008). C2H2 zinc finger, as the largest group of DNA-binding TFs in eukaryotes, was previously reported to have undergone lineage-specific gene duplications and gene losses (Seetharam and Stuart, 2013). Our results further evidenced the evolutionary plasticity and diversity of TFs. Due to the functional redundancy in many gene families, we postulate that *H. parviporum* virulence variation is mostly based on variation in the robustness of the involved regulatory network during infection rather than the changes in the number of protein-coding genes.

4.2 Functional study of a small secreted protein in *H. parviporum* (HpSSP) (II)

In this study, four small-secreted proteins (SSPs) were selected from *H. parviporum* 96026 reference genome, with one of them chosen for further study based on the observed induced cell death effects on *N. benthamiana* and the expression level during infection of host seedlings. The expression of several defense-related genes in both *N. benthamiana* and Norway spruce seedlings in response to the infiltration of selected SSP and infection with *H. parviporum*, respectively, was further explored.

4.2.1 Selection of HpSSPs by transient expression in *N. benthamiana* and expression level during infection of host seedlings

The four predicted *H. parviporum* SSPs (HpSSPs) were singled out on the basis of their high level of protein sequence similarities (> 86%) to four of the eight *H. irregulare* SSP candidates that have been previously shown capable of inducing chlorosis and cell death in *N. benthamiana* (Raffaello and Asiegbu, 2017). These four HpSSPs had no predicted domains and were named as HpSSP6.141 (269 amino acids), HpSSP27.89 (271 amino acids), HpSSP35.8 (177 amino acids), and HpSSP43.64 (230 amino acids) according to their location on scaffolds, and the arrangement of genes in individual scaffolds. The transient expression assay of the four HpSSPs in *N. benthamiana* leaves infiltrated by the mediation of *Agrobacterium tumefaciens* showed that HpSSP35.8 could induce strong plant cell death with completely compromised leaf tissues at 4 days post infiltration (dpi) **(II, Figure 1a).** HpSSP6.141 and HpSSP27.89 were not able to induce any evident cell death. HpSSP43.64 caused a certain level of cell death, characterized by discoloration and thinning of the infiltrated area **(II, Figure 1a)**, similar to the symptoms caused by its homolog in *H. irregulare* within the same incubation time.

Consistently, the gene encoding HpSSP35.8 was highly expressed relative to ungerminated conidiospores (around 30 fold higher) within the infected roots of Norway spruce seedlings at 2 dpi **(II, Figure 1b)**. No significant inductions were observed for the genes encoding HpSSP6.141 and HpSSP27.89. The expression of HpSSP43.64-coding gene had 2 to 3 folds increase at 2 to 3 dpi **(II, Figure 1b)**. Consequently, HpSSP35.8 was selected for more detailed studies.

The infection of host seedlings was repeated with increased number of replicates (5 replicates) in a time course experiment (sampling every 12 hours for four days post-inoculation) in order to assess the expression of HpSSP35.8-coding gene in a time-dependent manner. The visual symptoms on seedling roots challenged with *H. parviporum* hyphae were monitored closely

during the course of infection. The appearance of browning in infected roots was observed at 2 dpi and the browning was drastically intensified at 3 dpi **(II, Figure 3a)**. The gene encoding HpSSP35.8 was shown to be induced at 24 hours post-inoculation (hpi), and attained the highest expression at 36 hpi, followed by a dramatic reduction at the onset of necrosis browning **(II, Figure 3b)**.

HpSSP35.8 displayed 93% sequence similarity to its *H. irregulare* homolog (HaSSP30), sharing the same signal peptide sequence and cysteine residue sites **(II, Figure 2c)**. Moreover, HpSSP35.8 was conserved in all *H. parviporum* homokaryotic isolates investigated in the first study, with only three SNPs (two synonymous SNPs) identified among two isolates (isolates S1 and S3). The high degree of conservation at both inter- and intra-specific levels, the capacity to induce strong and consistent necrotic cell death in *N. benthamiana,* and the substantial induction during the host infection make HpSSP35.8 an important virulence candidate in the early stage of host-pathogen interaction.

4.2.2 The effect of HpSSP35.8 on photosynthesis in *N. benthamiana* by chlorophyll fluorescence imaging

The damaging effects of HpSSP35.8 on *N. benthamiana* leaves were also assessed by chlorophyll fluorescence imaging (CFI). CFI is a sensitive and non-destructive method to monitor and quantify changes in plants photosynthesis (Guidi and Degl'Innocenti, 2011, Murchie and Lawson, 2013). CFI has been widely used in the studies of plant-pathogen interactions for pre-symptomatic diagnosis of infection and in the investigation of pathogen-induced perturbations in host metabolism (Rolfe and Scholes, 2010). It is known that the light energy absorbed by chlorophyll molecules is dissipated in three ways: driving photosynthesis (photochemistry), being remitted as light (fluorescence), and being remitted as heat (non-photochemistry) (Murchie and Lawson, 2013). These three processes compete with each other. Thus, the yield of chlorophyll fluorescence provides valuable information on heat dissipation and on the efficiency of photochemistry, ultimately the photosynthetic productivity (Guidi and Degl'Innocenti, 2011, Murchie and Lawson, 2013).

In this study, three common fluorescence measurement parameters were calculated. These parameters were the maximum quantum yield (QY_{max}) indicating the maximum photochemical efficiency of photosystem II (PSII), operating efficiency of PSII photochemistry ($Ø_{PSII}$) representing the proportion of absorbed light energy being used in linear electron transport (photosynthesis), and non-photochemical quenching of fluorescence (NPQ) linearly related to heat dissipation

(Murchie and Lawson, 2013). The HpSSP35.8-infiltrated leaves showed continual decrease of QY_{max} compared with that of the empty vector-infiltrated control leaves. The QY_{max} discrepancy between these two treatments could be noted as early as 9 hpi and further enlarged as the infection progressed **(II, Figure 4a)**. The differences of both $Ø_{PSII}$ and NPQ between the two treatments were observed at even earlier time points. The $Ø_{PSII}$ of HpSSP35.8-infiltrated leaves was lower than that of the control at 3 hpi, whereas the NPQ of HpSSP35.8-infiltrated leaves exhibited higher value than that of the control from 3.75 hpi until 13 hpi **(II, Figure 4a)**. As the infection progressed, both $Ø_{PSII}$ and NPQ of HpSSP35.8-infiltrated leaves were reduced markedly **(II, Figure 4a)**. The observed decrease in QY_{max} and $Ø_{PSII}$, and the initial increase in NPQ followed by its rapid decline are typical host responses described in many other plant-pathogen interactions (Rolfe and Scholes, 2010). Therefore, the different responses of these parameters at early time point make CFI a sensitive tool for screening SSP candidates, including those unable to induce visible symptoms. The rapid decline of these three parameters at later infection stage suggested that the infiltrated HpSSP35.8 might have caused damage to photosynthetic apparatus, thereby influencing the photosynthetic activity.

4.2.3 HpSSP35.8 triggers defense response during the infection of non-host *N. benthamiana* leaves

The defense response in the non-host *N. benthamiana* leaves infiltrated by HpSSP35.8 over a three-day time course was investigated using 12 selected marker genes, namely, *HINI* and *HSR203J* for hypersensitive response (HR) cell death, *ethylene response factor* (*ERF1a), WRKY12, PR3,* and *PR4a* for jasmonic acid (JA)/ethylene (ET)-dependent pathway, *Non-expressor of pathogenesis-related gene 1* (*NPR1)*, *PR1a, PR2,* and *PR5* for salicylic acid (SA)-dependent pathway, and two other PR genes (*endochitinase B* and *protease inhibitor*) (van Loon et al., 2006, Pieterse et al., 2012, Kim et al., 2014, Eulgem et al., 2000, Singh et al., 2002, Pontier et al., 1999).

The two HR marker genes showed significant induction at 1 dpi compared to the control with subsequent decrease to a level comparable to the control at 3 dpi **(II, Figure 5 a,b)**. The activation of both HR markers together with the appearance of visible cell death at 36 hpi **(II, Figure 4b)** indicated that HpSSP35.8 might at least trigger a form of programmed cell death at the site of infiltration in *N. benthamiana*.

The chitinase genes *PR4a* and *endochitinase B* were significantly induced at 2 dpi, whereas the two TF-coding genes *ERF1a* and *WRKY12* exhibited upregulation at 1 dpi **(II, Figure 5 c-f)**. It has been established that SA-dependent responses are typically effective against biotrophic pathogens, whilst JA/ET-dependent responses are generally associated with resistance to

necrotrophic pathogens (Pieterse et al., 2012). The *PR4a* is one of the JA/ET-inducible genes (van Loon et al., 2006). The gene *ERF1a* was demonstrated to positively regulate the expression of a basic chitinase *(b-CHI)* gene and the JA marker gene *PDF1.2* in response to the necrotrophic fungus *B. cinerea* infection in *Arabidopsis* (Berrocal-Lobo et al., 2002). Overexpression of the gene *WRKY12* was reported to mitigate disease symptoms and to increase the expression of *PR4* and *PDF1* in Chinese cabbage (*Brassica rapa*) infected by the necrotrophic bacterium *Pectobacterium carotovorum* ssp. *carotovorum* (Kim et al., 2014). Consistently, the sequential expression of *ERF1a, WRKY12,* and *PR4a* in *N. benthamiana* in our study suggested the activation of JA/ET-mediated defense responses upon the transient expression of HpSSP35.8 by the necrotrophic pathogen *H. parviporum*. Furthermore, the SA-response genes were not significantly upregulated during the experimental time course except for the gene *NPR1* which was significantly induced at 1 dpi **(II, Figure 5 h,i,j,l)**. NPR1 is a crucial regulatory protein, acting as a transcriptional coactivator of many defense-related genes in SA-mediated pathway (Pieterse et al., 2012). Cytosolic NPR1 was shown to play a role in the SA-mediated suppression of JA pathway (Spoel et al., 2003). However, the function of NRP1 is not restricted to SA-dependent responses. In *N. attenuata*, NPR1 was proposed to suppress SA production, which minimized the SA-JA antagonistic effects, thereby eliciting JA-mediated defense against herbivore attack (Rayapuram and Baldwin, 2007). Hence, the functional picture of NPR1 is complex, and in *N. benthamiana,* it might also help fine-tune the defense responses against HpSSP35.8 in a yet unknown manner, one of which could be by restraining SA production to retain the JA-dependent response.

4.2.4 *H. parviporum* triggers the up-regulation of defense-related genes in host seedlings

Similarly, we also examined the defense response in the roots of the host Norway spruce seedlings subjected to *H. parviporum* infection during the 1-9 dpi period. We used five markers associated with JA/ET-mediated pathway (i.e., *PR4*, *ERF1a*, *ERF1b*, *WRKY12* and *Lipoxygenase 1 [LOX1])* and three markers involved in SA-mediated pathway (i.e., *LURP1*, *PR1* and *Phenylalanine ammonia lyase 1* [*PAL1]*) (van Loon et al., 2006, Arnerup et al., 2011, Pieterse et al., 2012, Arnerup et al., 2013).

The chitinase gene *PR4* showed strong and robust upregulation as early as 36 hpi, and the TFs-coding genes *WRKY12, ERFa,* and *ERFb* reached peak expression at 24 hpi, 72hpi, and 84 hpi, respectively **(II, Figure 6 a-d)**. The *LOX1* gene, involved in the regulation of JA production, showed induction at 36 hpi followed by its rapid downregulation compared to the control **(II, Figure 6 h)**. These data collectively reflected the involvement of JA-mediated pathway in the defense response of Norway spruce against the necrotroph *H. parviporum*. On the other hand, the gene

LURP1, which was considered as the regulon of the SA marker gene *PR1* in *Arabidopsis* (van Loon et al., 2006, Knoth and Eulgem, 2008), was co-expressed as *PR1* **(II, Figure 6 e,f).** These data presumably indicated the accumulation of SA in the infected seedlings, which was further reflected by the expression of the gene *PAL* implicated in SA production **(II, Figure 6 g)**. The parallel induction of SA- and JA/ET-mediated pathways has previously been reported in the bark tissue of Norway spruce in response to *H. parviporum* inoculation and wounding, and no obvious antagonism between these two defense signalling pathways against *H. parviporum* could be pinpointed (Arnerup et al., 2011, Arnerup et al., 2013). Our data further reinforced this notion, and could form a basis for future efforts in the mechanistic understanding of host defenses in the *H. parviporum*-Norway spruce pathosystem.

4.3 DNA methylome and transcriptomic profiles in the lifestyle strategies and asexual development of *H. parviporum* (III)

In this study, the transcriptomic profiles of the dual lifestyles of *H. parviporum* reference isolate 96026 and its lifestyle transition were investigated. To do so, *H. parviporum* 96026 pre-colonized sawdust was used as inoculum to infect 20-30 years old Norway spruce trees under the field condition. Hyphae recovered from the sawdust and from surrounding invaded necrotic stem tissues represented the fungal saprotrophic (SAP) and necrotrophic growth (NECT), respectively. The asexual lifecycle stages of *H. parviporum* 96026 conidiospores (SPORE) and derived mycelia (MYCEL) grown in axenic culture were also subjected to RNA-seq. Also, DNA cytosine methylation patterns in these four conditions (SAP, NECT, SPORE, and MYCEL) were obtained by whole-genome bisulfite sequencing (BS-seq). Transcriptomic and methylome profiles from these different conditions were described and compared. The resulting transcriptomic and methylome variations associated with different lifestyle strategies and fungal developmental stages were highlighted.

4.3.1 Genome-wide DNA methylation pattern and DNA methyltransferases

Whole-genome BS-seq was applied on the genomic DNA of *H. parviporum* 96026 from four conditions with three biological replicates per condition. Methylation could be measured and compared among samples by methylation density (reflecting methylation broadness) and methylation level (denoting methylation deepness) (Su et al., 2011). Methylation density reflects the proportion of methylated cytosine (mC) sites among all cytosine sites in a given DNA segment. Methylation level represents the fraction of methylated reads covering a cytosine site over all reads covering the same site. A global methylation level (i.e., total C sites divided by total cytosine sites in all reads) of 3.3% to 5.2% was found among the 12 samples, with higher global methylation levels in CpG nucleotide context (6.7-9.3%) than in non-CpG context (CHG and CHH, 2.0-3.7%) **(III, Supplementary Table S1)**. The pronounced preference of methylation in CpG sites was also shown by both its higher methylation density (mCpG/total genomic CpG) **(III, Figure 2b)** and average methylation level **(III, Figure 3)**.

The mC sites were not evenly distributed across the *H. parviporum* genome, but clustered generally in TE-rich and gene-poor regions in all conditions **(III, Figure 4)**. TEs also demonstrated remarkably higher methylation level in comparison to other genomic features (exons, introns, gene transcribed regions, and 1.5 kb up- and 1.0 kb downstream of transcribed regions) particularly in CpG context **(III, Figure 2c)**. The mC sites were also identified in gene transcribed regions, and a sharp increase in methylation level was observed in their flanking regions **(III,**

Figure 2d). This pattern was analogous to other fungal species, such as *M. oryzae* (Jeon et al., 2015)*, C. militaris* (Wang et al., 2015), and *C. parasitica* (So et al., 2018).

Principal component analysis (PCA) of the methylation level of all mC sites clustered SPORE and MYCEL together, with SAP and NECT being grouped individually **(III, Supplementary Figure S1a).** Compared with SPORE (11.60-13.68%) and MYCEL (11.02-13.32%), the number of mC sites was lower in SAP (9.79-10.23%) and NECT (6.61-7.64%) samples. However, the number of mC sites in NECT might be slightly underestimated due to the smaller number of genomic cytosines covered by BS-seq reads, **(III, Table 1)**. Yet, it is also likely that DNA methylation of *H. parviporum* had undergone substantial changes with the transition to different lifestyles**.**

In *H. parviporum* 96026 reference genome, three genes (evm.scaffold1.83, evm.scaffold1.1154, and evm.scaffold4.208) encoding putative DNA (C-5)-methyltransferases (DNMTs) were identified with the evidence of RNA transcripts. The predicted signature domains enabled the division of these genes into two groups, followed by separated phylogenetic analysis. The DNMTs encoded by evm.scaffold1.83 and evm.scaffold4.208 clustered with Mas2 of *Ascobolus immersus*, Dnmt1a and Dnmt1b from the Basidiomycota species *Coprinopsis cinerea* and *Laccaria bicolor*. Conversely, the DNMT encoded by evm.scaffold1.1154 shared the same clade with Dnmt2 of *C. cinerea* and *L. bicolor* **(III, Supplementary Figure S5a,b)**. The two putative DNMTs from Dnmt1 family appeared differentially regulated, with evm.scaffold1.83 being slightly more active in MYCEL and SPORE samples, and evm.scaffold4.208 in NECT and SAP samples. The DNMT from Dnmt2 family, however, remained constitutively expressed in all conditions **(III, Supplementary Figure S5c**). It is worth mentioning that one gene (evm.scaffold16.93) was also found to contain the DNA methylase domain as well as the SNF2_N domain and Helicase_C domain, which enables the assignment of this gene to Rad8 subfamily with yet unknown function (Huang et al., 2016).

4.3.2 Transcriptomic profiles associated with lifestyle strategies and fungal development

Reads from RNA-seq conducted on *H. parviporum* 96026 from the four conditions were mapped onto the reference genome and quantified on the gene level. Contrary to what has been revealed by the PCA of the methylome, PCA of the transcriptome grouped NECT and SAP together, separated from both MYCEL and SPORE groups. The dramatically smaller number of differentially expressed genes (469 genes) in NECT vs SAP compared to NECT vs MYCEL (3663 genes) or NECT vs SPORE (5565 genes) further corroborated the similar transcriptomic profiles of NECT and SAP samples. Therefore, we extracted those genes significantly upregulated in both NECT and SAP samples compared with both MYCEL and SPORE samples, and named these genes as *in planta*-

expressed genes (896 genes). Highly induced genes in a specific condition compared with all the other remaining conditions were considered as condition-specific genes. The number of NECT-, SAP- and SPORE-specific genes was 56, 108, and 1321, respectively.

The significantly enriched GO terms and KEGG pathways in the condition-specific and *in planta*-expressed genes were summarized in **Table 3**. Germination of spores marks the breakdown of dormancy, which normally associates with the coordinated activation of a series of metabolisms upon sensing appropriate nutrient conditions. *H. parviporum* conidiospores could readily form germ tube after 18-hour incubation in culture media (Li et al., 2006). The significantly overrepresented GO terms and KEGG pathways together with other main KEGG pathways (pathways involving at least 8 genes; such as Ribosome, Biosynthesis of amino acids, and Protein processing in endoplasmic reticulum) in SPORE-specific genes collectively suggested that the major biological processes, including nucleotide synthesis, transcription, translation, protein processing and degradation, and metabolism of an array of small molecules and secondary metabolites were intensively occurring in preparation for germ tube emergence, which were possibly driven by the energy produced by oxidative phosphorylation. The increased primary cellular activities and energy demand during *H. parviporum* conidial germination were generally congruent with other filamentous fungi such as *F. oxysporum* (Sharma et al., 2016), *A. fumigatus, A. niger,* and *A. oryzae* (Hagiwara et al., 2016).

Table 3. Significantly enriched GO terms and KEGG pathways of selected gene sets (III, Table 2).

IDs	Annotation	Type[1]	p-value	Tested genes
GO:0044281	Small molecule metabolic process	BP	4.7E-05	SPORE-specific
GO:0019748	Secondary metabolic process	BP	0.0014	SPORE-specific
GO:0006457	Protein folding	BP	0.0093	SPORE-specific
GO:0008135	Translation factor activity, RNA binding	MF	0.0038	SPORE-specific
Kegg:hir00190	Oxidative phosphorylation	-	1.3E-04	SPORE-specific
Kegg:hir03040	Spliceosome	-	9.0E-04	SPORE-specific
Kegg:hir03020	RNA polymerase	-	0.0018	SPORE-specific
Kegg:hir00240	Pyrimidine metabolism	-	0.0046	SPORE-specific
Kegg:hir00230	Purine metabolism	-	0.0063	SPORE-specific
GO:0003700	DNA binding transcription factor activity	MF	3.0E-04	SAP-specific
GO:0005975	Carbohydrate metabolic process	BP	7.4E-06	NECT-specific
GO:0016798	Hydrolase activity, acting on glycosyl bonds	MF	1.0E-05	NECT-specific
GO:0005975	Carbohydrate metabolic process	BP	1.6E-12	*In planta*-expressed
GO:0055085	Transmembrane transport	BP	0.0081	*In planta*-expressed
GO:0016798	Hydrolase activity, acting on glycosyl bonds	MF	5.1E-06	*In planta*-expressed
GO:0022891	Substrate-specific transmembrane transporter activity	MF	0.0010	*In planta*-expressed
GO:0043167	Ion binding	MF	0.0079	*In planta*-expressed
GO:0006259	DNA metabolic process	BP	9.2E-09	Amenable genes

[1]The GO categories, BP: biological process; MF: molecular function.

SAP-specific gene list was dominated by genes implicated in transcriptional regulation and signal transduction. The former process could be exemplified by the genes encoding putative TF-related DNA binding domains (e.g., Zn(2)-C6 fungal type DNA binding domain and Zinc finger C2H2 type) (Shelest, 2008) and genes sharing high similarity to the subunits Set1 and Ash2 of yeast Set1/COMPASS complex specialized in histone H3 lysine 4 methylation (H3K4me) for active transcription (Krogan et al., 2002, Shilatifard, 2012, Freitag, 2017). The latter process could be illustrated by genes encoding kinase-like proteins, tyrosine- and serine/threonine-specific phosphatases mediating phosphorylation/dephosphorylation cycles (Brautigan, 2013), and genes coding for guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP) activating/inactivating GTPases of Rho family (Chhatriwala et al., 2007, Harris, 2011). Rho GTPases were previously reviewed to be deployed for morphogenetic events, such as formation of septum and polarized hyphal growth in the Basidiomycota *U. maydis* and *C. neoformans* (Harris, 2011). One protein that might be assigned to septation in *H. parviporum* was also found to be highly induced and to contain the Usd1 (Up-regulated during septation protein 1) domain, which was originally reported as the highest-fold upregulated protein during the onset of septation in fission yeast (Bicho et al., 2010). Furthermore, genes associated with stress tolerance in *A. nidulans* also appeared in our SAP-specific list, such as an Arrestin-related gene putatively involved in ambient pH signaling pathway in response to elevated pH (Herranz et al., 2005) and a gene encoding one subunit of COP9 signalosome complex mediating transcriptional and metabolic reactions in response to oxidative stress and cell wall rearrangement (Nahlik et al., 2010). Not surprisingly, genes encoding transporters (one ammonium and one sugar transporter), proteases (one secreted aspartyl protease and one metallopeptidase), and guanine deaminase (hydrolyzing guanine to xanthine and ammonia) were highly induced and might be relevant nutrient acquisition. Taken together, it is postulated that *H. parviporum* in sawdust inoculum has triggered massive developmental reprogramming (e.g., morphogenesis) associated with nutrient deficiency and stress resistance by vigorous employment of complex signaling and regulatory networks, features that could be of relevance for lifestyle transition.

By contrast, genes involved in carbohydrate metabolic process were found enriched in the NECT-specific gene list. In total, 15 out of 56 genes encoded putative CAZymes, among which 12 genes have predicted signal peptide. These CAZymes were predicted mainly to degrade plant cell wall polysaccharide complex, including three cellulose-active genes (two GH12:cellulases and one AA9:lytic polysaccharide monooxygenase), two hemicellulose-active genes (one GH10:β-1,4-Endoxylanase and one GH115:α-glucuronidase), six pectin-active genes (one PL1:pectin/pectate lyase, one PL4:rhamnogalacturonan lyase, one GH28:polygalacturonase, one GH105:unsaturated

rhamnogalacturonyl hydrolase, and two GH43:endoarabinanase and galactan 1,3-β-galactosidase), and two genes (AA1:laccases) for lignin modification. Similarly, genes associated with nutrient assimilation such as two sugar transporters and one hydroxyisourate hydrolase (involved in purine catabolism) were also present in the NECT-specific list. Genes encoding a secreted cytochrome P450, a glutathione S-transferase, and three transporters from MFS family might be upregulated for detoxification of xenobiotic substrates or toxic plant metabolites. Additionally, the possible involvement of mitochondrial functions in *H. annosum* s.l. necrotrophic phase was also demonstrated in our study by the two genes related to NADH dehydrogenase, presumably for mitochondrial energy production. More intriguingly, three genes encoding secreted hypothetic proteins and one gene having 97% similarity to a nonribosomal peptide synthetase-like gene of *H. irregulare* TC 32-1 might contribute to fungal pathogenesis.

In planta-expressed genes were upregulated in both SAP and NECT conditions compared with MYCEL and SPORE. The dramatically larger size of this gene set (896 genes) compared to the individual SAP- (108 genes) and NECT-specific (56 genes) gene sets implied that *H. parviporum,* following dissemination from sawdust to surrounding living tree tissues, might tend to elicit mostly overlapping physiological responses and biochemical processes. These processes affect *H. parviporum* pathogenesis at multiple aspects and include plant penetration (e.g., plant cell wall degrading CAZymes), nutrient acquisition and transportation (e.g., proteases, sugar and amino acid transporters), stress tolerance and detoxification (e.g., cytochrome P450s, glutathione-S-transferases, and MFS general substrate transporters), fungal cell wall reorganization and biogenesis (e.g., hydrophobins and fungal cell wall degrading CAZymes), signaling and transcriptional regulation (e.g., kinases, phosphatases, and TFs), and expression of other virulence factors (e.g., secreted hypothetic proteins).

4.3.3 Associations of TE expression with DNA methylation

As epigenetic silencing has been widely perceived to control TEs proliferation across different eukaryotic organisms, and mC sites were found prevalent in *H. parviporum* TEs, we then inspected the association of expression of predicted TEs with their methylation status. In *H. parviporum* 96026 genome, long terminal repeat (LTR) retrotransposon was the most dominant categorized class, covering 14.68% of the genome with *Gypsy*-like LTR retrotransposon being the most frequent superfamily (13.36% genome coverage). As expected, the majority of TEs including *Gypsy*-like LTR retrotransposons were transcriptionally inactive, yet methylated in all four conditions **(III, Figure 6b,d)**. The TE expression level was negatively correlated with TE CpG methylation levels in the four conditions (*Rho* = -0.72 to -0.73, $p < 0.001$) **(III, Supplementary**

Figure S3b). The negative trend was also present in each TE (sub)class (except for the "Other" LTR retrotransposons that were all unexpressed with average methylation level of 87.4%-97.1%) (**III, Supplementary Figure S3a).**

Furthermore, a group of genes (23 genes) containing TE-related domains, such as reverse transcriptase domain, integrase catalytic core domain, and ribonuclease H-like domain were found to be extensively methylated (CpG methylation level ≥ 75%) and barely expressed (TPM ≤ 1) (called amenable genes in this study, **see section 4.3.4**). This further supports their effects on repressing TEs and the maintenance of genome stability exerted by DNA methylation in *H. parviporum*.

4.3.4 Associations of gene expression with DNA methylation

We also examined the associations of gene expression level with the DNA methylation level of the gene transcribed regions and upstream regions till 1.5 kb. Genes were classified into a silent group (1st group) and the lowest 25% to the highest 25% of expressed groups (2nd to 5th groups) in the four conditions. The silent group showed remarkably higher methylation levels (gene body: 60.9%-64.1%; gene upstream: 51.1%-56.0%) than the expressed groups, while the most highly expressed group (5th group) presented the lowest methylation levels (gene body: 0.45%-0.57%; gene upstream: 1.10%-1.44%) **(III, Figure 7c,d).** Therefore, it is likely that DNA methylation may play a repressive role in the expression of that small group of genes. Consequently, the distribution of methylation levels of the silent gene group was checked. The majority of silent genes were either extensively methylated (methylation level ≥ 75%) or barely methylated (methylation level ≤ 5%) **(III, Figure 7e,f)**. Therefore, these heavily methylated (methylation level ≥ 75%) and non- or lowly expressed genes (TPM ≤ 1) were defined as amenable genes and extracted for further inspection.

A total of 79 amenable genes have predicted InterPro domains with 58 genes extensively methylated in both their transcribed and upstream of transcribed regions. Aside from TE-related genes mentioned in section 4.3.4, genes containing helicase domains (11 genes), particularly DEAD/DEAH box helicase domain, and zinc finger of C2H2-type domain (6 genes) were relatively more abundant and basically silent in four conditions. DEAD-box proteins constitute the largest family of RNA helicases. They possess multiple properties such as RNA binding and unwinding, ATPase activity, and promoting RNA folding, thereby, being involved in various processes of RNA metabolism such as ribosome biogenesis, pre-mRNA splicing, and transcriptional regulation (Sarkar and Ghosh, 2016, Jarmoskaite and Russell, 2011). C2H2 zinc finger is the largest group of

DNA-binding TFs in eukaryotes (Seetharam and Stuart, 2013). Therefore, the silence of these genes found in our study suggested they are redundant or non-functional, and that DNA methylation could either be the cause or the consequence of their silence.

To further uncover the potential of DNA methylation in regulation of gene expression in response to different conditions, the differentially methylated cytosine sites (DMS) in comparisons of NECT vs MYCEL, SAP vs MYCEL, and SPORE vs MYCEL were identified. Genes harboring at least six DMS in either their transcribed regions or upstream till 1.5 kb of transcribed regions were designated as DMS-associated genes. Expression levels of the resulting DMS-associated genes in the individual comparisons were then explored, based on which, three methylation patterns were generalized. DMS-associated genes fulfilling methylation pattern I (18 genes) and pattern II (5 genes) were significantly upregulated in NECT and SAP when compared with MYCEL. Genes of methylation pattern I included 13 amenable genes and were heavily methylated in all four conditions with slightly lower methylation levels in NECT and SAP **(III, Figure 8a).** It is speculated that the expression of such genes, particularly those amenable genes (e.g., genes encoding a GMC oxidoreductase, an ATPase, and a secreted serine protease S53), might be silenced by DNA methylation in *H. parviporum* mycelia growing on artificial plates (MYCEL) and somewhat de-repressed upon switching to saprotrophic or necrotrophic growth (NECT and SAP) as required. Genes belonging to methylation II (e.g., gene coding for a cytochrome P450) were slightly more methylated in NECT and SAP, yet having generally very low methylation levels (< 10%) **(III, Figure 8b)**. As it is unclear if certain threshold needs to be reached for DNA methylation to be efficient, the role of DNA methylation in the expression of this group of genes remains obscure. The third methylation pattern was presented by downregulated genes in NECT and SAP when compared with MYCEL. Their lower transcript abundances in NECT and SAP coincided with the higher methylation levels in these conditions **(III, Figure 8c)**. However, unlike genes of methylation pattern I, this group of genes (e.g., genes encoding a DNA-directed RNA polymerase and an acetyl-CoA synthetase-like protein) were expressed constitutively in all conditions, which might be attributed to their low to intermediate methylation level (< 75%) that fluctuated in the four conditions. Moreover, genes encoding WD40 repeat-containing domain, Ankyrin repeat-containing domain, and F-box domain were found to possess methylation pattern I and III, implying their proneness to be affected by DNA methylation. As these domains are known to act as sites for protein-protein interactions (Xu and Min, 2011, Jonkers and Rep, 2009, Voronin and Kiseleva, 2008), it is likely that their methylation might enable more efficient regulation of biological processes by mediating the interplay among different proteins.

Notably, our classified condition-specific genes had very low or undetectable methylation levels. Additionally, we could not find clear evidence that could link any DMS-associated, differentially expressed genes to germination of conidiospores (SPORE vs MYCEL) or to transition of saprotrophic growth in sawdust to necrotrophic growth (NECT vs SAP), which undoubtedly needs further investigation.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, we used a hybrid sequencing strategy to present the first reference genome of a *H. parviporum* isolate, which displayed the highest virulence among all 15 studied isolates. The remaining 14 *H. parviporum* isolates were also sequenced. Intraspecific comparative genomic analysis revealed a remarkable level of polymorphism with a notable bias in CpG to TpG mutations and helped to identify candidate virulence genes for future functional studies. Read mapping coverage analysis identified two genomic regions exclusively present in the reference isolate and putatively contributing to its virulence. Genes enriched for copy number variations (duplication and deletion) and nucleotide polymorphisms were found to be associated with oxidation-reduction processes and transcription factors, and could be exemplified by the cytochrome P450-coding genes and C2H2 zinc finger domain-containing genes, respectively. It is highly likely that many layers rather than a single molecule determine *H. parviporum* virulence, and expression of many genes might collectively influence the ultimate manifestation of its virulence. Indeed, transcriptomic data yielded from mature host trees infected by *H. parviporum* sawdust inoculum under the field conditions underlined several well-known functional categories that appeared crucial for *H. parviporum* necrotrophic interaction with its host. These include genes encoding proteins relevant for plant cell wall degradation (e.g., CAZymes), nutrient acquisition (e.g., proteases and sugar transporters), and stress tolerance and detoxification (e.g., cytochrome P450s). The lists of highly induced secreted hypothetic proteins could present novel aspects in assisting *H. parviporum* pathogenic activity, and definitely merits further study. The growing list of candidate genes urges the need for the development of an efficient DNA transformation system or alternate system to provide direct evidence of their implication in *H. parviporum* pathogenesis.

With the annotated *H. parviporum* genome, SSPs were selected based on their ability to induce necrotic cell death in *N. benthamiana* leaves and their transcriptional dynamics over the course of infection on host Norway spruce seedlings. One SSP (HpSSP35.8) caused strong, fast, and consistent cell death in *N. benthamiana,* accompanied by diminished photosynthetic activity and activation of several defense-related genes involved in JA/ET-dependent pathway and hypersensitive response. Additionally, the HpSSP35.8-coding gene showed significant upregulation in the early stage of infection on host seedlings, which displayed concomitant induction of defense-related genes in SA- and JA/ET-mediated pathways. This SSP constitutes the first example of *H. parviporum* virulence candidate, whose transient expression triggered rapid plant cell death and defense responses in *N. benthamiana*, and might be important for the initial stage of host disease development. However, its mechanism of action is still unclear, and the lack

of any predicted domain makes inferences about its function challenging. Future efforts could be focused on producing its recombinant protein for direct evaluation of its effects on seedling roots and identifying the receptors in both *N. benthamiana* and Norway spruce interacting with this particular SSP, which could help elucidate its molecular function.

Our generation of the reference genome of *H. parviporum* has paved the way to undertake the first DNA methylation study in this species adopting distinct lifestyles and undergoing different asexual stages. The presence of DNA cytosine methylation, and their obvious preference for CpG dinucleotide context and TE-rich regions were demonstrated by whole-genome bisulfite sequencing. Negative correlation of TE expression with TE methylation levels, regardless of the studied conditions, is in line with the consensus of DNA methylations as repressive marks for TE activities. Combined analysis of gene expression and methylation levels resulted in the identification of small groups of genes (e.g., genes encoding a GMC oxidoreductase and a DNA-directed RNA polymerase) that might be amenable to DNA transcriptional regulation during saprotrophic and necrotrophic growth relative to mycelial growth in axenic culture. It seems that DNA methylation played negligible roles in the conidiospore germination and transition from saprotrophic growth to necrotrophic growth in the present study. However, no absolute conclusion can be drawn until the identified DNA methyltransferase-coding genes are disrupted and the corresponding transcriptional and methylation levels are re-profiled, which again necessitates the needs for a feasible gene inactivation approach in this forest pathogen. Alternatively, treatment of samples under conditions of interest with the suitable concentration of the demethylating agent 5-azacytidine followed by RNA-seq and BS-seq could be explored.

Overall, there is no doubt that with ever-improving methodological advances, better mechanistic understanding of pathogenesis and lifestyle changes in *Heterobasidion*-conifer pathosystem will be achieved.

ACKNOWLEDGEMENTS

This thesis was carried out at the Department of Forest Sciences at the University of Helsinki during 2015-2019. The studies included in this thesis were funded by the Academy of Finland (project number 276862). I would like to thank the Doctoral Programme in Sustainable Use of Renewable Natural Resources (AGFOREE) at the University of Helsinki, Alfred Kordelin Foundation and Niemi Foundation for financial support during these years.

My sincere gratitude goes to my supervisor, Prof. Fred Asiegbu, who brought me in the field of forest pathology. He is always optimistic and enthusiastic about research and patient with me. His projects have provided me with great opportunities to dive into the "omics" world. He was very supportive when I wanted to attend the courses organized outside our university and to upgrade my computer. His encouragement has motivated me to keep learning and developing my bioinformatic skills.

I am very grateful to my thesis pre-examiners, Prof. Louis Bernier and Prof. Ari Hietala, for their prompt, thorough and insightful comments on how to improve my thesis. I also would like to thank my follow-up group Petri Auvinen and Minna Pirhonen for their constructive advice on the setup of the whole PhD projects.

My sincere appreciation extends to my excellent collaborators Zilan Wen, Tommaso Raffaello, Andriy Kovalchuk, Hui Sun, Jiayao Wu, Mengxia Liu, Eeva Vainio, Sébastien Duplessis, Emmanuelle Morin and Mirko Pavicic for their valuable comments and contributions to make the listed publications possible. I especially want to thank Sébastien Duplessis for hosting me in his lab, giving me inspiring ideas and suggestions, and teaching me how to do manual genome annotation.

My special gratitude goes to Kean-Jin Lim and Jinhui Wang for their kindly shared experience in bioinformatic data analysis and skilled help with troubleshooting in various programs.

I wish to express my cordial thanks to the current colleagues in the MPAT group: Risto Kasanen for the great help in the field work, Zilan Wen, Mengxia Liu, Jiayao Wu, Ximena Silva, Kashif Muhammad and Mukrimin for being such wonderful company in the journey of doctoral study; and also the former members: Lihua Zhu, Hui Sun, Fei Ren, Juliana Quintana, Hongxin Chen, Arnaud Pascal, Tommaso Raffaello, Susanna Keriö, Eeva Terhonen, Emad Jaber, Abbot Oghenekaro, Jaeyoung Choi for all those treasured memories of parties, conference trips and profound discussions. I also wish to extend my particular thanks to my master's thesis research group: Prof.

Kistrina Lindström, Petri Penttinen, Lijuan Yan, Janina Österman and Abdollah Mousavi. Their amazing research has incented me to pursue my own doctoral study.

I also would like to thank my dearest friends Alejandra Culebro and Junko Sugano for all the laughers, tears, jokes and lively discussions about science and life. They made me feel secure and accompanied in Finland. I feel very lucky to have been surrounded by a group of other awesome friends over the years: Xuan Zhou, Man Hu, Mengzhen Zhang, Yiyang Ding, Wei He, Chao Zhang, Chunxiang Li and her lovely cat, Petri Penttinen, Lijuan Yan, Yunfan Wang, Arati Poudel, Wenfei Liao, Petri Nummi and many others. All these cheerful and memorable chats over lunch and coffee breaks, board games, badminton, mushroom picking and parties have made my life so joyful.

I wish to express my deepest gratitude towards my parents. Their unconditional love and support have enabled me to focus on the study and keep on achieving goals throughout all these years. Finally, I want to thank Tuure for coming into my life, always having faith in me, and helping me from all aspects of life.

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Helsinki 2019 **ISSN 2342-5423** **ISBN 978-951-51-5175-9**