Ecological transitions and evolutionary genomics of the invasive brown rot fungus *Serpula lacrymans*

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How many DNA markers are needed to reveal cryptic fungal species? Balasundaram SV, Engh IB, Skrede I, Kauserud H (2015). Fungal Biol. 119: 940-5 DOI: 10.1016/j.funbio.2015.07.006.

Paper II

The fungus that came in from the cold: Dry rot's pre-adapted ability to invade buildings. Balasundaram SV, Hess J, Brandström MD, Moody S, Thorbek L, Progida C, Grigoriev IV, Barry K, Boddy L, Högberg N, Kauserud H, Eastwood DC, Skrede I (2018). ISME J. 12: 791-801 DOI: 10.1038/s41396-017-0006-8.

Paper III

Niche differentiation and evolution of the wood decay machinery in the invasive fungus *Serpula lacrymans.* Hess J, Balasundaram SV, Bakkemo RI, Drula E, Henrissat B, Högberg N, Eastwood DC, Skrede I (2021). ISME J. 15: 592-604 DOI: 10.1038/s41396-020-00799-5.

2. Summary

2.1. Summary in English

Ecological transition is a vital process by which organisms adapt to environmental changes or colonize new environmental niches. Through various ecological transitions, fungi have adapted to occupy a tremendous number of niches. Ecological transitions can be studied through evolutionary genomics, aiming to understand which genetic changes that underpins the transitions. The major focus of this thesis is to explore the ecological transitions and evolutionary genomics of the dry rot fungus *Serpula lacrymans* and closely related species within the family Serpulaceae. We used phylogenetics and evolutionary genomic approaches, in combination with experimental analyses of wood decay and competition abilities, to better understand how *Serpula lacrymans* has transitioned from living in nature to the built environment.

In Paper I, we analyzed the number of genetic markers required to identify cryptic species in *Serpula*. We analyzed ten different DNA markers, comprising eight nuclear and two mitochondrial DNA markers, which were obtained from several cryptic *Serpula* species. The incorporation of at least five DNA loci gave the cryptic species a highly confident separation within *Serpula.* The genetic marker ITS, the universal fungal DNA barcode marker, did not perform as well in identifying the cryptic species as some of the other loci. The DNA loci *tub*, *hsp*, *rpb2*, and *tef* generally provided the highest support in single-gene trees for the different cryptic species. We draw the conclusion that, in addition to the common DNA barcode ITS, analysis of a few independent DNA loci, including *tub*, *hsp*, *rpb2*, and *tef*, may provide a reliable signal of the occurrence of cryptic species in fungi.

In Paper II, we studied the ecological adaptations of the destructive house-invading fungus *Serpula lacrymans* var. *lacrymans* to woody substrates inside the built environments. Owing to its threats to the building structures, resulting in substantial economic damages and cost implications on house owners, there has been a long-lasting interest in perceiving how var. *lacrymans* became adapted to human-made environments. In this study, we aimed to understand the genetic and physiological factors that have made the dry-rot fungus the most invasive wood decay fungus in the built environment. Here, we did comparative genome analyses on the house-dwelling var. *lacrymans* against its close relative and nature-living var. *shastensis* and to its sister species *Serpula himantioides*, to obtain knowledge about which genomic changes that have accompanied the transition from nature to buildings. Var. *lacrymans* displayed very effective wood decay whereas *S. himantioides* showed better competitor abilities. The latter might be because *S. himantioides* encounters more fungal competitors than *S. lacrymans* in its natural habitat. Analyses of selection across the lineages indicated that there have been selection on genes involved in intracellular transport mechanisms and decay, which might be key feature making var. *lacrymans* a successful and destructive decomposer of timber in houses, compared to its close relatives. Overall, our analyses indicate that the dry rot fungus var. *lacrymans* is an ecological specialist with poor competitive ability against other fungi.

Further, in paper III, we analyzed the evolution of the wood decay machinery in various *Serpula* lineages in relation to niche breadth. Through transcriptomics analyses, we compared the decay machinery across four *Serpula* lineages, including European var. *lacrymans* (SL200), Japanese var. *lacrymans* (SL198), var. *shastensis* (SHA17-1), and *S. himantioides* (MUCL38935) on three different substrates (spruce, pine, and fir). The analyses confirmed that var. *lacrymans* is more specialized to a rapid decay of specific substrates (spruce), while

S. himantioides seems to be more of a generalist. Our results indicate that the specialist var. *lacrymans* have less dependency on nitrogen-intensive enzymatic degradation, in contrast to the more generalist relative *S. himantioides*.

2.2. Sammendrag på Norsk

Arters evne til å tilpasse seg endringer i miljøet og kolonisere nye habitater og nisjer er viktig for arters overlevelse og videre evolusjon. Gjennom ulike evolusjonære endringer har sopp, i løpet av flere hundre millioner år, tilpasset seg et enormt antall ulike nisjer. For å forstå hvilke genetiske endringer som utgjør basisen for tilpasninger til nye habitater, kan man bruke komparative genomiske metoder. Hovedfokuset i denne oppgaven er å utforske de økologiske tilpasningene hos den destruktive soppen ekte hussopp, *Serpula lacrymans,* som angriper trematerialer i hus, og nært beslektede arter innenfor familien Serpulaceae. Fylogenetiske og genomiske analyser, i kombinasjon med eksperimentelle studier av soppenes råtemekanismer og konkurranseevne, ble brukt for å bedre forstå hvordan *Serpula lacrymans* har gått over fra å leve i naturen til å leve i menneskeskapte bygninger.

I Artikkel I analyserte vi antall genetiske markører som kreves for å identifisere kryptiske arter inne slekta *Serpula*. Vi benytta ti forskjellige DNA-markører, bestående av åtte nukleære og to mitokondrielle DNA-markører, for å studere forekomst av kryptiske arter i slekta og hvordan de kan avgrenses. Ved bruk av minst fem DNA-markører ble flere kryptiske arter separert med god statistisk støtte. Den genetiske markøren ITS, som er den universelle DNA-strekkodemarkøren for sopp, hadde ikke en like god evne til å identifisere de kryptiske artene som de fleste andre markørene. DNA markørene *tub*, *hsp, rpb2* og *tef* gav generelt den høyeste støtten i enkeltgentrær for de forskjellige kryptiske artene. Vi konkluderer med at analyser av noen få uavhengige DNA-markører, inkludert *tub, hsp, rpb2* og *tef*, kan avdekke kryptiske arter i sopp. Videre kan man konkludere med at den genetiske markøren ITS har begrensninger i dens evne til å skille mellom nært beslekta arter.

I Artikkel II studerte vi de økologiske tilpasningene ekte hussopp har til trebaserte byggematerialer. På grunn av skadene den påfører, og de store kostnadene dette medfører for huseiere, har det vært en langvarig interesse for å forstå hvordan soppen har tilpasset seg til menneskeskapte miljøer. Gjennom vekstforsøk og komparative genomanalyser mellom husboende ekte hussopp (var. *lacrymans*) og nære slektninger som vokser i naturen, ønsket vi å bedre forstå de genetiske og fysiologiske faktorene som har gjort soppen til den mest aggressive råtesoppen i bygninger. Forsøkene viste at ekte hussopp har en svært effektiv råte, mens søsterarten tømmernettsopp (*S. himantioides)* er mer konkurransedyktig. Det siste kan skyldes at denne (*S. himantioides)* er tilpasset til sterkere konkurranse i sitt naturlige habitat. Ekte hussopp derimot, møter trolig mindre konkurransen i hus eller i dens naturlig habitat. Seleksjonsanalyser indikerte at det har vært seleksjon på gener involvert i intracellulære transport og råte, noe som kan være nøkkeltrekk som har gjort ekte hussopp til en mer aggressiv nedbryter av tre-materialer i hus, sammenlignet med sine nære slektninger. Samlet sett tyder våre analyser på at husboende ekte hussopp (var. *lacrymans*) er en effektiv råtesopp, men samtidig en økologisk spesialist med dårlig konkurranseevne sammenliknet med andre sopp.

Videre, i Artikkel III, analyserte vi utviklingen av råtemekanismer hos ulike *Serpula*-arter og så dette i lys av deres nisjebredde. Gjennom transkriptomikk-analyser sammenlignet vi råtemekanismene på tvers av fire *Serpula* individer på tre forskjellige vedtyper (gran, furu og edelgran). Analysene bekreftet at husboende ekte hussopp (var. *lacrymans*) er mer spesialisert på nedbrytning av spesifikke substrater (gran), mens tømmernettsopp (*S. himantioides*) ser ut til å være en større generalist som kan bryte ned ulike substrater. Våre resultater indikerer at den spesialisten ekte hussopp (var. *lacrymans*) er mindre avhengig av nitrogenintensiv enzymatisk nedbrytning, i motsetning til slektningen tømmernettsopp, og at denne

tilpasningen kan være viktig for dens vekst og effektive nedbrytning av trematerialer i nitrogenfattige miljøer i bygninger.

3. Introduction

3.1. The fungal kingdom

The Fungal kingdom is among the largest and most diverse groups of heterotrophic eukaryotic organisms (Choi and Kim, 2017). It includes both unicellular microorganisms like yeasts and microsporidia, and multicellular groups such as mushrooms, truffles, lichens, and molds (Stajich, 2017). Fungi have a very old evolutionary history, and the oldest known fossils date back a billion years (Loron et al., 2019). They are vital for the ecosystem as they play several significant roles in regulating the nutrient cycle as saprotrophs and symbionts in both terrestrial and aquatic environments, apart from being pathogens and sources of food for other organisms (Sun et al., 2020, Barzee, 2021). The diversity of fungi is estimated to comprise between 2 and 11 million species (Phukhamsakda, 2022). One of the major sub-kingdoms of fungi is Dikarya, which is further divided into two large phyla, the Ascomycota and Basidiomycota. Basidiomycota constitutes the second-largest phylum of fungi, with approximately 41,000 described species (He, 2019). Within Basidiomycota there are three subdivisions where Agaricomycotina includes most species, approximately 30,000, of which 98% are present in the monophyletic class Agaricomycetes (He, 2019).

3.2. Fungal ecology

Fungal species are adapted to a vast diversity of habitats. Some fungi have narrow niches and small geographic ranges (specialist) while others are more ubiquitous (generalist). Generalist fungi typically occupy a wide range of habitats and may be more resistant to disturbances. In contrast, specialist fungi are less tolerant to environmental disturbance and have a narrow ecological niche, and can only use a restricted number of resources (Wang et al., 2021a). Generalists with wide niches face more competition while specialist usually faces fewer competitors as they may be adapted to more extreme environments.

Fungi have established three major ecological roles, namely saprotrophism, parasitism, and mutualism (Kendrick, 2011). Saprotrophic fungi are efficient in the biomineralization of wood and other dead plant materials through the decomposition process (Bödeker, 2016). Wood is a recalcitrant fibrous material and is composed of lignocellulosic components such as lignin, hemicellulose, and cellulose. In nature, lignin is one of the most abundant biopolymers conferring strength, rigidity, and resisting compression (Tribot, 2019). Cellulose is the key component of the plant cell wall, contributing to the largest extent of the dry weight of wood (Young, 1985). Hemicellulose is a biopolymer that mainly facilitates fiber's moisture absorption, biodegradation, and thermal degradation due to its lower resistance (Patel, 2018). Wood decay is a biological process caused mostly by fungi, but there are other kinds of degradation as well, caused by insects, bacteria, and ultraviolet rays (Johnston et al., 2016, Tlaskal et al., 2021, Ulyshen, 2016).

Wood decay fungi have been classified into white rot, brown rot, and soft rot (Schultz, 2008). However, recent findings have shown that there is no longer a clear distinction between these different types, indicating that the classification of rot types has to be further refined (Riley et al., 2014). Since the brown vs. white rot paradigm is inadequate, the idea of "gray rot" has emerged and provides a separate variable to link with gene contents (Schilling et al., 2020). White rot fungi are more often associated with hardwood decay and have a bleached appearance. This is characterized by the occurrence of spongy/stringy mass on the wood (Del Cerro et al., 2021). Soft rot fungi degrade damp woods with higher moisture and lower lignin content. With the secretion of cellulases, it can create distinctive cavities in the wood (Sahu et al., 2021, Goodell et al., 2008). Brown rot is considered a specifically important mechanism of fungal wood decay in temperate forest biomes, where wood is the major form of sequestered carbon. Brown rot fungi are prevalent in coniferous boreal woods and are

resulting in degraded wood that is brown and crumbly and is degraded via both enzymatic and non-enzymatic systems (Floudas et al., 2012, Sigoillot, 2012).

Brown-rot fungi are often considered more destructive as they may be more efficient than white-rot fungi. Brown-rot fungi decompose cell wall polysaccharides (hemicellulose and cellulose) but it also modifies lignin without substantial removal (Arantes et al., 2012, Rayner and Boddy, 1988). Recent findings have revealed that these fungi depend on a combination of two mechanisms, lignocellulose oxidation (LOX) and polysaccharide hydrolysis (Zhang et al., 2016). Fenton reactions play a key role in brown rot producing highly oxidizing hydroxyl radicals ($H_2O_2 + Fe^{2+} \rightarrow OH^- + Fe^{3+} + \bullet OH$) (Zhang et al., 2016). These oxidants modify the lignocellulose to make it more susceptible to enzymatic degradation by the limited set of glycoside hydrolases (GHs), which the brown rot fungi have retained in their genomes (Zhang et al., 2016).

The fungal networks (mycelium) transport mineral nutrients, including nitrogen, phosphorus, potassium, calcium, and magnesium (Jentschke et al., 2001). The physiology and underlying cellular machinery of both ectomycorrhizal and saprotrophic translocation do not correspond to a fundamental taxonomic or phylogenetic separation in basidiomycetes (Tlalka et al., 2008). In some basidiomycetes, rhizomorphs and hyphal cords, which are special transport organs, have evolved. Rhizomorphs and hyphal cords (mycelial cords) are linear aggregations of parallel-oriented hyphae. When they are melanized, as is the case for rhizomorphs produced by e.g. *Armillaria* spp., they may look like plant roots.

Within a resource, numerous wood decay basidiomycetes coexist in a diverse and dynamic community, and interactions affect species composition and the rate of wood decomposition

consequently (Hiscox et al., 2018). Within the wood, wood-decay fungi experience intra- and interspecific competition. Fungal competition can be divided into two: primary and secondary. During the primary competition, fungi colonize unoccupied territory/resource and exclude other fungi by capturing the resources first. During secondary competition, the nutrients and territory are captured from other fungi that have already colonized a resource, and hence, a replacement happens (Boddy and Hiscox, 2016, Boddy, 2000). Combative mechanisms comprise hyphal interference, gross mycelial contact, and mycoparasitism, and also include antagonism at distance (Boddy, 2000).

3.3. Study organism

3.3.1. Family Serpulaceae

One of the largest orders of Agaricomycetes is Boletales, which include mushrooms with fleshy tubes and are represented in most forest ecosystems worldwide (Margulies et al., 2005, Binder and Hibbett, 2006). Boletales have different nutritional modes including ectomycorrhiza fungi (ECM) associated with plant roots, saprotrophs, as well as parasites (Sato and Toju, 2019, Neuhof et al., 2007, Wu et al., 2021, Binder and Hibbett, 2006, Hibbett and Binder, 2002). Several studies have indicated that wood decay by saprotrophy is the plesiomorphic nutritional mode (Binder and Hibbett, 2006), while ECM has evolved independently several times within the Boletales (Tedersoo et al., 2010, Binder and Hibbett, 2006). Within the order of Boletales is the small family Serpulaceae. Molecular phylogenetic analyses have indicated that the family Serpulaceae, as currently defined, includes three genera, i.e. *Serpula* (Pers.) Gray, *Austropaxillus* Bresinsky & Jarosch, 1999, and *Gymnopaxillus* E. Horak, 1966 (Hibbett et al., 2000, Skrede et al., 2011). *Serpula* produces brownish, and resupinate basidiocarps, *Austropaxillus* produces stipitate-pileate fruiting bodies and a lamellate hymenophore, while *Gymnopaxillus* encompasses truffle-like

hypogeous (underground) fruit bodies (Skrede et al., 2011, Claridge et al., 2001). *Austropaxillus* and *Gymnopaxillus* form ECM with roots, whereas *Serpula* consists of several saprotrophic taxa that mainly degrade conifer wood, resulting in brown rot (Skrede et al., 2011). Hence, within Serpulaceae, there has been one major ecological transition from saprotrophy, still present in *Serpula*, to ECM symbiosis, present in *Austropaxillus* and *Gymnopaxillus* (Skrede et al., 2011) (Fig 1).

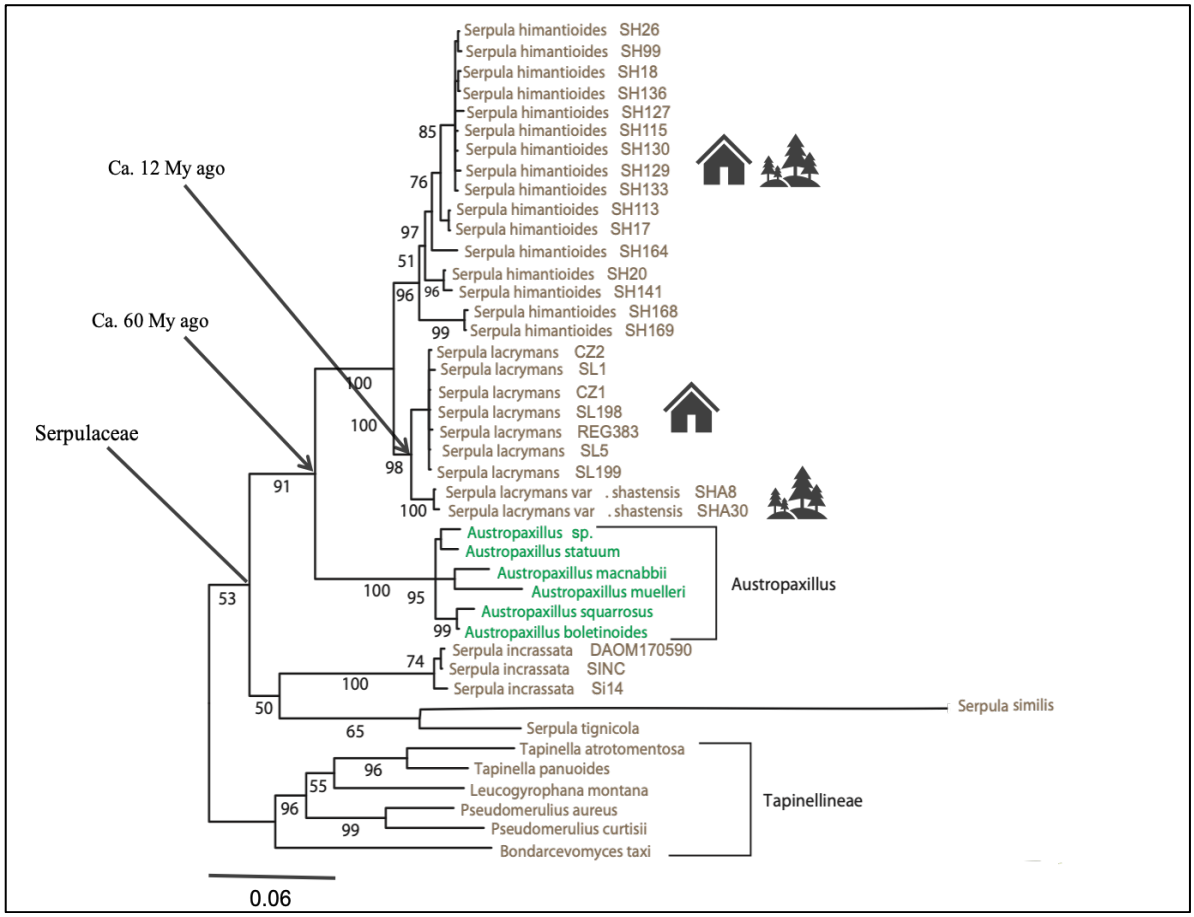

Fig 1. Phylogenetic tree describing the relationship in the family Serpulaceae with the out-group Tapinellineae. Bayesian posterior probabilities of more than 0.9 and parsimony Jackknife above 50% are superimposed (Modified from (Skrede et al., 2011)). The brown text indicates brown rot taxa, and the green text indicates ectomycorrhizal taxa.

3.3.2. Serpula lacrymans

The most well-known species in Serpulaceae is the infamous dry rot fungus *Serpula lacrymans*. It causes extensive damage to buildings in temperate regions. The sister species, *S. himantioides*, can also be found in buildings but causes less aggressive rot in both nature and buildings (Kauserud et al., 2007c). The morphotaxon *Serpula lacrymans* has a worldwide distribution of temperate and boreal regions and produces pancake-like fruit bodies (basidiocarps) of thickness 2-20 mm (Kauserud et al., 2007c, Kauserud, 2004). *Serpula lacrymans* includes two varieties, var. *lacrymans*, and var. *shastensis* (Fig 2).

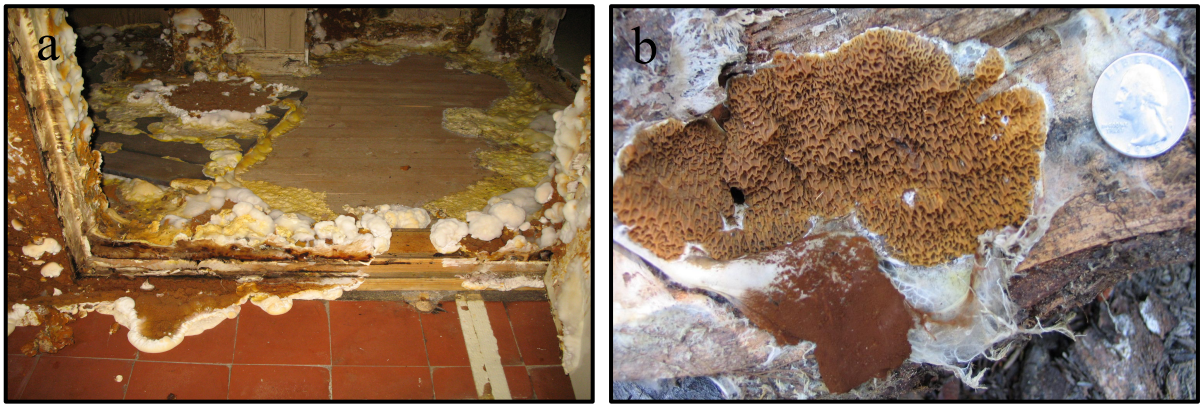

Fig 2. a). The aggressive form of *S. lacrymans* var. *lacrymans* from a house in Sweden, b). The non-aggressive form of *S. lacrymans* var. *shastensis* on a natural substrate (Photo credits: Mycoteam AS & Kauserud et al. 2007)

Var. *shastensis* has a natural distribution mainly in the Cascades mountain range in North America (Cooke, 1955, Harmsen, 1960), while var. *lacrymans* has a natural distribution in North East Asia (Bagchee, 1954, White et al., 2001), from where it has spread to Australia, New Zealand, Europe, and North and South America (Hallenberg and Eriksson, 1985, White et al., 2001, Kauserud et al., 2007c). In addition, another invasive *Serpula lacrymans* population is found in Japan, showing significantly higher levels of genetic diversity

(Kauserud et al., 2007c, Skrede et al., 2021, Engh et al., 2010b). The worldwide spread-out of var. *lacrymans* has apparently happened recently, as very little genetic variation occurs in the founder populations (Kauserud et al., 2007c). The sister species of *S. lacrymans*, *S. himantioides*, which is a species complex consisting of multiple cryptic species (Carlsen et al., 2011), also has a worldwide distribution in temperate regions.

The domestic lineage of *S. lacrymans* has evolved a uniquely aggressive mode of brown rot wood decay in houses in temperate regions. Var. *lacrymans* typically cause extensive damage in attacked buildings, being the most severe decayer of human-made wooden constructions (Skrede et al., 2011, Kauserud et al., 2007c). The optimum temperature for *Serpula* growth is about 19-21°C (Jennings and Bravery, 1991) and dies in temperatures exceeding 32 °C (Bech-Andersen, 1995). *Serpula lacrymans* produces hyphal cords that may be up to 2 cm in diameter (Fig. 3), which are used for the transport of water and nutrients from one part of the genet to another. Every year, this fungus causes damages worth millions of euros in Northern Europe (Bech-Andersen, 1995, Palfreyman et al., 1995).

Fig 3. Thick hyphal cords (Photo credits: Mycoteam AS)

3.4. Fungal genomics

3.4.1. Sequencing

Genome sequencing of fungi provides pivotal information about fungal evolution, cellular functions, and degradation mechanism. DNA sequencing technologies have made a paradigm shift in understanding the genomic structure, genome diversity, genome content, and expression profiles. The sequencing technologies have successfully provided the opportunity to obtain a vast amount of high-quality genomic information with a relatively modest cost and effort. First-generation sequencing, Sanger sequencing (also known as dideoxy or capillary electrophoresis sequencing) was developed in 1975 and was the most widely used sequencing method for many years (Sanger et al., 1977, Swerdlow et al., 1990, Hunkapiller et al., 1991). Around 2005, the second generation, also called Next Generation Sequencing technology (Shendure and Ji, 2008) emerged and it included Roche/454 FLX pyrosequencing (van Dijk et al., 2014), Illumina/Solexa Genome sequencing (Bennett, 2004), Ion Torrent sequencing (Rothberg et al., 2011) and SOLiD sequencing (Porreca et al., 2006). These technologies lead to proficient higher sample throughput and yield deep sequencing with high accuracy and speed at lower costs (Liu et al., 2012, Salk et al., 2018).

Though the second generation sequencing techniques demonstrate groundbreaking advantages, they have a major setback in producing short reads, which requires highly precise downstream bioinformatic analyses to deal with transposable elements and highly repetitive regions (Athanasopoulou et al., 2021). To overcome this read length disadvantage, third-generation sequencing technologies (also known as long reads sequencing) were developed. These sequencing technologies can read much longer DNA. It also has the advantages of single-molecule sequencing, and simplified library preparations reducing time and labor (Au, 2022). Single-molecule sequencing in real-time (SMRT) sequencing implemented by Pacific

Biosciences (PacBio) provides exceptional reads lengths of 10 - 25kb average with highly accurate sequences and uniform coverage without any GC bias (Hon et al., 2020). Oxford Nanopore Technologies (ONT) technology uses the nanopore proteins (a-hemolysin) and current to read and sequence the DNA, unlike the other sequencing techniques which use DNA polymerases (Wang et al., 2021b). ONT has multiple advantages of small sequencer size, real-time sequencing, and ultra-long read lengths despite the lower accuracy (Amarasinghe et al., 2020).

3.4.2. Genomics

Fungal genomics is a rapidly growing research arena. According to the updated Genomes Online Database, 26,117 ongoing eukaryotic genome projects are from the fungal kingdom (Mukherjee et al., 2017). The US Department of Energy (DOE) Joint Genome Institute (JGI) designed MycoCosm, which is a large integrated fungal genomics resource that has been important for the progression in this research field (Grigoriev et al., 2014). Over 2410 complete fungal genomes have been publicly released as of December 2022 at MycoCosm (http://genome.jgi.doe.gov/fungi/fungi.info.html) (Nordberg et al., 2014). Hence, there is a vast amount of sequence data available for fungal comparative genomics. Comparative genomic analyses help to understand the role of genomic structure and gene content in the ecological adaptation of fungi and its significant role in ecosystem functioning. Compared to other eukaryotes, fungal genomes usually have fewer repetitive elements, ranging from 1 – 25% of the total genome size (Castanera et al., 2016). Although there are fewer repetitive elements compared to other eukaryotes, they are still important for the functional evolution of fungal genomes (Castanera et al., 2016).

By comparative genomics techniques, such as comparing gene content and expression levels, it is possible to comprehend the genomic basis of adaptation in non-model organisms. Comparative genomics studies have been used to study the evolution of basic fungal traits, such as the fungal hyphae and the evolution of novel gene families in early diverging fungi (Kiss et al., 2019). Comparative genomic analyses of ectomycorrhizal fungi have shed light on the genomic mechanisms underlying host specificity. For example, the comparative genomics of *Suillus*, a specialist genus of ectomycorrhizal fungi, revealed the deactivation of reactive oxygen as a key mechanism for the host specificity (Lofgren et al., 2021). Comparative genome analyses of the ectomycorrhizal milk-cap fungi *Lactarius* also uncovered a large diversity in gene repertoires and genomic landscapes and suggested that symbiont host specificity may be connected to species-specific genes, such as secreted sedolisins (Lebreton et al., 2022). Also, numerous comparative genomics studies have been carried out to better understand the evolution of different decay mechanisms. For example, a comparison of the genomes of two white rot fungi *Phanerochaete carnosa* and *Phanerochaete chrysosporium* revealed that *P. carnosa* specializes in decomposing softwood, and has a higher number of genes encoding degrading enzymes (Suzuki et al., 2012). Similarly, a comparison of *Cylindrobasidium torrendii* and *Schizophyllum commune* revealed that these fungi exhibit characteristics intermediate between white-rot and brown-rot fungi, resembling soft rot. Both species cause weak decay and have genes related to carbohydrate degradation.

Additionally, *Fistulina hepatica* displays features of brown rot but also possesses genes related to cellulose degradation, suggesting a transition towards a brown-rot lifestyle. These results highlight the diverse mechanisms involved in wood decay, challenging initial assumptions about the process (Floudas et al., 2015). Furthermore, a genome-wide analysis of carbohydrate-active enzymes and oxidative enzymes in 11 Polyporales species revealed

that brown-rot polyporales lack cellulases and lytic polysaccharide monooxygenase genes, while white-rot polyporales maintain a greater enzymatic diversity, supporting their efficient attack on lignocellulose (Hori et al., 2013). In a broader context, a comparative genomic analysis and phylogenetically informed principal-components analysis of 33 basidiomycete genomes, found a continuum between white and brown rot modes, necessitating a more nuanced categorization (Riley et al., 2014).

Comparative transcriptomics analyses have also been used to understand the metabolic pathways and gene functions involved in fungal development and pathogenesis. For example, comparative transcriptomics of *Gymnosporangium* species identified highly expressed genes and metabolic pathways in teliospores that may serve as potential pathogenesis-related effectors (Tao et al., 2019). Comparative transcriptome analysis of fruiting body development revealed mechanisms for environmental adaptability and the development of abnormal symptoms in the commercially important mushroom *Lentinula edodes* (Yan et al., 2021). The comparative transcriptome and secretome analysis of *Rhodonia placenta* and *Phanerochaete chrysosporium* revealed that while *P. chrysosporium* employs a range of extracellular glycosyl hydrolases, *R. placenta* predominantly secrets hemicellulases, with only a few potential cellulases. Interestingly, this observation aligns with an upregulation of genes in *R. placenta* involved in iron acquisition (Vanden Wymelenberg et al., 2010).

Another comparative transcriptomic analysis explores in to the fungus *Cerrena unicolor*'s behavior when growing on birch, ash, maple sawdust, and control liquid medium. This study identified significant expression differences between the media, with upregulated genes particularly for cellulases and hemicellulases, being more abundant in sawdust medium (Janusz et al., 2018). Additionally, a comparative transcriptomics study focused on the impact

of wood acetylation on *Rhodonia placenta* and *Gloeophyllum trabeum.* This study uncovered clear differences in decay strategies between the two fungi, emphasizing the importance of identifying key genes to facilitate decay detection, identification, and biomarker selection (Kölle et al., 2021).

4. Research aims

The overarching objective of this PhD thesis was to investigate how and why the dry rot fungus *Serpula lacrymans* has become a successful invader of human-made indoor habitats. More specifically I aimed to:

- obtain a better knowledge of the phylogenetic relationships of the study organisms, and the number and quality of genetic markers needed to identify cryptic species (Paper I)

- identify features associated with the transition from living in nature to occupying buildings, by comparative genomics of *S. lacrymans* var. *lacrymans*, *S. lacrymans* var. *shastensis,* and *S. himantioides* (Paper II)

- investigate whether the transition could be related to divergent physiological characteristics, including decomposition ability and competitive interactions with other fungi (Paper II)

- identify how gene content and gene expressional differences affect wood decomposition efficiency in indoor *Serpula lacrymans* strains, compared to its relatives growing in nature (Paper II and III)

- study the evolution and expression of the decay machinery of *Serpula lacrymans* variants across three different substrates, to investigate its niche breadth. (Paper III)

5. Materials and methods

Here, the methods implemented in Paper I-III are briefly outlined. I will start by outlining the included sequencing techniques, followed by a brief description of bioinformatics approaches and growth experiments.

5.1. Sequencing

We employed Sanger sequencing (Sanger et al., 1977) for the sequencing of ten DNA loci in study I. In contrast, for papers II and III, we used Illumina sequencing technology as we were sequencing the entire genome/transcriptome. We sequenced the three *Serpula lacrymans* strains (SL198, SL200, and SHA17-1) using an Illumina 108 paired-end library on an Illumina GAII (Gravina et al., 2013). We obtained the genome of *S. himantioides* (MUCL38935) from The Joint Genome Institute (JGI) for use in comparative studies. Furthermore, in study III, we pooled the RNA libraries and sequenced them with a 150 bp paired-end library on an Illumina NextSeq 500 instrument (Illumina, 2014). This technology is capable of yielding high-quality sequencing data with a relatively fast turnaround time. Overall, these sequencing methods were suitable for their respective research questions and allowed for the generation of high-quality sequence data for downstream analysis. The rapid evolution in sequencing technologies presents an opportunity to utilize long-read sequencing technologies, such as PacBio (https://www.pacb.com/) and Oxford Nanopore (https://nanoporetech.com/), in future studies. The third-generation sequencing techniques will offer the potential to generate more complete and contiguous genome sequences, which can help in the identification of genes, regulatory regions, and other functional elements.

5.2. Genome assembly

In papers II and III, we primarily filtered out bad-quality reads and removed adaptor sequences. Then, we used the Velvet de novo assembler (Zerbino and Birney, 2008) to assemble reads into contigs for the three *lacrymans* strains. Velvet utilizes the de Bruijn graph approach to handle a large volume of data and to adjust k-mer length for varying complexities. In paper II, we estimated the completeness of the genome assemblies by using CEGMA pipeline (Parra et al., 2007), which compared the selected assemblies to pre-selected reference protein-coding genes. In paper III, we used the improved version of CEGMA called BUSCO (Simao et al., 2015, Bradnum, 2015), with a Basidiomycota reference database. BUSCO has substantially faster run times and can evaluate both prokaryotic and eukaryotic species (Manni et al., 2021).

5.3. Gene predictions and annotation

We used a range of bioinformatics tools and methods to identify and compare genes, orthologous groups, and gene families across four different *Serpula* strains. We used these tools to infer changes in selective pressure and functional categories associated with gene family expansions and contractions. We employed gene models to predict genes and their functions. Additionally, we used the "Just Annotate My Genome" pipeline (https://github.com/genomecuration/JAMg), a genome annotation improvement tool to refine and update annotations for the genome sequences, leading to a more accurate and comprehensive understanding of the biological information encoded in the genome. Lastly in paper III, we utilized transcriptomic data to confirm the presence of predicted genes and refine their boundaries. For detailed information about these methods, see Paper II and III.

5.4. Phylogenetic analysis

In Paper I, we used phylogenetic analyses to assess how many markers were needed to obtain robust support for the different lineages within the Serpulaceae family. We used multiple genetic markers and phylogenetic analysis to construct trees that maximizes the likelihood of the observed data. We used the GTRCAT model in RaxML (Stamatakis, 2014) to capture heterogeneity across sites and 1,000 bootstraps to approximate the substitution pattern of the nucleotide sequences. Additionally, we used the Phylogenetic informativeness (PI) analysis to evaluate which markers were most informative at which phylogenetic level, ranging from basal to the terminal branches. In Paper III, we used a phylogenomic reconstruction pipeline for estimating Maximum Likelihood (ML) trees using RaxML (Stamatakis, 2014) and improving them using TreeFix (Wu et al., 2013). Later, we reconciled the corrected trees with species trees using DLCpar for accurate taxonomic relationships (Wu et al., 2014) to reconstruct the evolutionary history of gene families and to identify orthologous genes across the four *Serpula* genomes. This pipeline constructed phylogenetic trees to infer the evolutionary relationships between the genes.

5.5. Combative analysis

In papers II and III we designed an antagonistic experiment to test the combative ability of the four *Serpula* strains, and three other brown rot decomposer fungi (*Antrodia xantha*, *Fomitopsis pinicola*, and *Coniophora puteana*). The experiment involved placing two well-colonized blocks of different fungal strains side by side to quantify the interactions. We assessed the antagonistic behavior of the European isolate (S7) of var. *lacrymans*, Japanese var. *lacrymans* along with three other *Serpula* strains, using precolonized wood blocks of fir.

5.6. Decomposition analysis

Using decomposition experiments, we compared the decomposition abilities of three *Serpula* strains and other brown rot fungi (paper II). We ran the decomposition experiments for 60 days at 20 °C to measure the mass loss differences among the strains using three different substrates (pine, spruce, and fir). In paper III, we evaluated the decomposition abilities of various S. *lacrymans* and *S. himantioides* strains on pine, spruce, and fir to investigate their responses to different wood types, and hence, their potential niche breadths.

6. Results

The following paragraphs provide a description of the key findings of the papers in this thesis.

6.1. Paper I. A few accurate markers can sufficiently resolve cryptic species

This study aimed to determine the minimum number of genetic markers required to differentiate closely related cryptic species within the Serpulaceae family. In the study, we used ten genetic markers to first create a multi-gene phylogenetic tree via maximum likelihood, which supported the topology observed in a previous phylogenetic study of *Serpula*. Further phylogenetic analyses of different combinations of the markers, ranging from nine to single-locus phylogenies, showed that a combination of fewer DNA loci, including *tub*, *hsp*, *rpb2*, and *tef*, were sufficient to obtain reliable discrimination of the cryptic species in the Serpulaceae family. However, at least a minimum of five genetic markers were necessary to achieve 100% bootstrap support for cryptic species across all nodes. The four markers (*tub*, *hsp*, *rpb2*, and *tef*) exhibited the highest bootstrap support in single-locus analyses and had a maximum phylogenetic informativeness (PI) at the point where the three lineages (*S. lacrymans* var. *lacrymans*, *S. lacrymans* var. *shastensis* and *S. himantioides*) diverged. Our findings indicate that multi-locus analyses of four or five informative loci can provide good discrimination of closely related species.

6.2. Paper II. Genomic and physiological changes in Serpula lacrymans

Paper II aimed to investigate the genomic changes that occurred during the transition of *S. lacrymans* from its natural habitat to the domestic environment. The study compared var. *lacrymans*, var. *shastensis*, and the sister species *S. himantioides* in order to identify the physiological and genomic changes associated with the transition of *S. lacrymans* to a house-dwelling fungus. The study used genomic analyses and two growth experiments to identify

the decomposition efficiency of all three strains and their competitive abilities with other brown rot species. The combative results showed that *S. himantioides* was more competitive than var. *lacrymans* and var. *shastensis*, and both *lacrymans* variants had a lower ability to exclude other species in comparison to *S. himantioides*. The decomposition results indicated that both var. *lacrymans* and var. *shastensis* decomposed more of the spruce wood blocks than *S. himantioides*. All three *lacrymans* strains degraded spruce more quickly, but 50% of spruce and only 5% of pine mass were lost by var. *lacrymans*.

Genome sequencing and in-silico analyses revealed that several gene families related to the wood decay mechanisms were expanded/contracted, and the set of CAZymes encoded within the three genomes was very similar, with greater gene complement in *S. himantioides*. However, var. *lacrymans* had an Iron reductase with a CBM1 and CytB domain, which was not present in *S. himantioides* and has been proposed to have an electron transfer function targeting reduced iron to the cellulose substrate for effective chelator-mediated Fenton (CMF) chemistry leading to better utilization of carbohydrates in its environment. Paper II also discovered a selective shift in genes involved in intracellular transport, cell development, and rearrangement. Also, there was an accelerated gene evolution and loss of copies of glutathione-S transferase in var. *lacrymans* compared to *S. himantioides*, which may have played an important role in substrate specilization and adaptation to a different environment, i.e. the built environment.

6.3. Paper III. The molecular underpinnings for changes in wood decomposition efficiency in Serpula lacrymans

We analyzed the evolution of decay mechanisms in *Serpula lacrymans* variants on three different substrates to investigate their niche breadth in paper III. Four strains, including two

variants of *lacrymans* (European var. *lacrymans* and Japanese var. *lacrymans*), one individual of the closest relative, var. *shastensis*, and one individual of *S. himantioides* as an out-group, were used. We examined the four strains through decomposition experiments on different wood resources (pine, fir, and spruce), and distinct wood type-dependent responses were shown among them. The fungal inocula on wood blocks consumed 0-69% of biomass after 60 days of growth. The generalist *S. himantioides* consumed, on average, 15% of the biomass of pine, 28% of the biomass of fir, and 34% of the biomass of spruce. In contrast, both the European and Japanese var. *lacrymans* and var. *shastensis* showed specialist behavior and a strong preference for spruce with average rates of 45% mass loss for both strains. In fir wood, the two var. *lacrymans* and var. *shastensis* strains exhibited a mass loss of less than 40%. However, in the case of pine wood, var. *shastensis* failed to establish while the var. *lacrymans* from Europe showed an average mass loss of less than 5% of the wood blocks. At the same time, var. *shastensis* growth was observed on pine wood shavings in the RNA analysis. On the other hand, the Japanese var. *lacrymans* caused an average mass loss of 23% in pine wood. In the most extreme cases, European var. *lacrymans* demonstrated up to 69% mass loss of spruce, significantly higher than the decomposition rate of *S. himantioides*, suggesting a shift in decay strategy from generalist to specialist. We also evaluated the antagonistic behavior of *Serpula* strains towards three common fungal decomposers through competition assays. The competition assays revealed that *S. himantioides* was a strong competitor. Also, Japanese var. *lacrymans* showed strong antagonism, particularly towards species outside the Serpulaceae family.

The transcriptomic profiling showed a shift in decay machinery in all four strains during the wood decay of different substrates. The generalist *S. himantioides* exhibited a largely consistent gene expression pattern. In contrast, European var. *lacrymans*, Japanese var.

lacrymans, and var. *shastensis* displayed distinct expression reactions to the different types of wood. Our functional analysis of differentially regulated genes in spruce and pine focused on the expression of carbohydrate-active enzymes (CAZymes) involved in the degradation of plant cell wall (PCW) material. The analyses revealed strong differentiation in the expression of key PCW decomposing enzymes between spruce and pine, with relatively few CAZymes specifically induced in spruce compared to pine.

We found that the spruce-specific CAZymes are characterized by accessory CAZymes, which play a role in the digestion of oligosaccharides into simple sugars (b-glucosidases GH1 and GH3 and b-mannosidase GH2) or debranching enzymes digesting hemicellulose side chains (CE16 acetylxylan esterase). This suggests that the spruce transcriptomes of var. *lacrymans* and var. *shastensis* represent more advanced stages of decay than *S. himantioides.* The evolutionary analysis of wood-induced gene sets revealed expression of certain genes on both spruce and pine in all four strains studied. The observed decay differences among the species are due to genetic bias as indicated by gene expression analyses, variable decay rates and also the gene content on spruce and pine. These results suggest a fundamental shift in decay strategy in the last common ancestor of var. *lacrymans* and var. *shastensis.* Var. *lacrymans* and var. *shastensis* showed a significantly higher rate of substrate-dependent decay and decreased effectiveness to decompose different substrates.

7. Discussion

Fungi occupy specific habitats in nature (Boer et al., 2005), as symbionts, pathogens or saprotrophs. In addition, humans have inadvertently re-created similar habitats for certain fungi in agricultural environments or in the built environment. In case these fungi are exposed to the actual human-made habitats, they may become invasive fungi. This is the case for the decomposer fungus *Serpula lacrymans,* which found and occupied a habitat in human-made construction materials that resembled its natural habitat in mountain regions (Schmidt and Czeschlik, 2006). Despite being a comparatively recent development in evolutionary terms, microbial decay of buildings follows a similar pattern to the natural degradation of organic matter in ecosystems. Fungal decomposition is a crucial biological process, essential for maintaining nutrient recycling by breaking down complex organic matter and releasing nutrients back into the environment.

Hence, understanding fungal decomposition processes is important for comprehending ecosystem function. In buildings, fungi can change the materials physical and chemical qualities as they colonize and deteriorate buildings, causing rot, discoloration, and weakening of the structures (Woo-Yang Chung et al., 1999). Hence, also from this perspective, it is important to understand how degradation of wood structures in building constructions happens (Goodell et al., 2020). *Serpula lacrymans,* our study organism, is of high significance due to its aggressive high degradation ability of built structures (Watkinson and Eastwood, 2012). Which genomic and physiological traits made *S. lacrymans* such an aggressive invader of the built environment is the main focus of this thesis. In the following, I first discuss the presence of cryptic species within *Serpula* and then discuss which traits and strategies that may have made *S. lacrymans* a successful invader of the human habitat.

7.1. Cryptic species and species delimitation in Serpula

Defining species and delimiting species boundaries is a basic first step in biology that most biological disciplines depend on. However, this can be challenging in many cases due to e.g. morphological similarity across species. Cryptic species are morphologically very similar and often closely related and can often only be distinguished by their genetic information. Cryptic species seem to be highly widespread in the fungal kingdom, maybe because relatively few morphological characters, mainly those associated with the fruiting bodies, are easily available. Although morphologically similar, cryptic species may differ in other features, like pathogenicity and virulence factors (Hirakawa et al., 2015), reproductive isolation (Stengel et al., 2022, Balajee et al., 2009), and physiological and biochemical traits. The identification of cryptic species is important for understanding species richness and conservation and preventing the overestimation of population size and the underestimation of the diversity of an ecosystem.

In this thesis, we studied the number of markers required for separating the cryptic species in *Serpula.* Although genome data is now available from many species, it is still a challenging task to produce full genome data, and this might not be needed for separating between cryptic species. According to previous studies (Taylor et al., 2000, Vilgalys and Sun, 1994, Rokas et al., 2003), multiple independent genetic markers, ranging from two to twenty, are required to accurately distinguish closely related fungal species. It was revealed earlier that 8–20 genetic loci were required to differentiate between several *Saccharomyces* species (Rokas et al., 2003). On the other hand, *Heterobasidion annosum* was separated into three distinct phylogenetic species using only four genetic markers (Johannesson and Stenlid, 2003), while in *Laccaria*, *Coniophora arida*, *C. olivacea*, and *C. puteana,* only three genetic markers were sufficient for delimiting cryptic species (Kauserud et al., 2007a, Kauserud et al., 2007b,

Sheedy et al., 2013). In contrast, *Pleurotus ostreatus* requires only two genetic markers to provide significant support for species differentiation (Vilgalys and Sun, 1994).

Our study supports that there are multiple cryptic species in *Serpula* and that the two sub-species of *Serpula lacrymans* represent well-differentiated lineages with no gene flow in between. Our study also suggested that at least five markers were required to identify the cryptic species in *Serpula*, with high statistical support. Among the ten genetic markers considered, the internal transcribed spacer (ITS), the main DNA barcoding marker for fungi (Schoch et al., 2012) performed the worst along with SSU (the small subunit) and mtSSU (mitochondrial small subunit). It should be noted that the number of markers supported in our study is not universally applicable to all fungi. This is due to the fact that different markers in different fungi have different levels of evolutionary rates, and that the splits between cryptic species of other groups are of different ages.

Though our methodology offers a snapshot of genetic variations, it may not manifest the species' complexity at the whole genome level. Amidst this, our methodology still offers several advantages such as being cost-effective, accessible and providing a rapid identification. There are several alternative ways for approaching cryptic species identification in fungi, including phylogenomics (Stengel et al., 2022) and population genomics (Kobmoo et al., 2019) analyses, where the entire genome or a large extent of the genomes are analyzed.

7.2. Mycelial growth and transport

Mycelium is the vegetative component of fungi and it plays various roles in nutrient absorption (Finlay, 2008), reproduction (Kues and Liu, 2000), biotic interactions (Klironomos, 2002), and decomposition (Talbot J. M. et al., 2008). The fungal hyphae that

makes up the mycelium grow and spread around and through the wood. Hyphal growth is reliant on secretory vesicles, cytoskeleton organization, and polarization. In study II, we observed that genes involved in endomembrane system functioning and hyphal growth had distinct selection pressure in *S. lacrymans* and *S. himantioides.* This indicated variations in resource translocation which may play a vital role in adaptation to various habitats. Some fungi, including *S. lacrymans*, produce rhizomorphs or mycelial cords for transportation purposes.

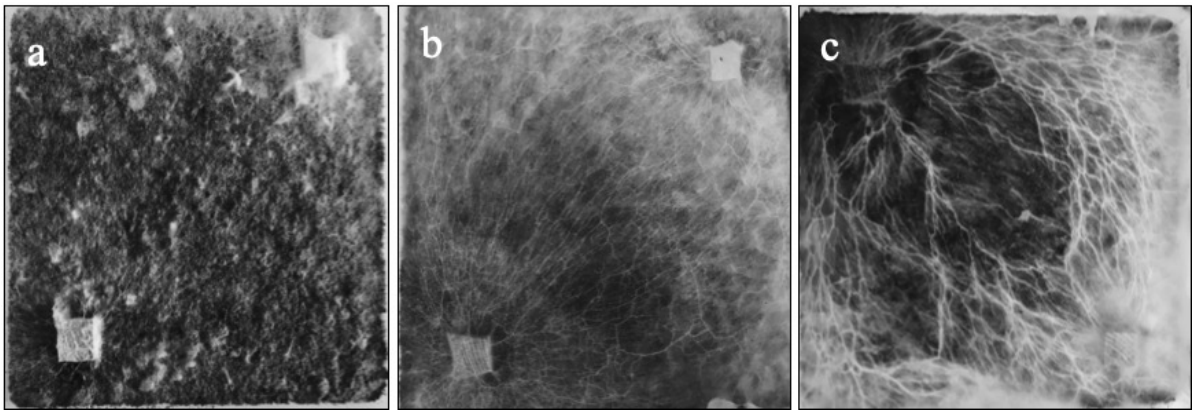

Fig 4. Mycelium and mycelial cords growth in the three different strains (a) *Serpula himantioides* (b) *S. lacrymans* var. *shastensis* (c) *S. lacrymans* var. *lacrymans.* The colonized wood blocks with three strains were confronted in paired combinations. The three strains act differently and var. *lacrymans* produce more thicker cords than the other two strains (Photo credits: Inger Skrede).

The mycelial cords are formed when several hyphae fuse together into a larger transport organ. *Serpula lacrymans* use the mycelial cords to transport nutrients and water from one point of colonization to another. When comparing var. *lacrymans* to var. *shastensis* and *S. himantioides*, there is a significant shift in selection pressure in genes involved in intracellular

transport, growth, and reorganization of the cell. This could play a crucial role and act as a key factor for its success in the built environment, where transport of resources, from one location to a new point of colonization, is of high importance. *Serpula lacrymans* var. *lacrymans* produce thicker mycelial cords compared with var. *shastensis* and *S. himantioides* (Fig. 4), permitting more efficient transport and translocation of resources (amino acids and water) to help colonize new wood substrates of the building (Watkinson SC et al., 2006).

7.3. Decay mechanism

Fungal decay involves colonization, substrate breakdown, and nutrient absorption. *Serpula* is capable of breaking down different wood structures through the use of specialized enzymes (Nurika et al., 2020) and a non-enzymatic Fenton reaction (Shimokawa T. et al., 2005, Presley and Schilling, 2017, Eastwood et al., 2011). The Fenton reactions is involved in the biodegradation of lignocellulose, as shown by the loss of enzyme-encoding genes in brown rot fungi (Nurika et al., 2020, Floudas et al., 2012). The growth experiments in both study II and III demonstrated *S. lacrymans* as an effective decomposer of coniferous wood but with a narrower niche breadth compared to *S. himantioides*. *Serpula lacrymans* is an effective decomposer of spruce and fir, but not so well for pine. The Japanese var. *lacrymans* caused significantly greater average weight loss on both spruce and pine. The RNA analysis specifically indicated the presence of var. *shastensis* on wood shavings derived from pine wood, leaving us with uncertainty regarding the reason. One possible reason might be the wood shavings' capacity to retain more moisture compared to solid wood, facilitating fungal growth. The increased surface area and aeration caused by the coarse quality of wood shavings may also impact its growth rate. Meanwhile, *S. himantioides* was also capable of decomposing pine to a greater extent. *Serpula lacrymans* decomposes suitable substrates faster and more efficiently than *S. himantioides*, possibly due to a more efficient CMF chemistry. The iron

reductase which is found in var. *lacrymans* but not in *S. himantioides* targets reduced iron directly for efficient CMF, highlighting its importance in early oxidative degradation steps (Eastwood et al., 2011). In study II and III, we observed that several gene families related to the wood decay, including CAZYmes and Cytochrome P450, were expanded and contracted, respectively. Both CAZYmes and Cytochrome P450 are involved in fungal metabolism and degradation (Pratiwi R A. et al., 2022).

Wood is a substrate with a low nitrogen content (Watkinson and Eastwood, 2012) compared to natural forest soils. Usually, the decay machinery uses a large amount of nitrogen for utilizing wood (Warner, 1999). The transcriptomic data in study III indicate that *S. lacrymans* has evolved to rely less on nitrogen-intensive enzymatic degradation, which might be an adaptation to the very low nitrogen content in wood. Upon the unavailability of adequate nitrogen, there is a shift in the decay mechanism to leverage the high carbon flux compounds such as oxalic acid. This decay adaptation strategy was found in var. *lacrymans* and var. *shastensis* (Hess et al., 2021). The results from paper II indicate that the dry rot fungus *S. lacrymans* is an ecological specialist and was preadapted to colonize buildings (Balasundaram et al., 2018). Population genomic analyses have shown that the fungus independently established in indoor environments in Europe and Japan and it is likely that these adaptations happened before the split of the European and Japanese populations (Skrede et al., 2021). European and Japanese isolates are highly divergent and split 3000-19,000 generations ago, likely predating human influence (Skrede et al., 2021).

7.4. Competitive ability

Competition among fungi is caused by their similar ecological needs (Dunrui C et al., 2023). When several fungi species grow together in the same environment, they will compete for nutrients, water, and space. Fungi can utilize a number of strategies to compete with one

another, including production of enzymes that decompose organic materials rapidly and/or secreting harmful compounds that prevent the growth of other fungi (Naresh and Aldred, 2008, Boddy, 2000). The experimental data, both from study II and III indicated that in a nutrient-poor environment, var. *lacrymans* and var. *shastensis* are poor competitors compared to other decay fungi. This does not come as a surprise, as organisms with relatively more extreme niches tend to shrink their antagonistic abilities by following the universal adaptive theory (Grime and Pierce, 2012). Competitive ability is more crucial under less extreme conditions, where competition for resources in general might be higher as more species can live under these conditions. The poor combative ability of var. *lacrymans* may be one of the reasons that it has rarely been able to move from its new building niche back into temperate and boreal woodlands. In the regions were it acts as an invasive fungus, it has only been observed a few times outdoors in the Czech Republic (Kauserud et al., 2012). Our experiments suggest that *S. himantioides* in general has a higher combative ability, which suits well with its appearance in less stressful environments. A higher competition for resources might be expected here.

The comparative genomic analyses in paper II revealed increased PFAM defense-related domains in *S. himantioides* compared to *S. lacrymans*, such as polyketide synthase (PKS) which are specialized metabolites with several functions and ATP-binding cassette (ABC) transporters (often involved in the efflux of small metabolites). Expansions for PKS and ABC transporters have also been observed in the extreme combative mycoparasites *Clonostachys rosea* and *Trichoderma virens,* indicating that these regions might be of relevance when it comes to the relatively higher combativeness in *S. himantioides.* Moreover, in a few cases, *S. himantioides* has also been shown to produce antifungal substances called himanimides (Aqueveque et al., 2002, Aqueveque et al., 2014). The experiments in paper III the competitive

behavior of *S. himantioides* compared to European and Japanese var. *lacrymans* and var. *shastensis*. However, the competition experiments in this study indicated that the Japanese var. *lacrymans* strain possess stronger competitive ability than European *S. lacrymans*, which might be connected to the greater genetic diversity in the Japanese population (Kauserud et al., 2007c, Engh et al., 2010a, Skrede et al., 2021).

7.5. Environmental stress

Many fungi are extremotolerant and can survive in extreme conditions with the aid of specialized enzymes, pigments, dormancy, and specialized structures. They thrive in conditions inhospitable for the growth of other fungi, e.g. psychrotrophic fungi that live in dark and cold Arctic and Antarctic conditions (Robinson, 2001). In its natural range of mountainous regions, *S. lacrymans* is capable of living in extreme conditions with cold winters, warm summers, as well as drought. *Serpula lacrymans* has likely evolved various traits to tolerate the stressful conditions that it also encounters within the building structures. This might include dormancy and reduced activity (Jennings and Bravery, 1991, Kauserud et al., 2012), which was not studied specifically in this thesis. Cellars and attics in buildings can be very dry places, and during summer they can also be warm. *Serpula lacrymans* has a maximum growth temperature of 28 °C, making it sensitive to higher temperatures. The investigation of *S. lacrymans* sensitivity indicated that *S. lacrymans* produces a GRoEL analogue, a crucial heat-shock protein that demonstrates a classic heat-shock response and could exhibit its ability to adapt to diverse temperature conditions (Sienkiewicz et al., 1997).

7.6. Var. lacrymans – the extremotolerant wimp

Taken together, the findings of this thesis suggest that *S. lacrymans* is an ecological specialist with extremely efficient brown rot degradation as well as efficient mechanisms for transportation, translocation, and growth. Our results indicate that the fungus largely was pre-

adapted to human habitats and became the most destructive wood decomposer in built constructions without evolving significant new traits. The species was well-suited for man-made building habitats due to its similarity to the natural habitat, which includes a combination of wood and mineral materials, dry conditions, high temperature variability, and a lack of competing fungi.

8. Future perspectives

Serpula lacrymans is among the most well-studied basidiomycete fungi. However, there are still open questions about its natural history and biology. The analyses of novel strains from different regions of the world, especially the natural range of *S. lacrymans* in Asia, is important to better understand how and when *S. lacrymans* colonized the human domain. The results in thesis are limited by the inclusion of only a few isolates. A higher number of isolates should be included in future studies to be able to conclude more safely on the generality of the results. Through comparative genomic analyses of newly sequenced *Serpula* genomes genes and metabolic pathways crucial for wood degradation can be pinpointed. To even better understand its physiology and the genetics underpinning this, more growth experiments, tentatively coupled to transcriptomics and proteomics analyses, should be conducted, emphasizing how it respond to different environmental conditions and stress. In addition to this, to gain a comprehensive understanding, additional experiments are necessary to map niche breadths considering multiple axes in dynamic niche spaces. For example, dormancy might be one important feature enabling its survival during cold and warm periods, which is so far little studied. An increased understanding of different life-history traits of *S. lacrymans* will also guide us to develop better strategies to control and manage *Serpula* damages within the buildings.

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How many DNA markers are needed to reveal cryptic fungal species?

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ABSTRACT

In the fungal kingdom there is a high prevalence of morphologically defined species that includes closely related 'cryptic' biological species with similar phenotypes. Due to evolutionary processes like incomplete lineage sorting and introgression through hybridization, several independent DNA markers are essential to resolve closely related fungal species. In this study we wanted to analyze how many independent loci are necessary to reveal the cryptic species, using the genus *Serpula* as a model system. DNA sequences from ten different DNA loci, eight nuclear and two mitochondrial DNA markers, were obtained from various cryptic species within *Serpula*. The inclusion of five loci gave a highly confident separation of the cryptic species. Several other loci performed better than the standard DNA barcoding marker ITS in separating the cryptic species. The DNA loci *tub*, *hsp*, *rpb2* and *tef* gave, on average, best support for the different cryptic species in single gene trees. We conclude that the analyses of a few but informative independent DNA loci, such as *tub*, *hsp*, *rpb2* and *tef* in addition to the standard DNA barcode ITS, may give a good indication about the existence of cryptic species in fungi.

Introduction

The fungal kingdom comprises high richness of species occupying diverse niches, the latest estimate being six million species (Taylor *et al.* 2014). Traditionally, fungal species have been separated based on their fruit body morphology. However, the fruiting structures represent only a short phase in the fungal life cycle. Moreover, many fruit bodies have a very simple structure and include limited number of characters, e.g. corticoid fungi with resupinate fruit bodies. This means that a limited number of characters often have been available for delimiting fungal taxa, as compared to e.g. animals and plants, where a larger part of the phenotype is available for characterization and species delimitations. This is may be one important reason why the prevalence of cryptic species, i.e. morphologically indiscernible biological/phylogenetic units present within taxonomic species, seems to be highly widespread in the fungal kingdom.

The adoption of molecular DNA based genetic analyses has revealed that cryptic species are highly prevalent in fungal morphospecies. Early in the 1990's, Vilgalys & Sun (1994) demonstrated that the oyster mushroom *Pleurotus ostreatus* includes high levels of phylogenetic divergence, where eight

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phylogenetic groups were detected. Eight cryptic species were identified in the genus *Neurospora* (Dettman *et al.* 2003) and in *Saccharomyces*, several cryptic species were identified by phylogenetic analysis of twenty genes across eight yeast taxa (Rokas *et al.* 2003). In the root rot fungus *Heterobasidion annosum* (sensu lato) a number of phylogenetic groups have been detected in Europe and in North America (Garbelotto *et al.* 1998; Johannesson & Stenlid 2003). The widespread wood decay fungi *Coniophora arida*, *Coniophora olivacea* (Kauserud *et al.* 2007a) and *Coniophora puteana* (Kauserud *et al.* 2007b) comprise a significant number of cryptic species. In the fungal kingdom, identification of cryptic species is essential for a better understanding of species richness and for proper conservation and management plans.

Phylogenetic species recognition relies upon the analyses of multiple un-linked DNA markers, where congruence in tree topology across markers is evaluated (Taylor *et al.* 2000). Groups that are stable and can be recognized across several markers likely represent evolutionary independent lineages that recombine within but not across groups. However, how many independent markers are needed to differentiate those groups? This question certainly depends upon factors like lineage age, demographic history and population size and, hence, vary from case to case.

In this study, we are focusing on cryptic species found in the family *Serpulaceae* (*Basidiomycota, Boletales*). The genus *Serpula* belongs to this family and consists of saprotrophic fungi, which are involved in wood degradation, resulting in brown rot (Binder & Hibbett 2006). In addition, the *ectomycorrhizal* genera *Austropaxillus* and *Gymnopaxillus* are grouping inside *Serpula* (Skrede *et al.* 2011). The dry rot fungus *Serpula lacrymans* is divided into two groups that represent genetically well-separated lineages (Kauserud *et al.* 2007c). One of them is *S. lacrymans* var. *shastensis*, residing naturally in North America, while the other is *S. lacrymans* var. *lacrymans*, which is colonizing buildings on all continents (Kauserud *et al.* 2007c). The sister species *Serpula himantioides* appears to include five cryptic species, some of them with a worldwide distribution (Carlsen *et al.* 2011).

Our aim in this study is to evaluate how many genetic markers are needed to resolve cryptic species, using *Serpula* as a model group. To address this question, ten different DNA markers were sequenced, two representing mitochondrial DNA (COX and mtSSU) and eight representing nuclear DNA (*gpd*, *hsp*, ITS, LSU, SSU, *rpb2*, *tef* and *tub*).

Materials and methods

Materials

In this study we analyzed 29 collections and isolates of *S. lacrymans* var. *lacrymans*, *S. lacrymans* var. *shastensis*, *S. himantioides*, *Austropaxillus* spp., and *S. incrassata* strains from the family *Serpulaceae* of the order *Boletales* (Table S1).

Molecular work

A small amount of fungal tissue was homogenized in a Mixer Mill (MM301, Retsch GmbH & Co., Haan, Germany) before we extracted total DNA following the 2 % CTAB miniprep method described by Murray & Thompson (1980) with minor modifications from Gardes & Bruns (1993). We dissolved the dried DNA pellet in 100-μL of sterile milli-Q H_2O, and used further dilutions for PCR amplification. Ten different DNA loci were PCR amplified using the primers and PCR programs listed in Table S2. Cycle sequencing was performed using the ABI BigDye Terminator sequencing buffer and v3.1 Cycle Sequencing kit (Life Technologies, Carlsbad, CA). Sequences were processed on an ABI 3730 DNA analyser (Life Technologies). Sequences were assembled and edited using BioEdit 7 (Hall 1999). All sequences have been deposited in GenBank, and accession numbers are given in Table S1.

Phylogenetic analyses

Single gene alignments were constructed for the ten different genetic markers. These were combined into a concatenated alignment using AIR-Appender (Kumar *et al.* 2009) with default parameters. Further, randomized concatenated alignments were constructed that included two to nine gene markers. For each number of loci (2–9) 20 replicate alignments were obtained. In total, 150 randomized gene alignments were constructed in this manner. All the sequences were aligned with ClustalW (Larkin *et al.* 2007) with default parameters and then manually corrected. In total, ten single gene trees, a single concatenated tree and 150 alternative trees were constructed. All phylogenetic trees were constructed using maximum likelihood analysis as implemented in the GTRCAT approximation in RaxML (Stamatakis 2006) running 1000 bootstraps. Phylogenetic trees were viewed in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and were manually inspected for tree topology and branch support for selected clades. All phylogenetic analyses were done on the Abel computing cluster from The University of Oslo (http://www.uio.no/english/services/it/research/hpc/abel/).

We analyzed phylogenetic informativeness (PI) to calculate an informativeness profile for each gene to resolve branching order against a particular epoch in a phylogenetic tree (chronogram). The online program PhyDesign (Lopez-Giraldez & Townsend 2011) was used to measure the PI of the ten loci. We calculated net and per site phylogenetic informativeness. The net PI resolved particular nodes in the phylogenetic tree to assess the utility of molecular markers, whereas the PI per site is relevant because it compares relative informativeness of genes without the confounding influence of sequence length (Townsend 2007; Fong & Fujita 2011). PI finds genes with an optimal rate of evolution during a given period of time on the phylogenetic tree.

Results and discussion

Multi-gene phylogeny

The best maximum likelihood tree based on the ten combined genetic markers is shown in Fig 1. The tree supports the topology observed in previous phylogenetic studies of *Serpula* (Carlsen *et al.* 2011; Skrede *et al.* 2011). Not surprisingly, the current tree, based on additional loci, provides higher support for all nodes. The ectomycorrhiza forming genus *Austropaxillus*

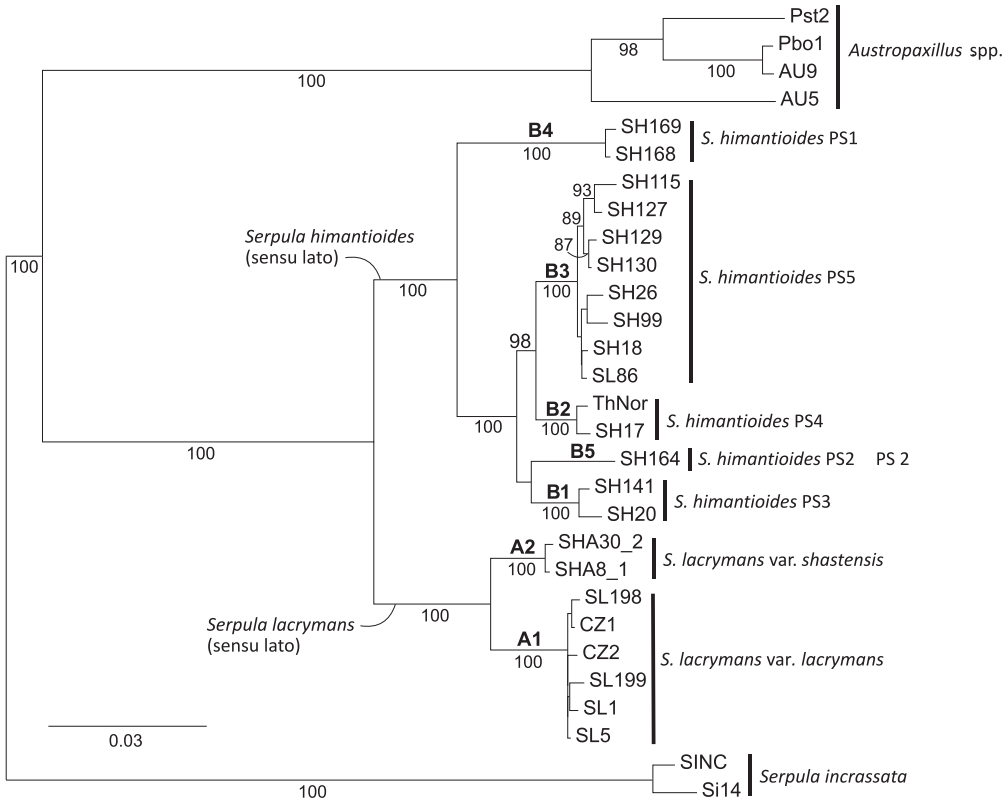

Fig 1 – Maximum likelihood tree inferred from ten genetic markers. Numbers below internodes denote bootstrap support from 1000 replicates. Node A1 and A2 refer to clades of *S. lacrymans* var. *lacrymans* and *S. lacrymans* var. *shastensis*. Nodes B1–B5 refer to five different *S. himantioides* species level lineages.

forms a monophyletic group nested within saprotropic genus *Serpula*. The *ectomycorrhizal Austropaxillus* makes up a monophyletic clade, while *S. lacrymans* and *S. himantioides* constitute another monophyletic clade. The monophyletic groups need revision for taxonomy based on a phylogenetic species concept. The split between *Austropaxillus* and *S. himantioides/lacrymans* was estimated to be approximately 50.4 million years ago, between the Late Cretaceous to Late Eocene (Skrede *et al.* 2011). Seven different sub-clades, likely indicating seven different cryptic species (Kauserud *et al.* 2007c; Carlsen *et al.* 2011) are indicated in the multi-locus tree (Fig 1). Node A1 and A2 refer to the two clades of *S. lacrymans* var. *lacrymans* and *S. lacrymans* var. *shastensis*, respectively. These two taxa diverged approximately 8.6 millions years ago and probably evolved allopatrically due to Beringan vicariance (Skrede *et al.* 2011). Nodes B1–B5 refer to five different species-level lineages within *S. himantioides*, B5 was represented by a single specimen (SH164) because, it is the only species of its kind to conclude as a distinct species (Carlsen *et al.* 2011). Bootstrap support could not be obtained for the B5 lineage.

How many DNA loci are needed to separate (cryptic) fungal species?

Multiple independent DNA markers are generally needed to resolve closely related fungal species (Taylor *et al.* 2000). This is due to evolutionary processes like incomplete lineage sorting, introgression of alleles across species boundaries (i.e. incomplete reproductive isolation) and because different genetic regions may be exposed to different selective regimes. In our case study of closely related cryptic species within *Serpulaceae*, 100 % BS support was obtained for all nodes when we included five markers (in different combinations) (Fig 2). When four markers were included, five out of six lineages received >95 % BS support, thus giving considerable support for species delimitations. In a classic study by Rokas *et al.* (2003), it was demonstrated that 8–20 loci were needed to separate between different species of *Saccharomyces*. Our data suggest that fewer informative loci were needed to resolve closely related cryptic species within the *Serpulaceae*. In *Heterobasidion annosum*, four genetic markers were used to separate three distinct phylogenetic species (Johannesson & Stenlid 2003). In *Laccaria*, *Coniophora arida*, *Coniophora olivacea* and *Coniophora puteana*, three genetic markers gave high support for the delimitation of cryptic species (Kauserud *et al.* 2007a, Kauserud *et al.* 2007b; Sheedy *et al.* 2013), whereas in *Pleurotus ostreatus* only two genetic markers gave good support for delimitation (Vilgalys & Sun 1994). The number of independent markers required to recognize closely related (cryptic) species depend on numerous factors, including; (i) time since reproductive isolation, (ii) demographic properties (e.g. population sizes), (iii) whether

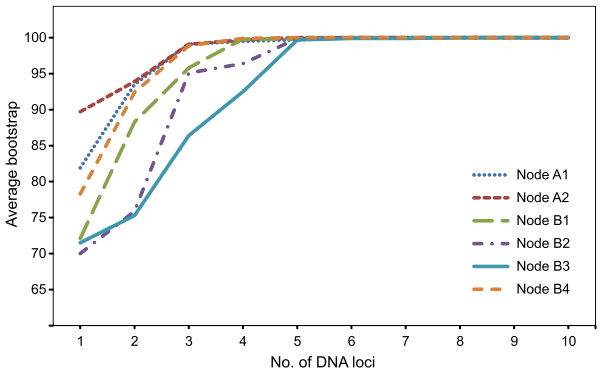

Fig 2 – Maximum likelihood average bootstrap values for six nodes (A1–A2, B1–B4) in *Serpulaceae*. 100 % BS was obtained for all nodes when five random markers were included.

Table 1 – Average bootstrap support and standard deviation values of ten genetic markers over the selected nodes (see Fig 1) of *Serpulaceae*. Numbers of supported nodes out of the six selected are listed, here using a bootstrap cutoff of 75 %.

Genetic marker	Bootstrap support		# of supported nodes
	Average	Std. dev	
COX	59.2	35.76	2/6
gpd	60.3	49.00	3/6
hsp	89.5	15.31	5/6
ITS	59.5	46.00	3/6
LSU	58.3	34.70	3/6
mtSSU	28.3	44.54	1/6
rpb2	83.3	40.82	5/6
SSU	22.3	34.60	0/6
tef	76.5	38.85	5/6
tub	94.6	4.80	6/6

introgression has happened between the lineages after the initial divergence, and (iv) various properties of the genetic markers (such as level of polymorphism and homoplasy, whether it is neutral or not, co-dominant inheritance, frequent occurrence in genome). Our data suggest that if a few suitable markers are used that include the right level of polymorphisms, only a few markers may be needed to resolve cryptic species.

Which marker is more suitable?

In Table 1, the average support values and number of supported nodes of various markers over the selected nodes are summarized. The marker *tub* gave, on average, best support (94.6 %) for the different lineages in a single gene tree, followed by *hsp* (89.5 %), *rpb2* (83.3 %), and *tef* (76.5 %). *Tub* also varied least across the different nodes. The rDNA ITS region, which has been selected as the main DNA barcoding region for fungi (Nilsson *et al.* 2008; Seifert 2009; Schoch *et al.* 2012) provided, in comparison, on average only 58.8 % support. Thus, it performed poorer than many of the other markers in the studied group (Fig S1). Which marker(s) that are most suitable will obviously vary from group to group, but there are now several examples that show ITS performs poorer than other markers. For example, in *Coniophora arida*, both *tub* and *tef* gave a better separation of the cryptic lineages (Kauserud *et al.* 2007a). A better separation of three cryptic species was also seen by *tef* and *tub*, as compared to ITS, in *Coniophora puteana* (Kauserud *et al.* 2007b). Moreover, *rpb2* performed best and ITS worst, when the three genetic markers (ITS, *rpb2* and *tef*) were used in phylogenetic species recognition (PSR) in *Laccaria* in Southern Australia (Sheedy *et al.* 2013). Moreover, LSU preformed better than ITS both in PSR in *Pleurotus ostreatus* (Vilgalys & Sun 1994) and *Helvella lacunosa* (Nguyen *et al.* 2013). The genetic markers *tef1* and *rpb1* had the highest sequencing success rate and performed the best whereas ITS and *rpb2* the worst, in species delimitation in *Trametes* (Carlson *et al.* 2014). One reason why ITS often performs worse than other markers such as *tub*, *tef* and *rpb2* could be due to a slower rate of allelic fixation of rDNA variants caused by its multi-copy nature. Even though ITS is often not the best marker for species delimitation, several other properties make it a good choice for DNA barcoding, including conserved primer sites, easy PCR amplification and a high number of reference sequences (Koljalg *et al.* 2013).

Phylogenetic informativeness

Phylogenetic informativeness (PI) correlates with the accuracy and robustness with which a gene sequence recovers the correct topology (Lopez-Giraldez *et al.* 2013). PI shows that genetic markers reached PImax (i.e. the point in time where PI is highest) at different times (Fig 3). The four markers that gave highest bootstrap support had PImax where the three lineages (*S. lacrymans var. lacrymans*, *S. lacrymans var. shastensis* and *S. himantioides*) diverge. Although the genetic marker *gpd* shows high PImax, the average bootstrap support in the single gene tree is low (60.3 %). This can be explained by the early PImax, before the divergence of the *Austropaxillus* and *S. himantioides/lacrymans* clades. mtSSU has a high PImax at the intraspecies level, thus this genetic marker creates noise in the dataset as it only separates between strains, with no phylogenetic signal. The markers *tub*, *hsp*, *rpb2* and *tef* show good average branch support and PI.

Conclusions

Our case study indicates that the analyses of relatively few DNA loci, such as *tub*, *hsp*, *rpb2* and *tef* may give a good indication about the existence of cryptic species in fungi. However, at least five genetic markers were required to resolve cryptic species with high confidence (100 % BS at all nodes). The fungal DNA barcode ITS performed to a large extent worse than other markers in delimiting different cryptic species.

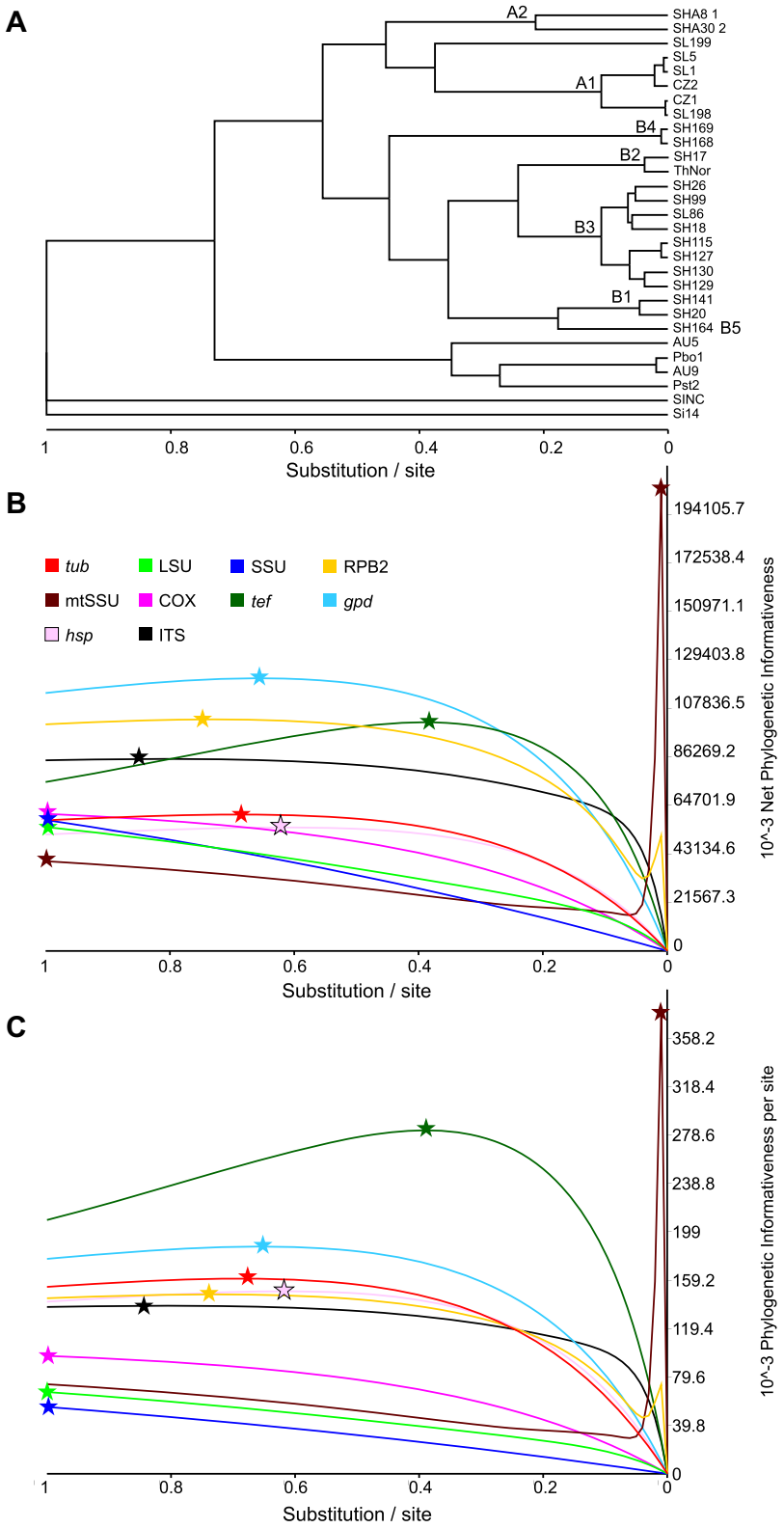

Fig 3 – Phylogenetic informativeness (PI) for ten genetic markers obtained from phylogenetic analyses of several species of *Serpulaceae*. (A) Ultrametric tree based on maximum likelihood tree inferred from all ten markers. Node names are from Fig 1. Branch lengths are relative to divergence time. Graphs of the net PI (B) and per cite PI (C), respectively, for each of the ten markers, relative to the divergence time in the tree. Star indicates the PImax.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.07.006.

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Paper II

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ARTICLE

The fungus that came in from the cold: dry rot's pre-adapted ability to invade buildings

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Abstract

Many organisms benefit from being pre-adapted to niches shaped by human activity, and have successfully invaded man-made habitats. One such species is the dry rot fungus *Serpula lacrymans*, which has a wide distribution in buildings in temperate and boreal regions, where it decomposes coniferous construction wood. Comparative genomic analyses and growth experiments using this species and its wild relatives revealed that *S. lacrymans* evolved a very effective brown rot decay compared to its wild relatives, enabling an extremely rapid decay in buildings under suitable conditions. Adaptations in intracellular transport machineries promoting hyphal growth, and nutrient and water transport may explain why it is has become a successful invader of timber in houses. Further, we demonstrate that *S. lacrymans* has poor combative ability in our experimental setup, compared to other brown rot fungi. In sheltered indoor conditions, the dry rot fungus may have limited encounters with other wood decay fungi compared to its wild relatives. Overall, our analyses indicate that the dry rot fungus is an ecological specialist with poor combative ability against other fungi.

Introduction

Species worldwide are negatively affected by anthropogenic habitat destruction. However, for those few species originally living in natural habitats that resemble the man-made ecosphere, the opposite is also the case. Animals like the Norwegian rat (*Rattus norvegicus*) and the German cockroach (*Blatella germanica*) have extended their distribution dramatically [1, 2]. Likewise, many plant pathogenic fungi have become extremely widespread as monotypic crop cultivation creates large habitats, and the trade and transport of these crops aid their dispersal [3–5]. A similar pattern is seen with the few wood decay fungi that have expanded their realized niche into the human built environment.

Probably the best-known example of a successful fungal invader of the built environment is the dry rot fungus *Serpula lacrymans* var. *lacrymans* (subsequently referred to as var. *lacrymans*), which is distributed in houses in temperate and boreal regions worldwide causing brown rot decay. It spreads with human transport of timber over long distances and colonizes new buildings in its vicinity by air-borne spores [6, 7]. Colonization of construction timber in buildings is characterized by rapid vegetative mycelial growth and formation of thick (up to 2 cm diameter, Fig. 1) mycelial cords that mediate the transport of nutrition and water to new wood substrates [8]. This allows quick growth and re-allocation of resources via the transport of nutrition and water to the new wood substrates [8, 9].

Comparative genomic approaches have shown that var. *lacrymans* and other brown rot fungi have a reduced set of plant cell wall hydrolyzing enzymes to decompose wood compared to the ancestral white rot fungi [10–14]. A recent study has suggested that the set of secreted enzymes

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Fig. 1 The dry rot fungus *Serpula lacrymans* and its habitat. *Serpula lacrymans* is one of the most devastating decomposer of houses in temperate and boreal regions worldwide. The species is known to form thick cords and a rapid decay of coniferous wood. In nature the species decompose large logs in dry mountain forests. Photo credits: Top left photo by H. Kauserud, the other photos by Mycoteam AS.

responsible for decomposition of var. *lacrymans* is even smaller than that of some other brown rot fungi [15]. The loss of enzymes by brown rot fungi is correlated with a strategy in which the initial attack of the wood is mediated by hydroxyl radicals produced by chelator-mediated Fenton (CMF) chemistry [10, 13, 16]. These initial attacks have been suggested to be controlled by differential gene expression of the fungi [14, 15]. The attacked wood structure is then further depolymerized by oxidizing and hydrolyzing enzymes that target cellulose and hemicellulose elements in the wood, while leaving modified lignin behind.

Var. *lacrymans* has a scattered natural range in high altitude mountain regions of North-East Asia, thriving in moraine-dominated habitats around the treeline where woody resources are heterogeneously distributed [6]. Human transport of infected wood appears to have facilitated the colonization in the human domain in temperate regions worldwide. It is widespread in buildings in Europe and Japan, and it is also found in buildings in temperate parts of North and South America (Chile), Australia and New Zealand, but with less abundance [7, 17]. The large European house-colonizing population of var. *lacrymans* has low genetic variation [7, 18], suggesting a severe population bottleneck during the colonization of the European built environment [6, 7]. *Serpula lacrymans* var. *shastensis* (subsequently referred to as var. *shastensis*) is a close relative of var. *lacrymans*, from high altitude mountain regions in the Cascade mountain range (North America), but has not been reported in the built environment [19, 20]. Although genetically well-separated, the two sub-species are able to form a dikaryotic mycelium when paired *in vitro* [7, 20, 21]. In the habitat close to the treeline in the Cascades (Fig. 1), var. *shastensis* colonizes and decays large logs of *Abies magnifica* [6, 22]. Both varieties of *S. lacrymans* appear to be ecological specialists, thriving in exposed mountainous habitats with patchy resource distribution.

In contrast to the confined niches of *S. lacrymans*, its sister species *Serpula himantioides* has a widespread circumboreal distribution in natural habitats in temperate and boreal regions [23]. As with *S. lacrymans*, *S. himantioides* causes brown rot of conifers, but decomposes wood more slowly, as shown on spruce [19], and produces smaller fruit bodies and smaller cords. *Serpula himantioides* is rarely found in buildings, and when it is, it decomposes wood more slowly than var. *lacrymans*. Unlike var. *lacrymans*, indoor colonization by *S. himantioides*, as with the majority of other wood decay fungi, represent random, and repeated colonizations from nature [6].

It is not evident which characteristics have made var. *lacrymans* such a successful invader of the built environment compared to its wild relatives. Pinpointing contrasting genomic differences among the lineages is a first step toward detecting the genetic basis of var. *lacrymans* invasiveness and persistence. In this study we, therefore, set out to reveal which genomic features separate var. *lacrymans* from its predominantly wild relatives. We analyzed which genes have undergone shifts in selective pressure and then, which gene families have expanded or contracted during divergence between variants or species. This was achieved by sequencing and de novo genome assembly of var. *lacrymans* and var. *shastensis* strains and comparing these to the genome of the sister species *S. himantioides*. Genomic analyses were complemented by two growth experiments investigating differences in decomposition ability and interspecific competition, to provide more direct evidence for how each of these factors may contribute to var. *lacrymans*' success in the built environment.

Materials and methods

Strains

Three strains were used for physiological experiments and genome comparisons in this study. The *S. himantioides* strain (MUCL38935) was cultured from soil in the UK in 1994, the var. *shastensis* strain (SHA17-1) was collected in California, US on *Abies* in 2004 and the var. *lacrymans* (SL200) was collected from a house in Poland in 1953. Since these strains have been maintained in culture for extended periods of time, caution should be used when interpreting the results as the strains may have changed their behavior through these years.

DNA extraction, sequencing, assembly and gene predictions

More details of the DNA extraction, library preparation, sequencing procedure, and gene prediction pipeline can be found in the Supplementary text. DNA of all three strains was extracted by a modified phenol-chloroform protocol available at the JGI webpage (http://jgi.doe.gov/collaborate-with-jgi/pmo-overview/protocols-sample-preparation-information/). All strains were sequenced using Illumina technology. The two *Serpula lacrymans* strains were sequenced on an Illumina GAII at the SNP&SEQ Technology Platform in Uppsala, Sweden, while *S. himantioides* was sequenced on an Illumina Hiseq 2000 at the JGI (http://genome.jgi.doe.gov/Serla_varsha1/Serla_varsha1.info.html).

The Velvet de novo assembler [24] was used to assemble reads into contigs for var. *lacrymans* and var. *shastensis*. JGI assembled *S. himantioides* with the AllPathsLG assembler [25]. The CEGMA pipeline was used [26] to estimate completeness of all assemblies (Table 1). Protein coding genes in the three *Serpula* strains were annotated using MAKER2 version 2.27 [27].

Functional annotation

Genes were given a preliminary description by BLAST alignment toward UniProt. InterProScan was used for functional annotation and classifications of protein families [28]. Protein sequences of var. *lacrymans*, var. *shastensis* and *S. himantioides* were obtained from the MAKER2 predictions.

OrthoMCL clustering

Homologous proteins of the three *Serpula* strains were clustered using the software OrthoMCL [29]. This tool clusters homologous proteins across the given species using Markov cluster algorithm to group orthologs and paralogs. In total 34,273 protein sequences from three different *Serpula* genomes were compared.

CAFÉ analysis

CAFÉ estimates a global birth and death rate of gene families and changes in gene family size across a phylogeny [30]. All orthoMCL clusters were used as gene families. CAFÉ was run using a global birth/death parameter (λ).

Table 1 Summary statistics of the genome assembly, annotation and CEGMA analyses of the three sequenced genomes of *Serpula lacrymans* var. *lacrymans*, *S. lacrymans* var. *shastensis* and *Serpula himantioides*

Species	Strain	# of contigs	# of scaffolds	N50	Genome size (Mpb)	Assembler	CEGMA	# of predicted genes
var. *lacrymans*	SL200	4534	1529	59,716	37	Velvet	97.6%	11,352
var. *shastensis*	SHA17-1	3839	1170	92,207	38	Velvet	97.2%	10,910
*S. himantioides**	MUCL38935	5964	4893	20,000	46	AllPathsL	89.5%	12,011§

*Sequenced by JGI, § Number of genes predicted by Maker annotation tool; however, the JGI annotation pipeline predicted 13,805 gene models

Rapidly evolving gene families were estimated using the best fit λ (0.002) at a p-value threshold of 0.01. The ultrametric three used for CAFÉ analysis was based on a multilocus maximum likelihood phylogeny of ten loci from [31] that was made ultrametric in the R package APE [32].

Selection pressure

Clusters of single copy orthologs were chosen to screen for branch specific changes in selection pressure. The clusters were aligned with the multiple sequence alignment program PRANK [33] with the 'codon' alignment mode, using the species phylogeny [21] as guide tree. PRANK has been shown to provide the most accurate alignments, with the lowest false-positive rates [34]. The Codeml from the PAML package [35] was used to identify changes in selection regime. For each group of orthologs, a single dN/dS ratio (ω) was estimated for all branches on the tree (H_0) and for three instances where each one of the species was allowed to evolve at a separate rate (H_1), The best fit model was determined using a likelihood ratio test and p-values were adjusted to control the false discovery rate (FDR) for multiple hypothesis testing using a $\alpha < 0.05$ [36]. All alignments with a significant shift in selection pressure between species were manually examined to remove questionable alignment regions if present and were then rerun in the above outlined analysis.

Functional enrichment analyses

Functional enrichment analysis was used to characterize the genes present in all the genomes compared to gene families that were inferred to be expanded or contracted by CAFÉ. A Python script was used to perform functional enrichment analysis of PFAM domains using Fisher's exact test (http://cgrlucb.wikispaces.com/Functional+Enrichment+Analysis).

Annotation of genes of specific functions of interest

To predict the secretome of each species, a bioinformatics pipeline consisting of SignalP 4.1 [37], TargetP 2.0 [38], TMHMM 2.0 [39], PS_scan [40] and WolfPSort v. 0.2 [41], was used, as implemented in Kohler et al. [42]. Besides the annotations generated for the entire proteomes (e.g., CAZymes and PFAM domains), the proteolytic enzymes present in each secretome were also annotated through BLAST searches against the MEROPS database [43]. Carbohydrate-active enzymes were predicted by searching predicted proteomes with the dbCAN tool [44, 45].

As cytochrome P450 (cytP450) is an important class of enzymes involved in specialized metabolism, the clusters annotated with cytP450 PFAM domains in Interproscan were manually curated. Only those of over 300 residues with both the EXXR and CXG motif were accepted as functional, according to the method of Syed and Mashele [46]. According to cytP450 nomenclature, a similarity of 40% was considered sufficient to classify a predicted protein into a particular family. A similarity of 55% would allow allocation to a sub-family. Those with < 40% similarity to named cytP450s were—with those that had no significant matches in the NCBI or UniProt databases—considered to probably belong to novel cytP450 families.

Data availability

All raw sequence reads, and assembled genomes are available on NCBI at Bioproject PRJNA412961. In addition, the *S. himantioides* MUCL38935 genome is available at the JGI genome browser (http://genome.jgi.doe.gov/Serla_varsha1/Serla_varsha1). The MAKER2 gene predictions, the OrthoMCL clusters and the alignments used as input to the Codeml analyses have been deposited in the Dryad Digital Repository: doi:10.5061/dryad.28sb6.

Combative ability

Var. *lacrymans*, which is found predominately, if not exclusively, inside houses in Europe, was hypothesized to show decreased ability to combat for limited resources since it faces few competitors in this environment. An antagonistic experiment was used to test this hypothesis, where the three *Serpula* strains of interest were confronted with each other and other brown rot decomposer fungi, pairswise, by growing two well-colonized blocks side by side (see Supplementary Text for detailed experimental setup). The three *Serpula* strains, and the three species *Antrodia xantha*, *Coniophora puteana* and *Fomitopsis pinicola* were used. All combinations were repeated 10 times. After the experiment, three small wood pieces from within the wood block were transferred to three new culture plates. The strains that were re-isolated from the wood piece were identified and reported. A Pearson's χ^2 Goodness of Fit test was used to test whether one species had significantly outcompeted another.

Wood decay

The specialized house-living var. *lacrymans* was expected to decompose spruce especially fast as it is mostly found on spruce in houses, where it is known to grow quickly [19]. To compare the decomposition ability of the three *Serpula* strains and *A. xantha*, *F. pinicola* and *C. puteana*, mass loss of wood was determined after 60 days colonization at 20 °C on the three tree species *Pinus sylvestris*, *Picea abies* and

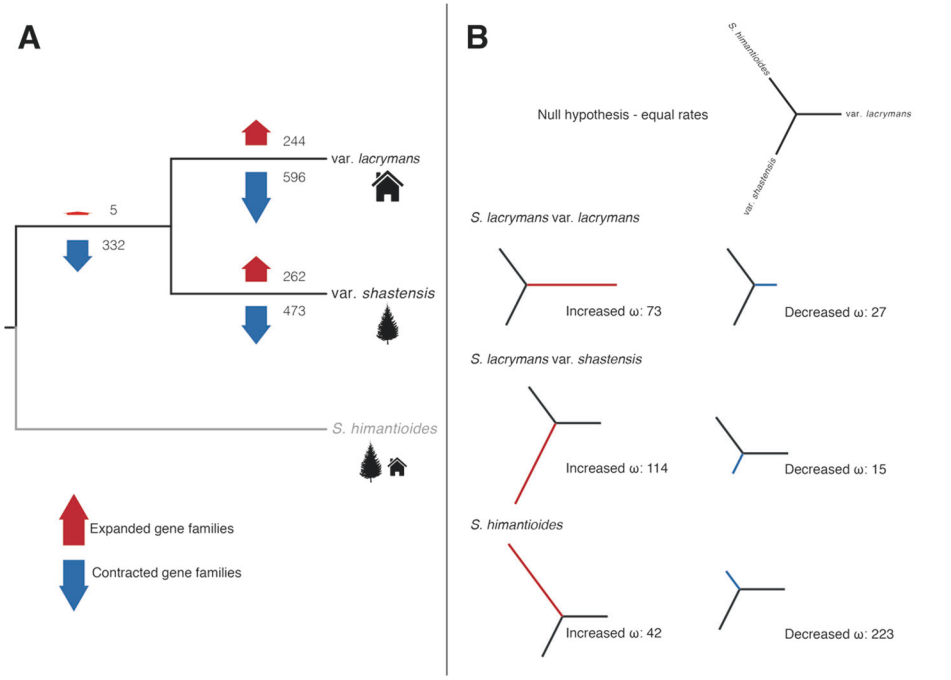

Fig. 2 The comparative genomic differences among the *Serpula lacrymans* var. *lacrymans*, *Serpula lacrymans* var. *shastensis* and *Serpula himantioides*. **a** The number of significantly expanded and contracted gene families, based on analyses using a birth-death model of gene family evolution on all gene clusters. The analyses use a rooted ultrametric tree from a 10 loci maximum likelihood analysis, where *S. himantioides* was the out-group. Thus, only changes in var. *shastensis*, var. *lacrymans* and the branch leading to these two, but not the *S. himantioides* branch were evaluated. **b** Phylogenetic sketch trees demonstrating the selection analysis. Each tree highlights a branch and the number of genes with significantly increased or decreased ω-values on that branch compared to the expected based on 5866 single gene clusters. The null hypothesis is equal rates on all branches

Abies lasiocarpa (see Supplementary Text for experimental setup; the three non-*Serpula* species were not tested on *Abies lasiocarpa*). The significance of the differences in mass loss among strains and among wood species was tested with ANOVA analyses using R [47].

Results

Genome summary

The gene prediction pipeline identified a total of 11,352 gene models in var. *lacrymans*, 10,910 gene models in var. *shastensis* and 12,011 gene models in *S. himantioides* (Table 1). Annotated genes were clustered into gene families, of which 6695 were shared among all three strains, corresponding to approximately 55 to 61% of annotated genes in each genome. Given the close relationship among the three species, the number of singleton clusters inferred for each species was surprisingly high. Of the predicted genes 18% in var. *shastensis*, 23% in var. *lacrymans* and 24% in *S. himantioides* were unique to each of the three lineages. Further analysis of singleton genes showed that singletons predominately represented cases where orthologs were absent in the other two species, either due to gene loss or absence of the corresponding coding region from the respective assemblies (results not shown).

Analyses of selection

The genome-wide estimates of selection yielded a mean estimate of $\omega = 0.137$ for *S. himantioides*, $\omega = 0.179$ for var. *lacrymans* and $\omega = 0.234$ for var. *shastensis* (gene clusters with $\omega > 2$ were omitted from these estimates).

Shifts in selective pressure on individual genes between species may pinpoint genes whose functions have contributed to adaptation by each species to their respective realized niches. For the analyses of shifts in selective pressure on a gene-by-gene basis, three series of tests were run, each one with a different species as the foreground branch. After correction for multiple testing, 100, 129, and 265 genes with significantly different ω between foreground and background branches in var. *lacrymans*, var. *shastensis* and *S. himantioides* were detected, respectively (Fig. 2). Among the sets of genes, 43% were annotated with PFAM domains while the rest were unannotated. Our functional analyses were only focused on the genes that had PFAM annotations. A full list of significant genes is provided in the Supplementary Material (Supplementary Table 1).

One of the most pronounced functional signatures detected was the selective shift in many proteins involved in intracellular transport (Table 2) with an elevated ω in *S. lacrymans* compared to *S. himantioides* (higher ω in one or both of the *S. lacrymans* varieties). Several of these proteins identified were involved in the transport of vesicles to the

Table 2 The gene families that are evolving at a significant different rate (p-value < 0.05 after FDR) among the different *Serpula* strains and includes a PFAM domain related to intracellular transport

Cluster.No	Description	PFAM ID	Test	*P*-value
2435	Domain_of_unknown_function_(DUF202), SPX_domain	PF02656, PF03105	H*l*, L*h*	0.00899, 0.00044
1272	Cofilin/tropomyosin-type_actin-binding_protein, Variant_SH3_domain	PF00241, PF14604	H*l*, L*h*	0.03537, 0.00589
6654	RasGEF_N-terminal_motif, RasGEF_domain	PF00618, PF00617	H*l*, L*h*	0.02021, 0.00370
6080	SNARE_domain	PF05739	H*l*, L*h*	0.00346, 0.00003
6147	RhoGEF_domain	PF00621	H*l*, L*h*	0.00899, 0.00573
3843	PX_domain	PF00787	L*l*, H*h*	0.01602, 0.00220
1940	Oxysterol-binding_protein	PF01237	H*s*, L*h*	0.01365, 0.00607
3226	PH_domain, FHA_domain, Kinesin_motor_domain	PF00169, PF00498, PF00225	H*s*	0.00279
6485	WD_domain, _G-beta_repeat	PF00400	L*h*	0.00683
2827	Sec1_family	PF00995	L*h*	0.02796
1406	FYVE_zinc_finger, TCP-1/cpn60_chaperonin_family	PF01363, PF00118	L*h*	0.01075

H indicates higher omega, L indicates lower omega. *l* symbolizes *Serpula lacrymans* var. *lacrymans, s* symbolizes *S. l.* var. *shastensis* and *h* indicates *S. himantioides*, thus H*l* indicates significant higher omega for var. *lacrymans*

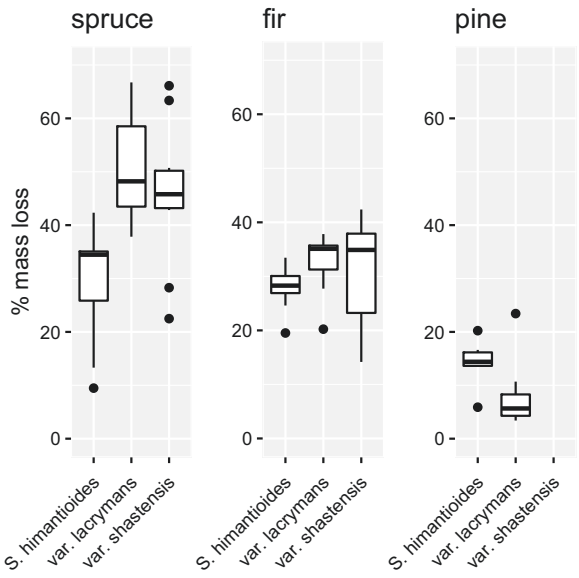

Fig. 3 Decomposition rate of *Serpula lacrymans* var. *lacrymans*, *S. lacrymans* var. *shastensis* and *S. himantioides* on different wood species. Percent mass loss of wood blocks from the three plant species fir (*Abies lasiocarpa*), pine (*Pinus syvestris*) and spruce (*Picea abies*) inoculated by var. *lacrymans*, var. *shastensis* and *S. himantioides* for 60 days. No successful growth was obtained for var. *shastensis* on pine

Golgi stack for secretion, (see Supplementary Text for details). In contrast, a protein involved in early endosomal membranes evolved faster in *S. himantioides* than in *S. lacrymans*. This, suggested a faster evolution of an endocytic pathway in *S. himantioides* vs. an exocytic pathway in *S. lacrymans*.

In addition to the genes related to membrane transport, two regulators of actin polymerization (the guanine nucleotide exchange factors, Rho GEF and Ras GEF) and a gene with a role in actin depolymerization (cofilin) evolved significantly faster in var. *lacrymans* than in *S. himantioides* (Table 2).

Expansion and contraction of gene families

All clusters in the data set and a rooted tree were used to infer 244 and 262 gene families that were expanded on the var. *lacrymans* branch and on the var. *shastensis* branch, respectively, compared to the rest of the tree (Table 2). Only 5 were expanded on the common branch leading to var. *lacrymans* and var. *shastensis*. Compared to the genomic background, CAFÉ inferred 112 and 135 gene families that expanded significantly faster than expected (based on all clusters) in var. *lacrymans* and var. *shastensis*, respectively (*P*-value 0.01). In turn, 596 and 473 gene families were contracted on the var. *lacrymans* branch and on the var. *shastensis* branch, respectively, and 332 were contracted on the common branch. Six (var. *lacrymans*) and four (var. *shastensis*) gene families showed significantly higher rates of contraction than the genomic background rate.

Functional enrichment of the expanded and contracted gene families demonstrated a change in copy number for gene families related to specialized metabolism amongst all three strains (Supplementary Table 2). In particular, expansions and contractions in a variety of polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) related PFAM domains were identified (Supplementary Table 2). One NRPS gene family (cluster 0012) was expanded in var. *lacrymans*, var. *shastensis* and their common branch. This gene family had nine gene copies in var. *shastensis* and var. *lacrymans*, but only one in *S. himantioides*. The opposite pattern was found for a putative PKS-NRPS hybrid protein gene family of unknown function (cluster 0005), where *S. himantioides* had ten copies, var. *lacrymans* eight and var. *shastensis* six copies. Copy

Table 3 Results from combat experiments with *Serpula lacrymans* var. *lacrymans*, *S. lacrymans* var. *shastensis*, *S. himantioides* and three other fungal species

	var. *lacrymans*	var. *shastensis*	*S. himantioides*	*C. puteana*	*A. xantha*
var. *shastensis*	0.450 (20)				
S. himantioides	0.689 (45)*	0.685 (27)			
C. puteana	0.430 (43)	0.500 (34)	0.155 (45)**		
A. xanta	0.400 (45)	0.355 (38)	0.136 (44)**	0.154 (39)**	
F. pinicola	0.978 (46)**	0.889 (45)**	0.156 (48)**	0.931 (29)**	0.292 (48)**

The proportion of plates with mycelia from the species named in the column after the confrontation test with the species in the row, i.e., read horizontally, higher than 0.5 wins the confrontation with the vertical strain Number of plates (n) used in parenthesis. *indicates significant different ($^*p < 0.05$, $^{**}p < 0.005$) from expected ($E = n/2$) by a Person's χ^2 Goodness of fit test, df = 1

number changes in ATP-binding cassette (ABC) transporters were also detected. These were reduced in var. *lacrymans* compared to var. *shastensis* and *S. himantioides*.

Cytochrome P450s showed expansion in *S. lacrymans* compared to *S. himantioides* (Supplementary Table 3). Eighty-nine, 91 and 109 predicted functional cytochrome P450s were identified in *S. himantioides*, var. *shastensis* and var. *lacrymans* respectively. Thus, both var. *shastensis* and var. *lacrymans* have experienced expansion of capacity compared to *S. himantioides*, with an extra five families represented in each. Var. *lacrymans* and var. *shastensis* had the same families except that var. *shastensis* uniquely had one member of CYP5145, and var. *lacrymans* had one member of CYP6001, a family that was not present in either of the other strains. Thus, in both var. *shastensis* and var. *lacrymans* the higher numbers of cytochrome P450 copies were predominantly the result of an increased number of genes from existing families.

Several gene families related to wood decay mechanisms were expanded or contracted (Fig. 2; Supplementary Table 2, Supplementary Text for details). Specifically, the set of CAZymes encoded within the three genomes was very similar, but with a somewhat greater gene complement in *S. himantioides* (see Supplementary Text for details; Supplementary Table 4). In contrast, an iron reductase with only a CBM1 and a CytB domain was found in *S. lacrymans*, but not in *S. himantioides*. (Supplementary Fig. 2).

Evaluating substrate preference

Both *S. lacrymans* varieties decomposed more of the spruce wood block than *S. himantioides*, under the conditions tested (50% and 45 vs. 30% mass loss, respectively; Fig. 3). There was no significant difference in the amount of decomposition between var. *lacrymans* and var. *shastensis* on spruce or fir (χ^2, $p > 0.05$). Var. *shastensis* failed to grow on pine, but it is unknown whether this is due to its inability to decompose pine, or due to other experimental factors, e.g., the experimental setup on moist perlite may not have provided enough minerals. Spruce was more readily degraded by all strains, and this was particularly pronounced for var. *lacrymans*, which caused a mass loss of 50% of spruce but only 5% of pine wood blocks. See Supplementary Material for the mass loss of the additional species (Supplementary Fig. 1).

Evaluating antagonistic behavior

Serpula himantioides was significantly more combative than var. *lacrymans* and var. *shastensis*, as well as the three other brown rot species under the conditions tested (Table 3). *Serpula himantioides* was present in 79% of the re-isolations from the confrontations against the other species (i.e., as 50% would be a deadlock, *S. himantioides* took the substrate of the other species in 29% of the cases). The two *S. lacrymans* varieties were less able to exclude the other species compared to *S. himantioides* in this experiment, (var. *shastensis* was found in 40% and var. *lacrymans* in 41% of the cultures following confrontations, i.e., both lost their substrate in about 10% of the cases). When var. *lacrymans* and var. *shastensis* were confronted with *C. puteana* and *A. xantha*, the outcomes were close to 50% (i.e., a deadlock), but both *S. lacrymans* varieties were excluded by *S. himantioides* and *F. pinicola* (Table 3).

Discussion

In this study we aimed to identify which features have made the dry rot fungus *Serpula lacrymans* var. *lacrymans* the most successful invasive wood decay fungus in the built environment by comparing its characteristics to its less invasive relatives. Since the successful establishment of an invasive species typically depends on a range of factors, we investigated the contribution of physiological factors (decomposition and combative ability), as well as underpinning genomic features. We detected numerous genomic signatures that may be linked to var. *lacrymans* invasiveness, including changes in selection pressure and evolution in gene families involved in hyphal growth, transportation,

defense and decomposition of wood. Our experimental data suggest that *S. lacrymans* has poor antagonistic abilities toward other brown rot fungi, but that it has high wood-decomposition ability compared to its largely non-invasive relative *S. himantioides*. This suggests that *S. lacrymans* is an ecological specialist while *S. himantioides* is more of an ecological generalist.

One of our main findings is the differences in genome-wide selection pressure, evaluated by changes in rates of non-synonymous to synonymous substitutions (ω). The ω-values suggested that on average the genes of *S. himantioides* experienced stronger purifying selection than those of var. *lacrymans*, and especially those of var. *shastensis*. However, even if ω-values can detect genes under selection, systematically increased ω at the genome-wide level, can also be the result of demographic history [48]. In organisms with small effective population sizes, selection is less effective in removing deleterious mutations which can lead to elevated genome-wide ω-values. Correspondingly, we suggest that var. *lacrymans* and var. *shastensis*, which have higher average ω overall in the genome, may have lower effective population sizes compared to *S. himantioides*. The differences in effective population size is expected as *S. himantioides* is distributed worldwide [23], while var. *shastensis* has limited current distribution and var. *lacrymans* has gone through a domestication process [7].

In more detail, the genomic analyses revealed a selective shift in genes with functions involved in intracellular transport, growth and reorganization of the cell. Our data suggest that evolutionary changes to these processes may underlie the increase capacity of transportation and growth in var. *lacrymans* which in turn is likely to be a key factor for its success in the built environment. Buildings are a dry habitat, where the water resources are the most limiting factor. Var. *lacrymans* can produce the thickest mycelial cords described in the fungal kingdom, up to 2 cm in diameter [8]. In comparison, var. *shastensis* and *S. himantioides* produce smaller cords, and *S. himantioides* has a slower growth rate [19]. The corded network permits the translocation of intracellular resources, e.g., amino acids and water through vacuolar and vesicle trafficking to ensure complete exploitation of large woody substrates [49].

Proteins associated with endomembrane system functioning and hyphal growth had different selection pressure between *S. lacrymans* and *S. himantioides*, indicating that changes in resource translocation are important in the adaptation to the different niches. Hyphal growth is dependent on both transport and fusion of secretory vesicles to the plasma membrane and on actin cytoskeleton organization and polarization. Indeed, actin is important for polarized growth and also represents the mechanism for the transport of secretory vesicles that contain materials for the synthesis of new cell wall and membranes in the growing tip [50]. We hypothesize that these genes play a role in the development and maintenance of the mycelial cords, possibly through mediating the re-grouping and re-allocation of resources.

To become a successful colonizer of wood, a fungus has to compete for resources with other decay species. However, the confrontation experiments, where the fungi were growing in fir blocks on moist perlite, revealed that var. *lacrymans* and var. *shastensis* have poor combative abilities compared to other wood decay fungi, at least in this nutrient poor setup (Table 3). Species inhabiting more extreme environmental habitats may reduce their antagonistic abilities, following the universal adaptive strategy theory [51]. Thus *S. lacrymans* inhabiting the dry treeline and built environments may have lost the capacity for broad antagonistic responses. This may also explain why var. *lacrymans* usually does not spread from colonized buildings into the natural environment, though a few exceptions have been noted in the Czech Republic [52]. In less stressful climates in the boreal and temperate zones, where *S. himantioides* is typically found, interspecific antagonistic interactions may be more important. Hence, under these conditions, it may have been more advantageous to evolve strong combative ability. This is supported by the increased numbers of PFAM domains possibly related to defense in *S. himantioides* compared to *S. lacrymans*, e.g. PKS and ABC transporters. PKS are large synthases particularly involved in the biosynthesis of specialized metabolites with many diverse functions. The gene families are known to expand and contract rapidly in response to adaptation to nutritional and environmental factors, pathogens or interactions with other organisms [53]. ABC transporters are often involved in the efflux of small metabolites [54, 55]. Furthermore, similar expansions of PKS and ABC transporters have been observed in the mycoparasites *Clonostachys rosea* and *Trichoderma virens*, and were suggested to be the reason for their extreme combative ability, by producing and transporting toxic compounds from the cells [54]. *Serpula himantoides* is known to produce antifungal substances, himanimides, that could increase its antagonistic ability [56]. It is unknown if var. *lacrymans* can produce these substances. More genomic analyses and experiments using different conditions are needed to pinpoint the exact function of the larger number of PKS and ABC transporters in *S. himantioides*, and whether any of these expansions are related to the previously detected himanimides.

Our growth experiments on wood substrates confirm earlier findings that var. *lacrymans* is a highly effective decomposer of coniferous wood [19]. In natural environments, *S. lacrymans* typically occupies large logs of *Abies* or *Tsuga* (Fig. 1) and has developed a unique capacity for rapid decay during a short season of favorable growth conditions. Resource availability and utilization of nutrients

involve a diverse chemistry for saprotrophic fungi. The varying levels of extractives, such as terpenoids and other phenolic compounds, and the recalcitrant nature of the carbohydrates of wood imply that specialization and adaptation to these conditions are essential to utilize this niche. Our findings suggest that *S. lacrymans* is a more successful decomposer of spruce and fir than pine, and is more specialized for these specific substrates than *S. himantioides*. A more narrow substrate range was also suggested in a recent study of var. *lacrymans* and *Gloeophyllum trabeum*, where they found gene expression of a wider CAZyme complement in *G. trabeum* than in *S. lacrymans* [15]. Furthermore, the speed and efficiency with which *S. lacrymans* decomposes spruce, compared to *S. himantioides*, could be related to a more efficient CMF chemistry. The iron reductase (with a CBM1 domain and a cytochrome B domain) found in var. *lacrymans* and var. *shastensis*, but not *S. himantioides* has previously been suggested to have an electron transfer function [57]. Thus, it can target reduced iron directly to the cellulose substrate for efficient CMF. In previous analyses of *S. lacrymans*, this iron reductase was specifically pinpointed as important in the early oxidative degradation steps of the CMF chemistry [10]. This could contribute to more efficient utilization of carbohydrates from its habitat.

The content of inhibitory extractives is greater in pine wood than in spruce wood [58], which makes pine a less favorable food source for fungi. Differential gene expression analyses of a white rot fungus (*Phlebiopsis gigantea*) grown on wood where extractives were removed showed several genes potentially related to the processing of extractives [59]. These differentially expressed genes encoded glutathione-S transferase, ABC transporters, lipases, cytochrome P450s and aldehyde dehydrogenase. We found accelerated evolution in *S. lacrymans* for aldehyde dehydrogenase, an ABC transporter, and cytochrome P450s, and loss of copies of glutathione-S transferase and ABC transporters. The ability to process a diversity of extractives found in wood and secrete their breakdown products may, therefore, also play an important role in substrate specialization and hence adaptation of *S. lacrymans* to a different habitat. Furthermore, the loss of laccases and the increase of cytochrome P450s in the branch leading to *S. lacrymans* could be related to both community interactions and the processing of toxic phenolic derivatives produced during the decomposition of lignocellulose. Brown rot fungi do not utilize lignin, however, they depolymerize lignin to gain access to the cellulose and hemicellulose. Thus, as part of adapting to a specialized niche *S. lacrymans* may have lost genes important for exploitation of some woody substrates in nature, but rather specialized for a more streamlined decomposition of specific substrates. Cytochrome P450s have been suggested to easily duplicate, and to be important in the colonization of new environments and in the breakdown of novel compounds [60]. Moreover, it has been suggested that the large gene repertoire of cytochrome P450s evolved in *Phanerochaete chrysosporium* increased its resource availability [61], thus the expansion of cytochrome P450s could be related to an expansion of biochemical capacity in var. *lacrymans* as it invades timber wood. Timber wood is similar to the wood encountered naturally by primary decay species, containing more plant-derived compounds than partially degraded wood that is often available in the forest.

The chemistry of defense and foraging is a recurring issue in our data set. However, without in-depth functional analysis, it is unclear whether the product moved by a particular ABC transporter or metabolized by a cytochrome P450 gene is of importance to the species' competitive ability and the decomposition of different substrates. Thus, further analyses of the increased set of cytochrome P450s in *S. lacrymans*, and the increased set of PKS and ABC transporters in *S. himantioides*, can pinpoint in which functions these gene expansions are involved.

Our results indicate that the devastating dry rot fungus is an ecological specialist that has developed highly effective brown rot decay and effective systems for transportation and growth. Common traits identified between genetically related var. *lacrymans* and var. *shastensis* when compared with the sister taxon *S. himantioides* suggest that var. *lacrymans* was pre-adapted to the built environment and that the requirements of the mountainous, dry, treeline habitat and the patchy nutrient environment of a house, including a blend of wood and mineral materials, share similar features important for *S. lacrymans*. This enabled var. *lacrymans* to opportunistically exploit the built environment when given the opportunity by human activity. Particularly, the evolution of the thick cords and rapid growth may be linked to its natural substrates, to maximize resource translocation and effectively decay the enormous logs. The lower combative ability, suggested from both physiological and genomic data and the narrower enzymatic assortment of our selected strains might explain why var. *lacrymans* rarely has been able to move from its new building niche back into temperate and boreal woodlands. As var. *shastensis* is very similar to var. *lacrymans* both in genetic and physiologic features, we conclude it has the potential to invade buildings, but has not done so because its native range has not been widely exploited by humans and so has not been transferred to the built environment.

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Author contributions I.S., J.H., H.K., D.C.E., N.H., and L.B. conceived and designed the research. L.T, I.S., and J.H. analyzed physiological properties. I.S. and N.H. extracted DNA., K.L., A.A., K.B., and I.V.G. sequenced and analyzed the *S. himantioides* genome at JGI., S.V.B., M.B.D., J.H., C.P., I.S. and S.C.M. analyzed genomic data. S.V.B., J.H., D.C.E,, H.K. and I.S. wrote the paper and all other authors discussed and modified the paper.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Paper III

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ARTICLE

Niche differentiation and evolution of the wood decay machinery in the invasive fungus *Serpula lacrymans*

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Abstract

Ecological niche breadth and the mechanisms facilitating its evolution are fundamental to understanding adaptation to changing environments, persistence of generalist and specialist lineages and the formation of new species. Woody substrates are structurally complex resources utilized by organisms with specialized decay machinery. Wood-decaying fungi represent ideal model systems to study evolution of niche breadth, as they vary greatly in their host range and preferred decay stage of the substrate. In order to dissect the genetic basis for niche specialization in the invasive brown rot fungus *Serpula lacrymans*, we used phenotyping and integrative analysis of phylogenomic and transcriptomic data to compare this species to wild relatives in the Serpulaceae with a range of specialist to generalist decay strategies. Our results indicate specialist species have rewired regulatory networks active during wood decay towards decreased reliance on enzymatic machinery, and therefore nitrogen-intensive decay components. This shift was likely accompanied with adaptation to a narrow tree line habitat and switch to a pioneer decomposer strategy, both requiring rapid colonization of a nitrogen-limited substrate. Among substrate specialists with narrow niches, we also found evidence for pathways facilitating reversal to generalism, highlighting how evolution may move along different axes of niche space.

Introduction

Adaptation to exploit a habitat can lead species to develop specialized phenotypes. Species vary greatly in the degree of specialization to environmental conditions, nutritional resources, and competitors they experience. Species specialized in inhabiting a narrow niche may face lower competition pressure [1], and they may be unable to adapt to changing environments due to their restricted range [2]. In turn, generalists with wide niches face more competition for resources and, in some cases, may experience trade-offs associated with being less well adapted to particular aspects of the environments they inhabit [3]. Niche breadth evolution therefore has important implications for evolutionary processes such as speciation and extinction, yet the underlying genetic architecture remains unresolved in many systems [4].

Fungi have adapted to colonize diverse habitats and their compact genome sizes make them ideal model systems to study the evolution of niche breadth in Eukaryotes on genome-wide scale. Many species are dependent on plants for their survival, either via biotrophic interactions (e.g., root and leaf pathogens, mycorrhizal fungi, and endophytes)

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or in the decay of dead plant material. Wood decomposition is dominated largely by Agaricomycete fungi [5] and constitutes an important part of the global carbon cycle. Exploitation of the plant cell wall (PCW) resource by these species provides a tractable system to study niche-driven specialism through genetic adaptation. PCWs of woody tissues are recalcitrant substrates made up of complex carbohydrates (cellulose, hemicellulose, and pectin) cross-linked to lignin, a polyphenolic substance that represents a strong barrier requiring a specialized decomposition machinery for microbes to access resources [6]. The evolution of lignin-depolymerizing class II peroxidases ~300 MYA probably led to the emergence of the white rot (WR) decay mode and facilitated the radiation of Agaricomycete fungi [7]. The brown rot (BR) nutritional mode, where decay-modified lignin is not catabolized, appears to have emerged independently from WR lineages multiple times, and is often associated with the decay of more lignin-rich softwoods [8]. Present day WR species generally have an expanded complement of peroxidases, associated oxidoreductases and carbohydrate active hydrolases to exploit the entire PCW resource [7, 9]. Whereas the BR mode is characterized by a refined suite of enzymes, where evidence strongly suggests the loss of class II peroxidase-driven delignification is replaced by a chelator-mediated Fenton (CMF) mechanism that modifies the lignin to access the carbohydrates of the PCW [10–12]. It is proposed that low pH iron oxidation by hydrogen peroxide generates reactive oxygen species that disrupt the lignin polymer releasing PCW carbohydrates for subsequent hydrolytic enzyme decomposition [13]. These processes are understood to take place in distinct stages, an early phase characterized by oxidative attack on the substrate, followed by a hydrolytic phase resulting in stage-specific expression of decay enzymes [14–16]. Similarly, nonenzymatic components of the decay machinery also show stage-specific expression, for example early decay in the BR fungus *Rhodonia placenta* includes a short window of high oxalate production [17] which may help to provide a chemically favorable environment for CMF reactions [18]. However, such detailed understanding of decay progression is currently limited to a handful of systems and classification of decay mode based solely on gene repertoires has proven challenging, highlighting a greater diversity of fungal decay strategies [19, 20].

Saprotrophic fungi offer tractable systems to allow the assessment of the additive effect of physiological and ecological factors in characterizing niche breadth and the mechanisms underlying species separation [21]. Fungal decay machineries show varying degrees of adaptation to different substrates, ranging from decay specialists restricted to single tree species, to generalists able to utilize both angiosperm and gymnosperm substrates [22]. Furthermore, the influence of ecological factors on the decay community structure is becoming apparent [23, 24]. Abiotic (water availability, temperature, pH) and antagonistic competition affect dispersal and structure of successional decay community members [25].

BR fungi in the Serpulaceae (Boletales) span a range of niche widths from substrate specialists with a narrow geographic range to a cosmopolitan generalist. *S. lacrymans* var. *lacrymans*, commonly known as dry rot, causes timber decay in houses. It is a niche specialist characterized by thick mycelial cords, relatively rapid primary decay habit with a strong substrate preference for spruce and low antagonistic ability [26]. It has a natural range in high-altitude regions in Asia from where it has spread to temperate and boreal regions world-wide [27]. Two separate invasions into the built environment have been suggested, one to Europe and one to Japan, where this species is largely restricted to houses and rarely found in nature [27, 28]. While European populations were likely established by a few founding members and have experienced a strong population bottleneck, the Japanese populations show much higher levels of genetic diversity [27, 28].

There are two variants of *S. lacrymans*: *S. lacrymans* var. *lacrymans* and *S. lacrymans* var. *shastensis* [29, 30]. The variant *shastensis* is found on large logs of *Abies magnifica* close to the treeline in the Cascade mountain range, but is not found in the built environment to our knowledge [31]. *S. lacrymans* var. *shastensis* provides an ideal comparator to var. *lacrymans* as it shares many of the niche characteristics, including a strong in vitro substrate preference for spruce and poor combative ability [26], yet these varieties split about 9 MYA ago [30]. *S. himantioides* is more commonly associated with natural environments and less frequently found in the built environment. This species is globally distributed and has a broad substrate range, associated with both hardwoods and softwoods [30, 31]. *S. himantioides* provides a more genetically diverse comparator and occupies a distinctive niche compared to its relatives, i.e., a generalist behavior with intermediate decay rates for a wider range of substrates [26, 32, 33]. It also is a stronger competitor suggesting that it may occupy later stages in the decay succession [26].

In this study, we used a combination of experiments, phylogenomic reconstruction, and expression profiling on three different substrates, *Picea abies* (spruce), *Pinus sylvestris* (pine), and sucrose-based media (Czapek Dox) to establish the niche breadth of *S. lacrymans* variants *lacrymans* and *shastensis*, and *S. himantioides* and study the evolution of their decay machineries within this context. In order to survey variation within var. *lacrymans* from different invasive populations with contrasting levels of diversity we also de novo sequenced the genome of a Japanese strain to distinguish the European and Japanese

var. *lacrymans*. Our analyses aimed to determine whether evidence supported a shift between generalist and specialist wood decay among the widely distributed *S. himantioides* that inhabits a broad host range, and the ancestor of vars. *lacrymans* and *shastensis* which are associated with narrow treeline ranges. Comparison of decay machinery with *S. himantioides* indicated an increased reliance on CMF and decreased reliance on PCW-degrading enzymes (PCWDEs) in *S. lacrymans* variants. However, decomposition ability and gene expression analysis on spruce and pine suggest a genetic basis for an underlying variability in decay between individuals of *var. lacrymans* and a possible route to reversal to generalism, but without the trade-off in decomposition rates seen in *S. himantioides*.

Materials and methods

Fungal material and experimental set-up

Fungal strains SL200 (*S. lacrymans* var. *lacrymans*), SL198 (var. *lacrymans*), SHA17-1 (*S. lacrymans* var. *shastensis*), and MUCL38935 (*S. himantioides*) were maintained on Malt extract agar in the dark at 20 °C. Growth experiment data were taken from Balasundaram et al. [26] and supplemented with experiments for var. *lacrymans* strains SL198 and S7. Briefly, decomposition abilities for each strain were assessed by measuring percent weight loss of fir, spruce, and pine wood blocks 60 days after inoculation. Competitive capacities were determined by confrontation experiments on fir wood blocks. Pre-inoculated wood blocks were tied together and incubated for 5 months on average. For every pair, proportions of each strain re-isolated from ten replicate pairs of wood blocks were scored by subculturing on three plates per block, yielding up to 60 observations (2 wood blocks × 3 re-isolated plates × 10 replicates: Fig. 1B, Supplementary Table S1). Results were assessed for deviation from the initial proportion of 0.5 using χ^2 tests. Both experimental procedures are described in detail in the Supplementary Material. To survey gene expression, we set up 150 mm diameter Petri dishes with 20 ml of Czapek Dox medium (SCD/control; [26]) or SCD without sucrose and glutamate but with a layer of wood shavings from either pine or spruce (pine—*P. sylvestris* and spruce—*P. abies*) in five replicates per treatment per strain. Wood shavings from untreated wood planks (H. C. Thaugland Trælastforretning, Oslo, Norway) were autoclaved twice at 121 °C for 30 min, leaving at least 24 h between treatments and stored at −20 °C until further use. The shavings were soaked in diH_2O and autoclaved for a third time before ~2 g of wet weight wood shavings were spread evenly across the inoculated Petri dishes. Five fungal inocula of 5 × 5 mm were placed onto a 30 µm polyamide mesh (Sefar Nitex, Sefar, Heiden, Switzerland) and each plug was supplemented with 200 µl of 0.001% glucose solution. All treatments were grown at 20 °C in the dark for 30 days prior to harvesting.

Nucleotide extraction, sequencing, and bioinformatic processing

Full details regarding DNA and RNA extractions, Illumina sequencing, de novo assembly and annotation of the SL198 genome and data processing for differential expression analysis are provided in the supplement (Supplementary Tables S2, S3 and Supplementary Data S1).

Differential expression analysis

Differentially expressed genes were determined on a species-by-species basis using EdgeR v.3.16.5 [34] with the GLM approach and the quasi-likelihood *F*-test, based on an FDR-corrected *P* value of less than 0.05 and absolute log fold change greater than 1 (Supplementary Text, Supplementary Fig. S1). To determine the common set of genes induced on both types of wood ("core") as well as those specific to spruce and pine, the following contrasts were implemented: (1) core = (spruce + pine)/2 vs. SCD, (2) spruce/pine = spruce vs. pine. Spruce-specific and pine-specific sets were determined by only retaining genes that were significantly upregulated in the respective wood type compared to the SCD control (contrasts 1a and 1b) as well as significantly different between the wood types in contrast 2 (Fig. 1B). Standardized expression levels for the purpose of visualization were obtained using the rlog function from DESeq2 [35] taking the median value within condition and obtaining a zero sum mean across the three conditions for each gene.

Evolutionary analysis

Predicted proteomes of all four strains were clustered into gene families using OrthoMCL v.2.0.9 [36] with BLASTP *e*-value cutoff $1e^{-5}$ and percentMatchCutoff = 25. The inflation value was set to 3. For a total of 1010 non-trivial clusters (>3 sequences, >1 gene/strain) we used a full phylogenetic approach to determine orthology relationships and gene ages within the cluster (see Supplementary Material).

Functional enrichment analysis

Enrichment of PFAM domains was conducted using Fisher exact tests corrected for multiple testing using the False Discovery Rate at threshold $\alpha = 0.05$. GO term enrichment analysis was conducted using topGO v2.26.0 (89) with the

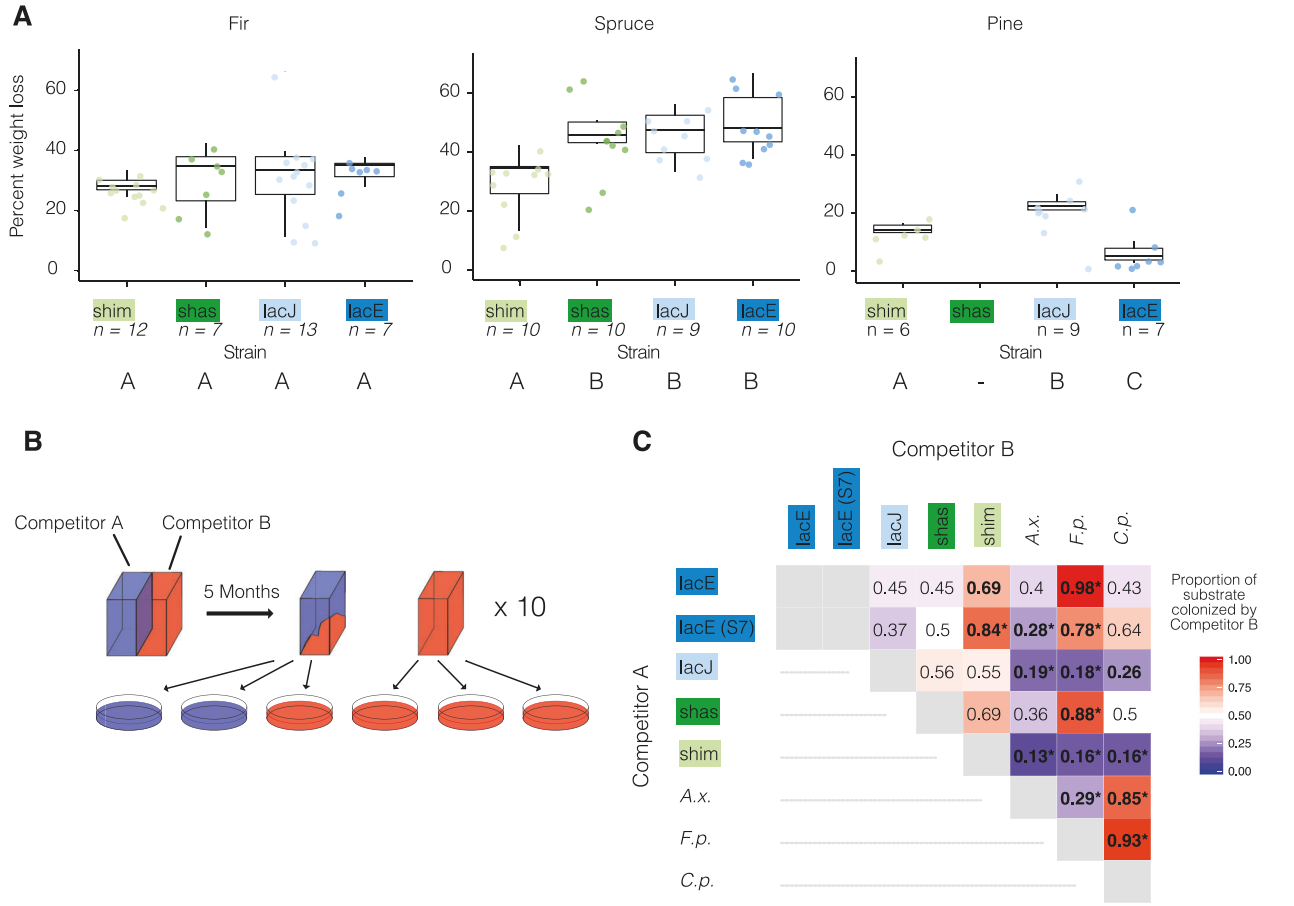

Fig. 1 Strain phenotyping to determine niche breadths. A Weight loss of target strains on different wood types. Percent weight loss indicates the dry weight lost after 60 days of inoculation. Significant differences are indicated according to Kruskal–Wallis rank sum test ($P < 0.05$). **B** Experimental set up of the competition experiment. **C** Proportions of competitors recovered from substrate in a pairwise competition setup containing equal amounts of wood blocks inoculated with strain pairs. A proportion of 0.5 indicates no gain or loss of substrate, while proportions > 0.5 indicate substrate gain by competitor B and proportions < 0.5 indicate substrate gain by competitor A. Significant deviation from equal outcome (0.5) was tested using a χ^2 goodness of fit test (df = 1). Bold: $P \leq 0.05$; Bold*$P <= 0.01$. A.x. *Antrodia xantha*, F. p. *Fomitopsis pinicola*, C.p. *Coniophora puteana*. Sample sizes for each pair ranged from 19 to 48 observations (Supplementary Table S1).

"weighted01" algorithm and the Fisher exact test with *P* value cutoff 0.01.

Results

Growth experiments indicate niche differentiation

Decomposition experiments on different wood resources (pine, fir, and spruce) show distinct wood type - dependent responses among the four strains examined, with 0–69% of biomass (dry weight) consumed after 60 days growth and clear differences in decomposition among different species and wood types (Fig. 1A). The widely distributed generalist *S. himantioides* (strain MUCL38935 = shim) consumed all three wood types at moderate levels, on average reducing biomass by 15% on pine, 28% on fir and 34% on spruce. In contrast, the average weight loss on spruce for var. *lacrymans* strains (strain SL200 = lacE and strain SL198 = lacJ) and var. *shastensis* (strain SHA17-1 = shas) was significantly higher than for shim (Kruskal–Wallis rank sum test, $\chi^2 = 15.471$, df = 3, P value = 0.001455; Fig. 1A), with lacE causing up to 69% reduction. On pine, shas failed to establish under the conditions tested and lacE consumed on average less than 5% of the wood blocks. Strain lacJ caused significantly greater average weight loss on both spruce and pine (45% and 23% respectively; Fig. 1A). Testing two additional var. *lacrymans* isolates from Europe and New Zealand confirmed a strong preference for spruce, while indicating that weight loss on pine varied among individuals (Supplementary Text). No significant difference was found when species were grown on fir.

Antagonistic behavior of the *Serpula* strains profiled also supports niche differentiation between species. Each

strain was competed against each other, a second *S. lacrymans* var. *lacrymans* European isolate (S7), and three common decomposer fungi (*Antrodia xantha, Fomitopsis pinicola*, and *Coniophora puteana*) using pre-colonized wood blocks of fir (Fig. 1B; [26]). The generalist decomposer shim emerged as a strong competitor as it defended its substrate against all other strains (Fig. 1C) and outcompeted five of the seven opponents (χ^2 goodness of fit test, df = 1, P value ≤ 0.05). In contrast, poor competitors lacE and shas did not outcompete any strain tested. The second slac European isolate (S7) confirmed this trend, but was marginally more competitive, by outcompeting *A. xantha* (Fig. 1C). In contrast, lacJ showed strong antagonism, especially towards species outside the Serpulaceae. By the end of the experiment, lacJ significantly increased its proportion of substrate occupancy from 0.5 at the beginning of the confrontation to 0.81, 0.82, and 0.74 when confronted with *A. xantha*, *F. pinicola* and *C. puteana*, respectively.

Transcriptomic profiling identifies conserved shift in decay machinery

Specialization on spruce in vars. *lacrymans* and *shastensis* was mirrored by a conserved shift in transcriptional response when grown on spruce or pine wood shavings. We determined significantly upregulated transcripts (log2 fold change >1; FDR-adjusted P value < 0.01) on spruce and pine, as well as "core" wood-specific transcripts that were upregulated on both spruce and pine compared to sucrose (Fig. 2A). We refer to these sets of upregulated genes as spruce, pine, and core modules, respectively.

The generalist species shim expressed largely the same set of genes on both types of wood, with 1551 significantly upregulated core genes, 86 pine-specific genes, and only 8 spruce-specific genes (Fig. 2A, Supplementary Data S2). In contrast, lacE, lacJ, and shas showed distinguished expression responses to the different wood types with induction of 572 (lacE), 711 (lacJ), and 561 (shas) genes in the core module, 569 (lacE), 400 (lacJ), and 416 (shas) induced only on spruce, and between 342 (lacE), 409 (lacJ), and 405 (shas) genes specific to pine (Supplementary Data S3, S4, S5).

Genome-wide phylogenomic reconstruction inferred fine-grained homology relationships between genes in the four genomes (Supplementary Text, Supplementary Fig. S2). Comparison of one-to-one orthologs indicated that the partitioning of the transcriptomic response in the three *S. lacrymans* strains (lacE, lacJ and shas) is evolutionarily conserved and driven by overlapping gene sets in all three modules (Fig. 2B). Conservation between the three *S. lacrymans* strains and shim was strongest for the core module while there was a smaller, but significant overlap between the pine modules. Results also indicate significant overlap between the core module in shim and the spruce- and pine-specific modules in lacE, lacJ, and shas, supporting partitioning of a more general transcriptomic response to wood in shim into resource-specific transcriptomes in the three *S. lacrymans* strains.

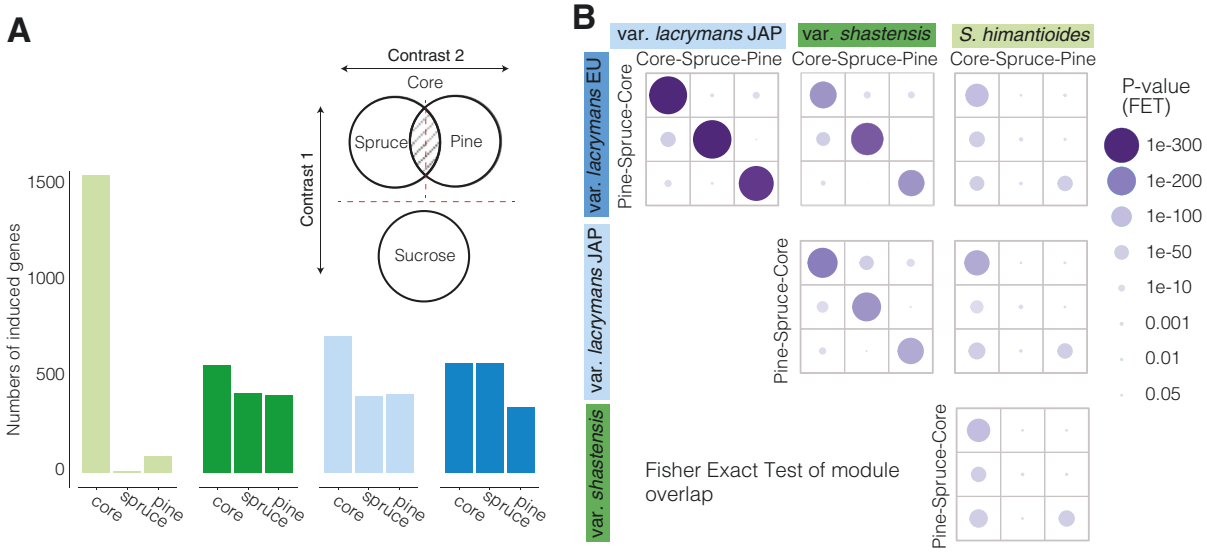

Fig. 2 Wood type-dependent gene expression. A Design of the RNA-seq experiment to determine core wood, spruce- and pine-specific induced genes as well as the numbers of significantly upregulated genes for each module (FDR adj. P value < 0.01 and log fold change >1). **B** Significance of gene overlap between expression modules among different species. Size and shading of the circles correspond to the P values of the Fisher exact test (FET) for each module comparison. P values were corrected for multiple testing using the Benjamini–Hochberg procedure.

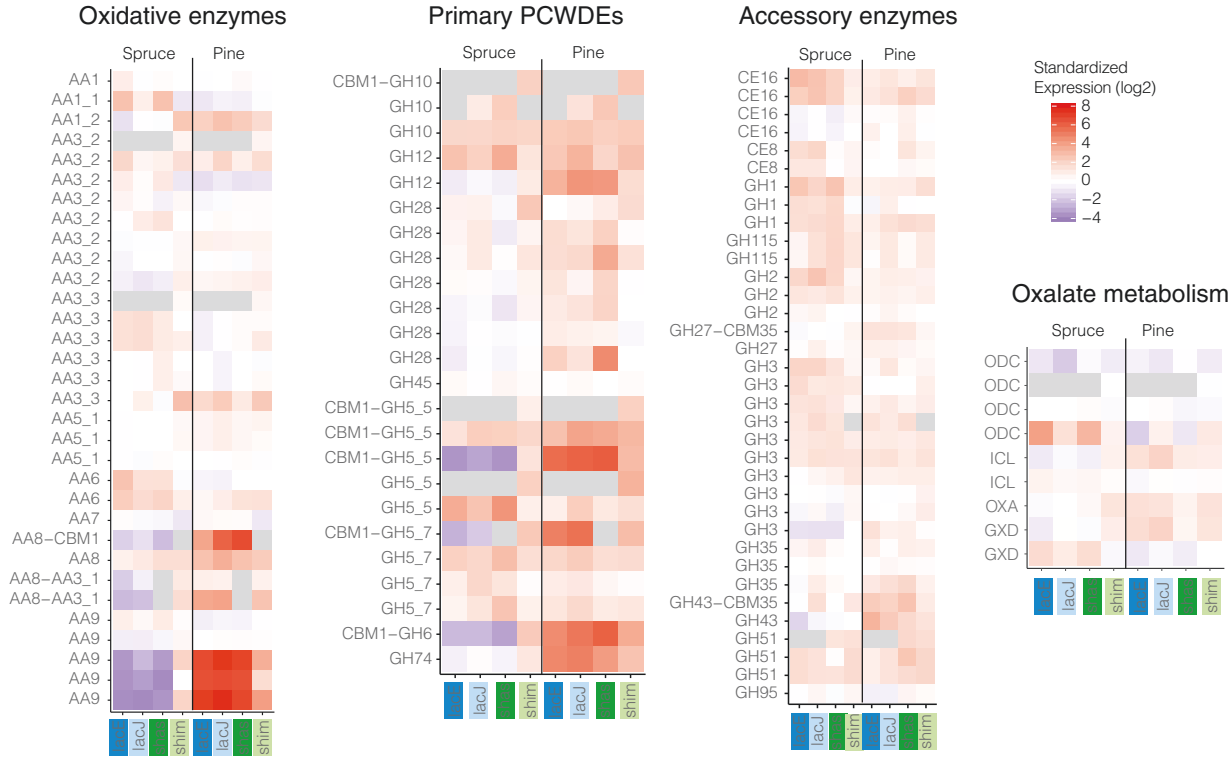

Fig. 3 Standardized expression (mean centered log2 counts per million reads) of carbohydrate active enzymes involved in plant cell wall decomposition and oxalate metabolism. PCWDEs plant cell wall decomposition enzymes.

Functional signatures of resource-specific expression suggest distinct decay stages

Carbohydrate active enzymes (CAZymes, including both hydrolytic and oxidative enzymes) known to be involved in the degradation of PCW material were mapped in each species into oxidative CAZymes, primary hydrolytic CAZymes acting on cellulose, hemicellulose and pectin, and accessory CAZymes metabolizing polysaccharides into simple sugars as well as assisting in PCW breakdown (Fig. 3, Supplementary Data S6). Many of the enzymes targeting crystalline cellulose (GH5_5 endoglucanases, GH6 cellobiohydrolase), hemicellulose (GH74 xyloglucanase, GH5_7 mannanase), and pectin (GH28 pectinase) were strongly upregulated on pine and repressed on spruce in lacE, lacJ, and shas. Similarly, several classes of oxidative enzymes known to be important for the depolymerization of lignocellulose, including three AA9 lytic polysaccharide monooxygenases, two AA8-AA3_1 cellobiose dehydrogenases with an iron reductase domain as well as an iron reductase domain fused to a cellulose-binding CBM1 module (AA8-CBM1) were strongly induced on pine and not detected on spruce in these strains. While some endoglucanases and mannases were induced on spruce, enzymes actively targeted to cellulose via a CBM1 were almost exclusively induced on pine in lacE, lacJ, and shas (Fig. 3, Supplementary Fig. S3). Although many of the key CAZymes mentioned above were also significantly upregulated on pine compared to spruce in shim (Supplementary Data S6), differences were subtle, and general patterns for both conditions closely mirrored the profiles found on pine in lacE, lacJ, and shas (Fig. 3, Supplementary Fig. S3).

Relatively few CAZymes were specifically induced on spruce for lacE, lacJ, and shas, between 12 and 18% of all wood-induced CAZymes, compared to a range of 47 to 53% on pine (Supplementary Data S6). Spruce-specific CAZymes were characterized as accessory CAZymes involved in digestion of oligosaccharides into simple sugars during more advanced decay stages, e.g., β-glucosidases (GH1 and GH3) and β-mannosidase (GH2), or debranching enzymes digesting hemicellulose side chains, e.g., CE16 acetylxylan esterase. No CAZymes were among the eight spruce-specific genes in shim.

Expression patterns of enzymes involved in oxalate metabolism also showed substrate-dependent differences (Fig. 3, Supplementary Data S6). We found strong upregulation of oxaloacetase (OXA) and one of two glyoxalate dehydrogenases, enzymes responsible for oxalate production, on pine in all four strains, while the same genes were repressed on spruce in lacE, lacJ, and shas. One of the four oxalate decarboxylases (ODC), which mediate the degradation of oxalate typical for later decay stages, was strongly repressed on pine in lacE and shas and induced on spruce in

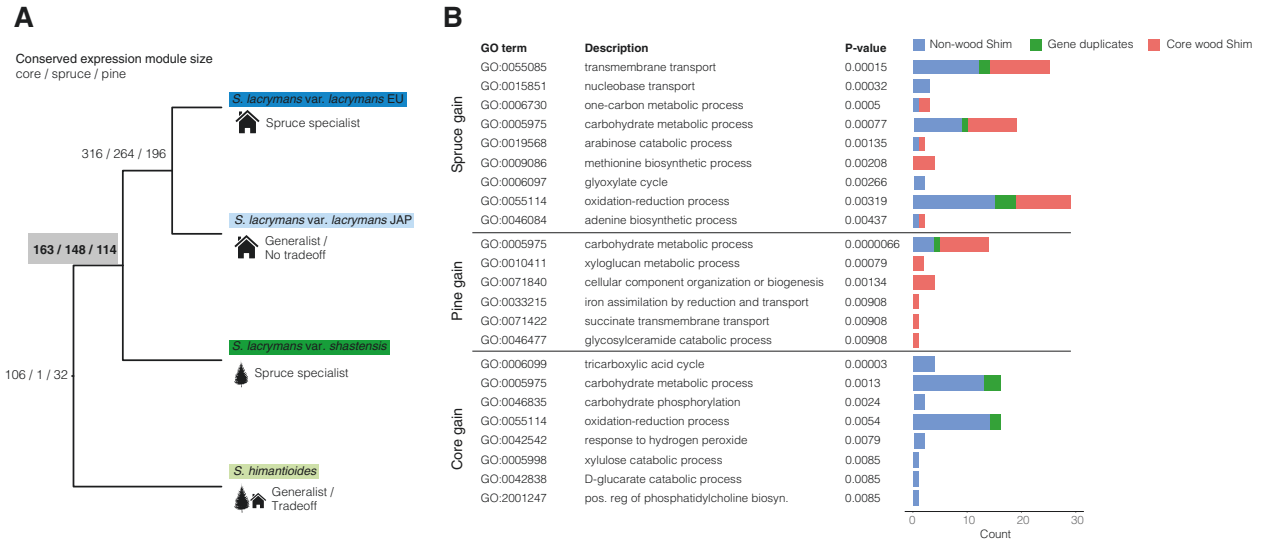

Fig. 4 Evolutionary analysis of wood-induced gene sets. A Reconstruction of conserved expression modules at internal branches. Branch labels indicate the numbers of conserved genes in the core, spruce, and pine modules. **B** Functional enrichment of genes recruited to the spruce, pine, and core expression modules in the last common ancestor of vars. *lacrymans* and *shastensis* (highlighted in **A**). Bar charts on the right indicate the number of genes in each term and whether they are induced on wood in *S. himantioides* (red), not induced on wood in *S. himantioides* (blue) or genes duplicated in the LCA of vars. *lacrymans* and *shastensis* (green).

all strains (Fig. 3). In lacJ and shim, the same ODC was induced on pine which is consistent with more advanced decay of pine for both strains (Fig. 1A; [17]).

Evolutionary analysis of wood-induced gene sets

We mapped evolutionary changes to the wood decay machinery coinciding with the shift in decay capability between shim and the last common ancestor (LCA) of lacE, lacJ, and shas towards spruce (Fig. 4A). A total of 106 genes showed conserved induction on both wood types ("core") in all four strains examined in this study, 32 showed conserved induction on pine only, while a single gene showed conserved spruce-specific induction in all strains. Spruce-specific genes increased disproportionally to 148 genes in the LCA of lacE, lacJ, and shas, while the core and pine modules gained 57 and 82 genes, respectively (Fig. 4A).

Functional enrichment analysis of the 147 genes added to the spruce module in the LCA of lacE, lacJ, and shas (Fig. 4B, Supplementary Data S7, S8) indicated broad changes to carbon and nitrogen metabolism. The term "carbohydrate metabolic process" GO:0005975 was significantly enriched, and genes driving enrichment of this term were predominately related to simple sugar metabolism and regulation of carbohydrate fluxes (Supplementary Data S8), mirroring targeted analysis of CAZymes (Fig. 3). Genes identified included enzymes involved in glycolysis, pentose-phosphate pathway, glyoxylate cycle, hemicellulose and pectin catabolism, and trehalose biosynthesis. A homolog of yeast SNF1 glucose-dependent catabolite regulation kinase was also identified.

Genes gained to the spruce module in the ancestor of lacE, lacJ, and shas were also enriched for uptake and metabolism of organic nitrogen. The strongest enrichment was for the term "transmembrane transport" GO:0055085 with 18 out of 25 associated genes involved in nitrogen transport e.g. oligopeptide transporters, permeases for nucleosides, purine and allantoate, three urea active transporters, and a nitrate transporter. Several terms involved in the biosynthesis of amino acids were also enriched (Supplementary Data S8). Many genes gained to the spruce module were not previously expressed on wood in shim (Fig. 4B, blue bars), and a large number of genes were recruited from the core module in shim to the spruce module in lacE, lacJ, and shas, suggesting a shift in the timing of expression of these genes (Fig. 4B, red bars, Supplementary Data S8). This concerned mainly genes involved in nitrogen metabolism and transport, xylose metabolism, and several GH18 chitinases.

Functional enrichment among genes gained to the pine module in the ancestor of lacE, lacJ, and shas included carbohydrate metabolism genes (GO:0005975), mostly CAZymes involved in the degradation of xyloglucan (GH12, GH31, GH27, GH16), pectin (GH28) and starch (GH13 and GH15), as well as an isocitrate lyase (EC 4.1.3.1), an enzyme linking the TCA and glyoxalate cycles. The term GO:0033215 "iron assimilation by reduction and transport" was also enriched, driven by an iron permease (Supplementary Data S7, S8). The majority of genes in the pine module of

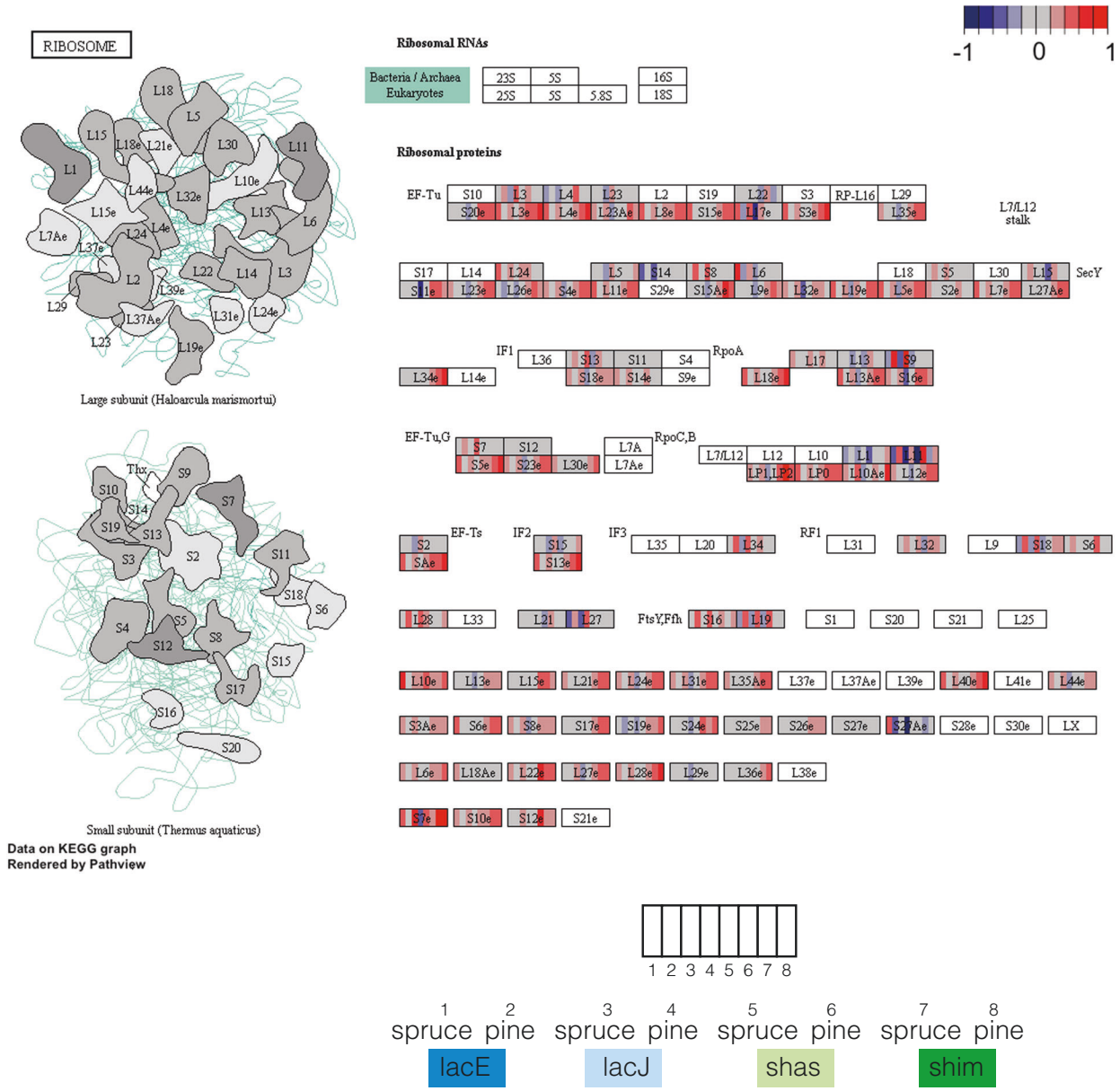

Fig. 5 Expression levels of ribosomal proteins (KEGG 3010). Odd columns indicate spruce expression, even columns pine. Spruce/pine pairs are grouped by species as indicated in the legend. Plots were produced using PathView [58].

lacE, lacJ, and shas were recruited from the core module in shim, reflecting partitioning of the existing response to wood into early and late decay rather than recruitment of new gene sets (Fig. 4B, barplot). A notable exception to this were genes involved in pectin metabolism which were not induced on wood in shim (Supplementary Data S8).

Functional enrichment analysis of genes gained to the pine module in lacJ, a strong pine decayer, highlights genes putatively related to detoxification of plant extractives (Supplementary Data S9, S10). Enriched GO terms include "cellular amide catabolic process" GO:0043605, "oxidation-reduction process" GO:0055114, and "transmembrane transport" GO:0055085. The PFAM domains PF01476 and PF00067, encoding a LysM domain and Cytochrome P450s, respectively, were also significantly enriched.

Loss of induction on wood in the LCA of vars. *lacrymans* and *shastensis*

Transcripts that were induced on wood in shim, but where conserved induction on either type of wood was lost in the ancestor of lacE, lacJ, and shas, were strongly enriched for ribosomal proteins and those involved in ribosomal biogenesis (Fig. 5, Supplementary Fig. S4; Supplementary Data

S11, S12). All of the 72 ribosomal proteins and snoRNAs identified (Supplementary Data S13) were significantly induced on both wood types in shim, while only 11 and 8 showed significant induction (core) in shas and lacE, respectively. Three and two showed core wood induction and spruce-specific induction, respectively, in lacJ.

The term "oxidation-reduction process" GO:0055114 was enriched among genes no longer induced on wood in lacE, lacJ, and shas, encompassing many cytochrome P450s and other putative components of the detoxification machinery. The term also includes one AA3_3 alcohol oxidase and four AA3_2 aryl alcohol oxidoreductases (Supplementary Data S12), all of which are H_2O_2 generating enzymes assisting oxidative degradation of PCW material [6]. Indeed, two of these genes were absent from the genomes of lacE, lacJ, and shas (see above, Fig. 3).

A streamlining of PCWDEs in lacE, lacJ, and shas was also suggested by targeted analysis (Fig. 3; Supplementary Data S6). Pine and core modules for shim included 118 CAZymes significantly induced during what we infer to be the active decay phase, when lignocellulose-targeted enzymes with CBM1s are expressed. In contrast, lacE, lacJ, and shas induced 98, 105, and 102 CAZymes, respectively, under the same conditions (Supplementary Data S6). Three CAZymes significantly upregulated in shim were lost from the genomes of lacE, lacJ, and shas (Fig. 3), including a GH10 endoxylanase with a CBM1 binding module (shim005171) and a GH5_5 endoglucanase (shim013482).

Discussion

Experimentally determined niche breadths mirror niche specialization in the wild

Decay experiments on different types of wood suggest that vars. *lacrymans* (lacE, lacJ) and var. *shastensis* (shas) are able to decompose at significantly higher rates compared to their close relative *S. himantioides* (shim), but that this is substrate-dependent (Fig. 1A, Supplementary Text). *S. lacrymans* var. *lacrymans* and var. *shastensis* inhabit a specialized niche in their natural ranges, where they grow on large logs at high altitude close to the tree line [27, 37]. Both varieties are pioneer species with a strong preference for *Abies* (fir) spp., although var. *lacrymans* has also been isolated from *Picea* (spruce) *smithiana* and less frequently from *Pinus* (pine) *wallichiana*, *Pinus sibirica* and *Cedrus deodara* [37]. *S. himantioides* which demonstrated a more generalist decay strategy (Fig. 1A; [26]), inhabits temperate forests globally and has been isolated from a variety of softwoods and hardwoods [33]. Competition experiments against other common forest fungi showed that the specialist decayers vars. *lacrymans* and *shastensis* are poor competitors, while *S. himantioides* showed an aggressive antagonistic behavior associated with widespread generalist species (Fig. 1B; [26]). Competition experiments using the Japanese var. *lacrymans* strain (lacJ), indicate that this strain also has superior combative abilities compared to lacE and shas (Fig. 1B). This suggests that the Japanese population may have an expanded niche compared to the European population (Fig. 1C). We hypothesize that this may be aided by greater genetic diversity in the Japanese population [27, 28].

Wood type - specific responses highlight distinct decay stages

Expression profiles of PCWDEs indicate decay stage-specific responses between substrate types, in line with existing temporal models of BR decay [14–17] and experimentally measured decay abilities (Fig. 1A). Pine-specific induction of primary PCWDEs, especially those targeted directly to lignocellulose via CBM1s (Fig. 3, Supplementary Fig. S3), iron assimilation (Fig. 4B) and oxalate production (Fig. 3) indicate that all four strains are in early decay stages. While shim appears in a similar stage also on spruce, the downregulation of primary PCWDEs and induction of oligosaccharide processing enzymes (Fig. 3; Supplementary Data S8) indicate a more advanced decay on spruce for lacE, lacJ, and shas. These results highlight the need for increased emphasis on temporal aspects of wood decay in comparative studies. Synchronizing cultures on complex, heterogeneous substrates such as wood is challenging, but further development of space-for-time setups [14] and molecular markers for different decay stages will greatly facilitate this task.

The decomposition machinery of each fungus reflects their respective niches

Evolutionary analyses of pine-induced genes and core modules, encompassing similar stages of decay and stage-independent genes, respectively, in all four strains, indicate a major shift in the transcriptomic response towards increased nitrogen use efficiency during wood decay in vars. *lacrymans* and *shastensis* (Figs. 3, 4). LacE, lacJ, and shas induced a smaller repertoire of CAZymes on pine compared to shim (Fig. 3, Supplementary Data S6), suggesting decreased reliance on nitrogen-intensive enzymatic decay. Three primary hydrolytic CAZymes, of which two (GH10-CBM1 and GH5-5) are among the top 10 induced genes in core and pine modules in shim, were lost from the genomes of lacE, lacJ, and shas altogether (Fig. 3, Supplementary Data S2). The strong induction of cellulose-targeted iron reductase during early decay in these strains

instead supports a greater emphasis on CMF-mediated degradation of lignocellulose (AA8-CBM1, Fig. 3; [11]). The AA8-CBM1 gene is absent from *S. himantioides* (Fig. 3), but conserved among a wider set of fungi [26] and has been experimentally shown to participate in the depolymerization of lignin and cellulose [38]. The presence of AA8-CBM1 could provide greater CMF-mediated iron cycling capacity by adding to the previously described hydroquinone-driven mechanism caused by benzoquinone reducatase activity on elevated 2,5-dimethoxyhydroquinone levels associated with *S. lacrymans* degraded wood [39]. Reciprocal gene losses of key decomposition genes in the *S. himantioides* lineage as well as the LCA of *S. lacrymans* varieties underline that based on the present data it is unclear whether substrate generalism is ancestral to the Serpulaceae or a derived characteristic of *S. himantioides*. This subject will require further investigation.

S. lacrymans produces copious amounts of oxalic acid compared to both distantly-related BR species and close relatives *S. incrassata* and *C. puteana* [40, 41]. Oxalic acid solubilizes iron from Fe(oxyhydr)oxide and chelates it at low pH, as is found in close proximity to the fungal hyphae [42, 43]. Consistent with a role for oxalate in iron accumulation and transport, high oxalate concentrations in *S. lacrymans* were linked to the ability to accumulate large amounts of iron in wood substrates [40, 41] which in turn may facilitate the above discussed CMF-based degradation [18]. Wood is an extremely nitrogen-poor substrate with a C:N ratio of up to 1250:1 [44], e.g., compared to most European forest soils which have C:N ratios of between 16:1 and 44:1 [45]. Transcription and translation of genes encoding the molecular machinery required to exploit woody substrates use relatively large amounts of cellular nitrogen [46]. The shift in decay mechanisms from a more nitrogen-intensive enzymatic strategy (as in shim) to one that leverages products of high carbon flux, such as oxalic acid (lacE, lacJ, and shas), therefore may present an adaptation to nitrogen-limited substrates.

Optimization of nitrogen use in lacE, lacJ, and shas is also reflected by increased partitioning of carbon and nitrogen uptake and metabolism in space and time (Figs. 3, 4B, 5). We found a subtle but coordinated reduction in expression of ribosomal proteins during enzymatic decay in lacE, lacJ, and shas, suggesting reduced overall protein production during wood decay (Fig. 5, Supplementary Data S13). Even small changes, such as alteration in codon usage in highly expressed genes, can have a large impact on the nitrogen budget of an organism [47], highlighting the potential significance of these changes to nitrogen use efficiency. Similarly, we found recruitment of genes involved in nitrogen transport and metabolism from the core module in shim to the spruce module in the ancestor of lacE, lacJ, and shas (Fig. 4B), suggesting a shift to a later decay stage and/or greater fine-tuning to the composition of the substrate compared to shim.

Resource-optimized decay and nitrogen transport may also enable vars. *lacrymans* and *shastensis* to rapidly colonize and thereby monopolize its substrate which can serve as a defense strategy for a pioneer species with limited combative ability [48]. To this end, upregulation of pectin metabolism and degradation of pit membranes during early decay in lacE, lacJ, and shas (Supplementary Data S8) also facilitates rapid advancement of colonizing hyphae between plant cells, in particular in conjunction with increased oxalic acid production [49]. Pectin in pit membranes is complexed with calcium ions, and oxalic acid has been shown to chelate Ca^{2+} from pectin, rendering it more amenable for hydrolytic degradation [49]. This is consistent with the formation of calcium:oxalate crystals around pit membranes during decay by *S. lacrymans* [50].

Decay machineries underpin substrate generalism

The shift to increased reliance on CMF in the LCA of vars. *lacrymans* and *shastensis* appears to represent a specialization for *Picea* spp. and *Abies* spp. as well as a small number of species from the genus *Pinus* (pine; see above). These species all share the common feature of low heartwood extractive content, in particular with respect to resin acids and pinosylvin stilbenes [51]. Differing extractive content may explain the substrate-dependent variation in decay rates and the poor decomposition rates of Scots pine wood by lacE and shas, in particular (Fig. 1). Pine wood is generally found more recalcitrant to decay than spruce in part due to extractives [52, 53]. Pinosylvins, the primary extractives found in Scots pine, are strong antioxidants and can inhibit BR decay by scavenging free radicals, preventing CMF reactions [54]. However, the enzymatic components of the BR decay machinery appear to be less affected by pinosylvins [55] and we hypothesize that the increased reliance on enzymatic decay in shim allows for more rapid decay of pine compared to the more CMF-heavy strategy of lacE and shas (Figs. 1A, 3).

In contrast, the increased weight loss caused by lacJ on pine compared to lacE, shas and shim may be due to a superior ability to detoxify pinosylvins or other inhibitory defense chemicals by this strain (Fig. 1A). Indeed, we found many cytochrome P450s and oxidoreductases among the pine-induced genes in lacJ but not lacE (Supplementary Data S9, S10). Cytochrome P450s have been found to degrade stilbenes in the BR *R. placenta* [56, 57] and are frequently implicated in response and adaptation to different wood types. Expansion of the detoxification enzymes, both by gene duplication and recruitment of existing genes,

therefore provides an alternative pathway to substrate generalism, and apparently without the trade-off associated with having a reduced resource-adapted decay machinery (Fig. 1A, C).

Taken together, our results provide a framework for understanding the evolution of fungal decay machineries in response to substrate and habitat pressures. Our conclusions are limited by the small number of isolates used in this study, and a more fine-grained mapping of niche breadths will require additional experiments, using a broader range of strains, substrates, and competitors, chosen to reflect the different habitats that these species are found in. Nevertheless, we discovered an evolutionarily conserved shift in decay strategy that coincides with increased mass loss and a more specialized niche. Integrative comparative systems, combining growth experiments and evolutionary genomic approaches provide powerful tools to understand how eco-evolutionary feedback mechanisms shape genome evolution and increasingly complex systems. Nitrogen limitation, substrate range, and fungal defense strategy in particular emerge as likely drivers shaping the decay machineries of *Serpula* spp., highlighting the importance of considering multiple axes in a dynamic niche space when interpreting genomic data.

Data availability

Raw reads and assembly of the SL198 strain, as well as RNA-seq data were deposited at NCBI under Bioproject ID PRJNA655420. Updated genome annotations, functional annotations, alignments, and gene trees underlying the phylogenomic reconstruction were deposited on Dryad https://doi.org/10.5061/dryad.4f4qrfj93. Supplementary Information is available for download on the ISME website.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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