Evolution of the dry rot fungus *Serpula lacrymans* and its allies

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[33] Herren talte til Moses og Aron og sa: [34] Når dere kommer inn i Kanaan, som jeg gir dere til eiendom, og jeg lar det komme sopp på et hus i deres land, [35] skal husets eier gå til presten og si: «Det ser ut til å være kommet sopp på huset.» [36] Da skal presten la huset rydde før han selv kommer for å se på flekkene, så ikke alt det som er i huset, blir urent. Så skal han komme og se på huset. [37] Finner han da at flekkene på veggene er grønnlige eller rødlige fordypninger som synes å ligge dypere enn veggen, [38] skal han gå ut av huset, låse døren og holde huset stengt i sju dager. [39] Den sjuende dagen skal presten komme tilbake. Finner han da at flekkene har bredt seg på veggene i huset, [40] skal han sette folk til å bryte ut de steinene som det er flekker på, og kaste dem på et urent sted utenfor byen. [41] De skal skrape huset rundt omkring innvendig, og leiren som er skrapt av, skal kastes på et urent sted utenfor byen. [42] Så skal en ta andre steiner og sette inn i stedet for de gamle og ta ny leire og pusse huset med. [43] Bryter flekkene ut igjen på huset etter at steinene er tatt ut og huset er skrapt og pusset, [44] skal presten komme og se på det. Finner han at flekkene har bredt seg, er det tærende sopp på huset. Det er urent. [45] Da skal huset rives ned, både steinene, treverket og all leiren, og føres til et urent sted utenfor byen.

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Summary

This thesis focuses on the natural history of the dry rot fungus *Serpula lacrymans* and closely related taxa within Serpulaceae. In the first study the phylogenetic relationships within Serpulaceae have been investigated using multi-locus sequencing. In the resulting phylogeny, two mycorrhiza-forming genera, *Austropaxillus* and *Gymnopaxillus*, form a monophyletic group nested within the saprotrophic genus *Serpula*. This confirms a transition from brown-rot to ectomycorrhizal life style that happened once in a monophyletic Serpulaceae, probably between 60 and 40 million years ago in western North America or, alternatively, in Southern temperate regions after long distance dispersal from North America.

The second study deals with cryptic speciation within the species complex *Serpula himantioides* which is the sister species to *S. lacrymans*. Evidence is provided for five cryptic species by four independent gene phylogenies. One of the phylogenetic species shows little phylogeographical structure at a global scale, indicating recent long-distance dispersal. Some of the lineages show adaptation to certain substrates. North and South America appear as the centre of divergence within this morphospecies.

In study III the origin and further worldwide spread of *S. lacrymans* have been analysed employing different molecular markers. Evidence is provided for that *S. lacrymans* is divided into two main lineages that probably represent well-differentiated cryptic species; one nonaggressive residing naturally in North America and Asia (var. *shastensis*), and another aggressive lineage including specimens from all continents, both from natural environments and buildings (var. *lacrymans*). Mainland Asia is pinpointed as the origin of the aggressive form var. *lacrymans*, and a few aggressive genotypes have migrated worldwide from Asia to Europe, North and South America and Oceania followed by local population expansions.

The fourth study provides a detailed survey of two major invasive populations of *S. lacrymans*; one from Japan and one from Europe. Both populations have gone through population bottlenecks prior to local expansion. The European population is extremely genetically depleted leading to the presence of only a few VC-types in Europe, while the Japanese population appears to be influenced by higher gene flow from the Asian source population and, correspondingly, more VC-types occur in Japan. Clonal dispersal seems very infrequent in both populations.

In study V, global distribution and richness of mating types (MAT A) in *S. lacrymans* was studied using a mating type linked genetic marker as a proxy. A high allelic richness and molecular variation was detected in the mating type linked marker as compared to other presumably neutral markers. Little geographic variation was observed in this marker as a contrast to other markers investigated earlier. We observed trans-specific polymorphisms as some alleles from the closely related species *S. himantioides* are more similar to those of *S. lacrymans* than other alleles from *S. himantioides*.

Altogether, this thesis illuminates the evolutionary background and the population genetics of the devastating dry rot fungus.

List of papers

I   Engh IB, Bendiksby M, Carlsen T, Binder M, Kauserud H (2010). Evolutionary history of Serpulacea (Basidiomycota): Molecular phylogeny, historical biogeography and evidence for a single transition of nutritional mode. *Manuscript*.

II   Carlsen T, Engh IB, DeCock C, Rajchenberg M, Kauserud H (2010). Multiple cryptic species with divergent substrate affinities in the *Serpula himantioides* species complex. *Accepted in Fungal Biology with minor revisions.*

III Kauserud H, Svegården IB, Saetre G-P, Knudsen H, Stensrud Ø, Schmidt O, Doi S, Sugiyama T, Högberg N (2007). Asian origin and rapid global spread of the destructive dry rot fungus *Serpula lacrymans*. *Molecular Ecology* **16**, 3350-3360.

IV   Engh IB, Carlsen T, Saetre G-P, Högberg N, Doi S, Kauserud H (2010). Two invasive populations of the dry rot fungus *Serpula lacrymans* show divergent population genetic structures. *Molecular Ecology* **19**, 706-715.

V   Engh IB, Skrede I, Saetre G-P, Kauserud H (2010). Frequency-dependent selection leads to high variability in a mating type linked region in the dry rot fungus *Serpula lacrymans*. *BMC Genetics* **11:64**.

Introduction

The fungal kingdom includes a vast number of species, being estimated to about 1.5 millions (Kirk et al. 2008), yet new species are described virtually every year. Many different ecological strategies have evolved back and forth within the fungal kingdom, including biotrophic parasitism, symbiosis and saprotrophism (James et al. 2006). Saprotrophic fungi degrade different types of organic compounds and are found interspersed throughout the entire fungal kingdom. These fungi have an important ecological function in nutrient cycling in many ecosystems. There are two main types of fungal wood decay; brown rot and white rot. Wooden materials contain a high level of lignocellulose that mostly consists of lignin, hemicellulose and cellulose. White rot fungi decomposes lignin and hemicellulose, whereas brown-rot fungi decomposes hemicellulose and cellulose but leaves the lignin (Rayner & Boddy 1988). Nevertheless, brown-rot fungi are often considered more destructive, as they may be more efficient than white-rot fungi. Brown-rot is considered the major mechanism of fungal wood decay in coniferous boreal and temperate biomes where wood is the major form of sequestered carbon.

Fungi also exhibit a vast variation in life history characteristics. Some fungi are capable of making huge and very long lived genets (Smith et al. 1992) while others only make microscopic thalli and have a very fast population turnover. Fungi also have many different ways of dispersal. Some are capable of vegetative spread by e.g. rhizomorphs, but most fungi spread most effectively through air with either asexual or sexual microscopic spores. Within the basidiomycetes, which is the second largest group of fungi including approximately 30.000 described species (Kirk et al. 2008), most species produce macroscopic fruit bodies from where meiospores are spread, mainly by air.

The basidiomycete life cycle includes a presumably short-lived monokaryotic mycelial stage after spore germination, followed by a more long-lived dikaryon phase. To establish a dikaryon, successful mating is required. After mating, a fruit body can be formed from the dikaryon, karyogami and meiosis takes place leading to the production of haploid meiospores. In basidiomycetes, the vegetative incompatibility system regulates fusion and self/non-self recognition between secondary (dikaryotic) mycelia (Rayner et al. 1984). The vegetative compatibility (VC) type of a dikaryon is governed by numerous independent vegetative incompatibility (*vic*) loci; vegetative incompatibility is associated with genetic dissimilarity in the *vic* loci (Malik & Vilgalys 1999). *vic* alleles are thought to be governed by inverse frequency-dependent selection ('rare allele advantage' as genets

including rare *vic* alleles to a greater extent is able to recognise self from nonself compared to genets harbouring common *vic* alleles (Cortesi et al. 2001).

A complex and unique genetic system for governing the mating process has evolved in basidiomycete fungi. Their mating system can be homothallic (non-outcrossing), bipolar or tetrapolar. Most basidiomycetes (50-65%) have a tetrapolar mating system where two separate gene complexes, MAT A and MAT B, govern the mating process and for mating to occur in tetrapolar species, different allelic versions must be present at both mating type loci. Successful mating requires the override of vegetative incompatibility by the mating compatibility system. Hence, the vegetative incompatibility system and the mating system operate in opposite ways; mating incompatibility is associated with genetic similarity in the mating compatibility (MAT) loci, whereas vegetative incompatibility is associated with genetic dissimilarity in the vegetative compatibility (*vic*) loci (Malik & Vilgalys 1999).

Fungi are in some cases able to disperse long distances by spores, even between continents (Brown & Hovmøller 2002). However, in most analysed species there seems to be clear barriers to gene flow between continents, leading to a geographic subdivision of intraspecific genetic variation (Taylor et al. 2006). However, many fungi have recently been spread by man over long distances, as symbionts or parasites of plants and animals, or growing as saprothrophs in wood materials. Many of these species, transported to areas where their natural enemies are absent, have become invasive species that has gone through rapid population expansions (Brasier & Buck 2001; Fisher *et al.* 2001; Pringle *et al.* 2005). Hence, while many fungi are negatively affected by the explosive human population growth, leading to destruction and transformation of various habitats and ecosystem, other fungi are positively affected by human activity. For example, many pathogenic fungi have co-evolved and adapted to their domesticated crop plants hosts (Stukenbrock et al. 2007). Many saprotrophic fungi have also probably experienced a population growth since their natural habitat is replicated by humans in buildings. The main focus in this thesis is to study the natural history of the devastating dry rot fungus *Serpula lacrymans* and analyse how it became such a widespread wood-decayer in human made habitats.

Study organisms

The boletes (Boletales) is a large group within the Agaricomycotina that includes mainly mycorrhizal-forming species but also saprotrophic and parasitic species (Hibbett & Binder 2002; Binder *et al.* 2005). The saprotrophic species of Boletales decay wood by brown-rot; white-rot is not present (Binder & Hibbett 2006). Different Boletales have different forms of fruiting bodies, ranging from stipitate-pileate forms with tubular hymenophore, to gasteromycetes, polypore-like, or resupinate forms (Binder & Hibbett 2006).

Serpula was traditionally placed in Coniophoraceae Ulbr.(Donk 1948), a family that includes most saprotrophic taxa that mainly degrade conifers, resulting in a brown-rot often termed Coniophoraceae-rot (Binder & Hibbett 2006). The family Serpulaceae Jarosch & Bresinsky (Boletales) was erected to include the genera *Serpula*, *Austropaxillus* Bresinsky & Jarosch and *Gymnopaxillus* E. Horak, based on chemical and molecular phylogenetical analyses (Jarosch 2001). *Austropaxillus* include species with stipitate-pileate formed fruiting bodies and a lamellate hymenophore, while *Gymnopaxillus* are secotioid, including hypogeous species (Claridge et al. 2001). *Austropaxillus* and *Gymnopaxillus* form ectomycorrhiza (ECM) with roots of trees from *Nothofagus* Blume and *Eucalyptus* L'Hér. and are restricted to the southern hemisphere (Oceania/South America).

The genus *Serpula* (Pers.) Gray was erected by Gray in 1821 (Gray 1821) to include species from Persoon's section *Serpula* of *Merulius*. *Serpula* species produces annual, brownish, and resupinate basidiocarps. They have a merulioid hymenophore and produce large amounts of smooth cyanophile spores (Falck 1912; Hallenberg & Eriksson 1985). Two of the most well-known species in *Serpula*, *Serpula himantioides* (Fr.) P. Karst. and *S. lacrymans*, have been described numerous times (Karsten 1885). Cooke (1957) treated these taxa as two varieties of *S. lacrymans*: the domestic *S. lacrymans* var. *lacrymans* and the wild form *S. lacrymans* var. *himantioides*. Based on mating studies and morphological characteristics, Harmsen *et al*. (1958) demonstrated that the varieties represented two biological species (see Fig. 1), recognised today as *S. lacrymans* and *S. himantioides*.

The morphospecies *S. himantioides* has a worldwide natural distribution and can be distinguished from *S. lacrymans* by the thinner and more tightly connected fruit bodies (Hallenberg & Eriksson 1985) and finer rhizomorphs. The species includes multiple

cryptic species (see below), most of them with a primary affinity to South and North America (Kauserud et al. 2006a)

The dry rot fungus, *S. lacrymans* (Wulfen) J. Schröt., has been an important study object for a long time, mainly because of its devastating wood rotting capabilities. It is one of the most well known and feared fungal species in North Europe, attacking houses and other wooden structural elements. Yearly the fungus causes damage of millions of dollars in northern Europe (Bech-Andersen 1995; Palfreyman et al. 1995). The dry rot fungus' bad reputation and impressive ability to damage manmade constructions have made people aware of its existence since old times. Even the Bible has a passage in Leviticus chapter 14 that could possibly refer to the dry rot fungus. The species was originally described as *Boletus lacrymans* in 1781 by Wulfen, at that time already well known to cause severe brown-rot both in houses and in sailing vessels (Ramsbottom 1937).

Serpula lacrymans includes two varieties, var. *shastensis* Harmsen and var. *lacrymans* (Wulfen) J. Schröt. (Harmsen 1960). Var. *shastensis* seems to have a natural distribution 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).
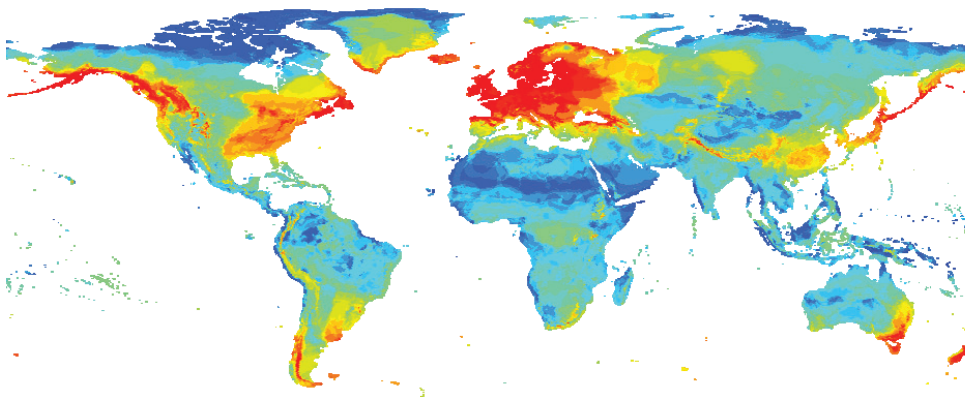

Figure 1. Habitat model of *S. lacrymans* on a worldwide scale, as modelled with the program openModeller implemented on the GBIF web portal (http://data.gbif.org). Dark red colours indicate suitable habitat areas, whereas dark blue indicates unsuitable habitat areas. The model is based upon nineteen climatic variables and geo-referenced records of *S. lacrymans* accessioned in GBIF. Noteworthy, although only GBIF records from Europe and New Zealand together with a single record from US were used for making the habitat model, the habitat model gives surprisingly good match with other localities where we know *S. lacrymans* exists such as Chile, North East Asia and the Himalayas.

Var. *lacrymans* is addition cosmopolitan in distribution; recorded from houses in temperate regions of Asia, Australia, New Zealand, Europe, and North and South America (Hallenberg & Eriksson 1985; White *et al.* 2001).

Serpula lacrymans produces pancake-like fruit bodies, 2–20 mm thick. Falck (1913) estimated that a 100 cm^2 basidiocarp can produce 50 million spores in 10 minutes. The dry-rot fungus is also capable of vegetative local dispersal by producing mycelial strands with the potential to grow several meters across inorganic materials in search of additional organic materials. It has also been observed that monokaryotic isolates produce arthrospores (Harmsen 1960; Schmidt & Moreth-Kebernik 1991).

The dry rot fungus causes brown-rot decay by incomplete ligninolysis. The optimum temperature for growth is about 19-21 °C (Jennings & Bravery 1991) and it will die at temperatures above 32 °C (Bech-Andersen 1995). This has been exploited in the fight against attacks in buildings. If constructions are not harmfully damaged by the dry rot fungus, the building can be heated to 50 °C for several hours, and the fungus is killed (Miric & Willeitner 1984). The life cycle of the heterothallic *S. lacrymans* includes a presumably short-lived monokaryotic primary mycelial phase succeeded by a predominant secondary mycelial dikaryotic phase, in which the fruit bodies are produced. In the tetrapolar *S. lacrymans*, two MAT loci govern the mating process (Schmidt & Moreth-Kebernik 1991).

Research aims

The aims of this thesis were:

- Analyse the evolution of Serpulaceae and *Serpula* by employing a multi-locus phylogeny
- Study the transition from saprotrophic to ectomycorrhizal life style within Serpulaceae and make some preliminary suggestions about the historical biogeography of *Serpula*.
- Use multi-locus sequencing to investigate the occurrence of cryptic species within *S. himantioides* and *S. lacrymans,* and to study the distribution and ecology (substrate affinity) of the cryptic lineages
- Analyse the geographic origin and further worldwide spread of the dry rot fungus *S. lacrymans.*
- Study the population genetics of two successful invasive populations of the dry rot fungus and investigate the level of variation and genetic structuring within and between the populations.
- Investigate the richness and distribution of mating types in *S. lacrymans* by using a mating type linked marker as a proxy, and infer whether this region is governed by frequency-dependent selection.

Methods

In this work a wide array of techniques and analyses have been employed; traditional culturing techniques, various DNA analyses and numerous statistical and phylogenetic inferences. I will in the following give a very brief introduction to these approaches.

Culturing of fungi

In vitro culturing of strains of *S. lacrymans* was performed in order to conduct different mating and vegetative incompatibility experiments. All strains were grown on Petri dishes with malt extract agar and incubated at 20 °C in the dark. In study IV, dikaryotic strains representing two well defined populations were confronted in all combinations to determine their VC type through mycelial interaction zones. When two strains belong to different VC types a zone will be formed between the two strains confronted against each other. When the two strains belong to the same VC type no such confrontation zones will be made.

Molecular markers

A diverse array of standard molecular techniques for analyses of genetic variation have been implemented in this work, including Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995) in study III, microsatellite analysis in study III and IV and multi-locus Sanger sequencing in studies I-V. Cloning of PCR amplified fragments were used in study III and V to separate between co-occurring alleles.

AFLP is a PCR-based method that produces anonymous dominant marker data resulting from restriction enzyme digestion of the whole genome. AFLP seems very suitable for population genetic analyses of fungi due to the relatively small genomes of fungi compared to e.g. plant genomes. Prior knowledge of the genome is not required as universal restriction enzymes are utilised in the process. Because it is difficult to discover contaminations using AFLPs and because of the risk of DNA degradation in herbarium specimens, only DNA extracted from living axenic isolates were used in the AFLP analyses.

Microsatellites are fast evolving neutral markers with a co-dominant nature, and are a natural choice in many population genetic studies. The development of microsatellite markers is relatively time consuming and expensive compared to e.g. AFLP analysis. However, DNA extracted from herbaria specimen and fruit bodies, as well as from

cultures, can be used as the amplification process is designed to be taxon-specific for each marker. However, there are some methodological drawbacks, including null-alleles that can cause problems for interpretation of results (causing false homozygotes). Furthermore, the correspondence between microsatellite based population genetic data and SNPs have been questioned (Väli et al. 2008). A set of fifteen polymorphic markers was developed during our work with the dry rot fungus (Högberg et al. 2006).

In all studies included in this thesis traditional Sanger sequencing have been employed. Sequences from the internal transcribed spacer (ITS) nrDNA region, small subunit (18S) and large subunit (28S) of nrDNA, parts of the beta tubulin (*tub*), glyceraldehyde-3-phosphate dehydrogenase (*gpd*), translation elongation factor 1α (*tef*), heat stress protein (*hsp*) regions and parts of the second largest subunit of the RNA polymerase II (RPB2) have been used in the different studies. In study III and V, cloning was combined with Sanger sequencing to separate between divergent alleles co-occurring in heterozygous dikaryons. Several challenges are associated with the cloning procedure; including introduction of PCR mediated mutations as well as PCR mediated chimeric sequences. In general, several 'replicate sequences' were cloned and analysed so we could check for these artefacts.

In study V we used a marker linked to the MAT A locus as a proxy to study the allelic richness and geographic distribution of mating alleles, and whether this locus is governed by frequency-dependent selection. We first used previously published primers to amplify a part of the non-mating type MAT-linked *mip* gene (James et al. 2004), and designed several new primers in order to amplify and sequence the various allelic versions in this marker.

Phylogenetic and population genetic analyses

To estimate divergence time for Serpulaceae within Boletales in study I we used Bayesian Evolutionary Analysis Sampling Trees (BEAST) 1.4.7 (Drummond & Rambaut 2007) on a concatenated data set consisting of five independent regions. The time estimation was based on secondary calibration points as there is no fossil record of Serpulaceae or close relatives in the Boletales; A fossil-based crown group age estimate of 55-35 million years for *Nothofagus* (Cook & Crisp 2005), a molecular clock based estimate of 60-35 million years for Suillaceae and 109-96 million years for Boletales was used (Bruns et al. 1998).

In the phylogenetic analyses performed in studies I, II, III and V we have employed MrModeltest 2.3 (Nylander 2004) and MrBayes (Huelsenbeck & Ronquist 2001;

Ronquist & Huelsenbeck 2003) using the Bioportal computer cluster at the University of Oslo. Parsimony analyses using TNT (Goloboff et al. 2008) and Jackknifing (Farris et al. 1996) have been performed in all our studies. In study III, haplotype networks were constructed from the haplophase sequence data sets using Arlequin (Excoffier et al. 2005). In the studies IV and V, tests for deviation from neutral evolution were performed using the program DnaSP (Rozas & Rozas 1999).

The AFLP data (study III) were analysed using Neighbor Joining (NJ) and phylogenetic tree length permutation test (PTLPT) using PAUP* version 4.02b (Swofford 1999).

In the studies III and IV, various population genetic analyses were conducted to analyse the population structure and genetic composition. A Bayesian clustering approach implemented in the program STRUCTURE version 2.2.3 (Pritchard *et al.* 2000), employing the computer cluster at the Bioportal at the University of Oslo, was used to infer population structure in the microsatellite datasets (Paper III and V). Structure is an unconstrained analysis without predefined groups. Standard population genetic statistics, including tests for Hardy Weinberg equilibrium and linkage equilibrium, was used as implemented in the program Arlequin. The ratio of the microsatellite allele numbers to the allele size range (M value) (Garza & Williamson 2001) was used to detect population bottlenecks as computed in the program M_P_VAL (study III and V).

Results and discussion

Phylogeny and historic biogeography of *Serpulaceae*

Our phylogenetic analyses in study I based on five loci show that the mycorrhiza forming *Austropaxillus* (and *Gymnopaxillus*) cluster within Serpulaceae and together with the included *Serpula* species form a monophyletic group corresponding to Serpulaceae, as earlier proposed by Jarosch (2001). Hence, *Serpula* is today a paraphyletic group. Within Serpulaceae there has been one transition from saprotrophy to ectomycorrhizal nutritional mode. Dating analyses using secondary calibration points indicated that this transition in life style happened 60 to 40 million years ago. Transition from a saprotrophy to an ectomycorrhizal life-form is a common ecological transition in the fungal kingdom (James et al. 2006; Tedersoo et al. 2010). Temperature decline and a drier climate in the mid to late Eocene may have promoted transition from saprotrophy to mycorrhiza in the common ancestor of *Austropaxillus* and *Gymnopaxillus*. The hypogeous fruit bodies of *Gymnopaxillus* also seem adapted to dry climates.

Our results indicate that the mycorrhizal *Austropaxillus/Gymnopaxillus* lineage diverged from the saprotrophic *S. lacrymans/S. himantioides* group about 60 (77-47) My ago and that the radiation of extant *Austropaxillus/Gymnopaxillus* species commenced about 37 (43-35) My ago. This largely corresponds with the radiation of the mycorrhizal suilloid group (Bruns et al. 1998). A 50 My old fossilised ECM, probably with a *Pinus*-host (LePage et al. 1997), demonstrates that ECM associations had evolved at least 50 My ago. It has been suggested that the radiation of ECM fungi happened as the obligate ECM hosts (Pinaceae and Fagales) became dominant in temperate forests as a consequence of drying and cooling from the Late Eocene (Bruns et al. 1998; Matheny et al. 2009).

In study I the results indicate a Late Cretaceous origin of extant Serpulaceae. The main host of Serpulaceae, members of the genus *Pinus*, probably evolved during the Cretaceous, between 155 and 87 My ago (Won & Renner 2006), and the fossil record confirms the presence of Pinaceae members in the high-latitude and high-altitude regions of North America during the early Tertiary (LePage 2003).

The initial divergence of extant *Austropaxillus* taxa into one largely southern South American clade and one Australian clade may depict Gondwanan vicariance as migration was probably possible between South America and Australia up until 28-32 My ago (McLoughlin 2001). The large distributional gap between North and southern South

American sister-taxa of *Serpula* favours long-distance dispersal to an explanation involving a historically continuous distribution and subsequent vicariance.

The divergence of the two varieties of *S. lacrymans* occurred about 12 (23-4) My ago. The presence of western North American and eastern Eurasian phylogenetic sister-taxa are indicative of a trans-Beringian distribution of their most recent common ancestor with subsequent vicariance, not the least given the timing of the divergence. About 14-3.5 My ago there was continuous boreal forests across Beringia and it seems probable that *S. lacrymans* also then had a continuous distribution from Northwest North America and into Eurasia and that var. *lacrymans* became differentiated from var. *shastensis* due to Beringian vicariance.

Cryptic speciation

Numerous studies conducted during the last 10-15 years have demonstrated that the occurrence of cryptic species is a common phenomenon in the fungal kingdom. Traditionally, almost all fungal species were defined based on morphological characters, but a conflict is very often seen between phylogenetic and morphological species identification (Taylor et al. 2000). The idea behind phylogenetic species recognition is to analyse the concordance of multiple gene genealogies from independent loci. A phylogenetic species can be recognised as a group of organisms all of whose genes coalesce more recently with each other than with those of any organism outside the group. Conflict among independent gene topologies can be caused by the exchange of genes among individuals within a species, and the transition from conflict to concordance determines the limits of species (Taylor et al. 2000). With the use of a phylogenetical species recognition concept, asexual (*Coccidioides* (Burt et al. 2001)) and unculturable fungi (*Pneumocystis carinii* (Cushion et al. 1991)) can be studied as well.

In the morphospecies *S. himantioides* our results (study II) indicate the presence of five phylogenetic/cryptic species with different geographic distribution patterns and substrate requirements. One phylogenetic species (PS1) is seemingly bound to South America, while the others (PS2-5) seem to have a primary affinity to North America. In *S. lacrymans* all our analyses point towards the presence of two species, today referred to as var. *lacrymans* and var. *shastensis* (study I, III and V). In study I, var. *lacrymans* and var. *shastensis* were hybridised *in vitro*, and a fruit body was induced. However, no viable monokaryotic mycelium was obtained from the fruit body, indicating that pre- or post-zygotic barriers exist between the two varieties. Furthermore, the hybrid dikaryon between

var. *lacrymans* and var. *shastensis* was grown under semi-natural conditions (figure 2). Noteworthy, the hybrid grew slower and was less viable than the parental isolates of var. *lacrymans* and var. *shastensis*, suggesting that the hybrid is less fit than the parents in a semi-natural environment (unpublished).
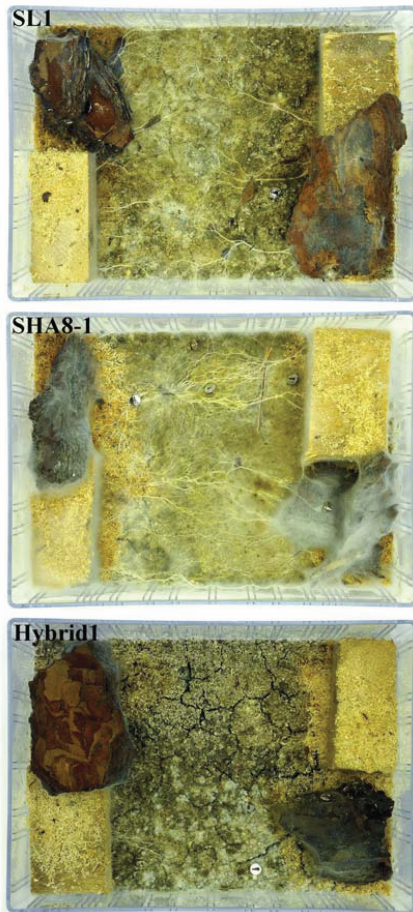

Figure 2. Growth experiment in semi-natural environments containing wood materials inoculated with *S. lacrymans* var. *lacrymans* (top, strain SL1), *S. lacrymans* var. *shastensis* (middle, strain SHA8-1) and a hybrid between the two varieties var. *lacrymans* and var. *shastensis* (bottom).

Var. *lacrymans* and var. *shastensis* show vigorous growth, while the hybrid displayed sparse growth and lower viability.

One underlying cause for the high prevalence of cryptic species in the fungal kingdom is probably that the morphospecies is mainly based upon a few characters associated with only one part of the fungi's life cycle, namely the fruit body. In comparison, in animals and plants, most of the organism's phenotype is used for species description. Furthermore, in plants and animals sexual selection might be an important driver for morphological diversification, but this aspect is mainly absent when it comes to fungi.

Phylogeography

Phylogeography was defined by Avise as a field of study concerned with the principles and processes governing geographic distributions and genealogical lineages, especially those within and among closely related species (Avise 2000). Hitherto, the phylogeography of fungi has been poorly studies compared to other organisms (Beheregaray 2008). Many fungal species are widely distributed across several continents, but recent molecular based phylogeographic studies have indicated that most fungi also experience barriers to gene flow. A distinct genetic differentiation is often observed between fungal populations from different continents (Taylor et al. 2006). A complicating factor in fungal phylogeographic studies is the presence of cryptic species, which must be sorted out in order to perform meaningful phylogeographic analyses.

In study III, a phylogeographic analysis of *S. lacrymans* was conducted based on genetic analyses of a global sample of cultures and fruit bodies. We first observed that the dry rot fungus is divided into two main lineages; the nonaggressive residing naturally in North America and Asia (var. *shastensis*), and the aggressive lineage including specimens from all continents, both from natural environments and buildings (var. *lacrymans*). Our population genetic analyses pinpointed mainland Asia as the most likely area of origin of the aggressive form var. *lacrymans*. A few aggressive genotypes have apparently migrated worldwide from Asia to Europe followed by local population expansions (cf. study IV). There has been a separate dispersal event to Japan, where the fungus holds a strong population in buildings but has not been found in nature. Further human mediated dispersal from Europe to North and South America and Oceania seems likely to have happened during the last centuries. The very low genetic variation in the founder populations indicate that they have established through recent founder events, for example by infected wood materials transported over land or sea (Ramsbottom 1937).

We obtained a fairly good sample from one of the cryptic lineages within the *S. himantioides* species complex (PS5 in study II), making us able to make some hypothesis about its phylogeographic structure. This cryptic species is more or less distributed globally, but most genetic variation is found in North America and North East Asia, which could indicate its natural range. Otherwise, the lack of a clear phylogeographic structure on a global scale within PS5 indicates that there have been several recent long distance dispersal events, for example to Africa and Oceania. PS5 might have spread together with introduced coniferous trees that are grown in plantations in these areas.

Population genetics in invasive populations of *S. lacrymans*

In study IV, the genetic structures and variation in two presumably invasive populations of *S. lacrymans* var. *lacrymans* from Europe and Japan, respectively, were investigated using co-dominant microsatellite markers and sequence data. The two populations were found to be highly differentiated, indicating that little or no gene flow has happened recently between the two populations. A very weak but significant isolation by distance effects were observed both in Europe and Japan indicating that some barriers to gene flow exist within the two areas. Lack of genetic sub-structuring has commonly been observed at comparable regional scales in basidiomycete fungi spread by airborne spores (Högberg *et al.* 1999; Kauserud *et al.* 2004), but these observations may partly be due to low resolution of the utilised genetic markers in these studies.

Higher genetic variation was observed within the Japanese population than within the European population, corresponding with an observed higher richness of vegetative compatibility (VC) types in Japan (38 VC types observed in 68 isolates), supporting the view that there has been a higher level of gene flow from the Asian source populations to Japan than to Europe. The European population is genetically more homogenous with only six detected VC types resulting from 67 individuals studied (Kauserud et al. 2006b). However, our analyses indicate that both the European and the Japanese populations have gone through population bottlenecks prior to population expansion (paper IV).

Our analyses indicated that little clonal dispersal occur in both the European and the Japanese populations since almost none identical multi-locus genotypes were observed. Furthermore, only low levels of linkage disequilibrium between microsatellite loci were observed. These results may indicate that the indoor populations of *S. lacrymans* spread mainly by basidiospores and to a less extent as clones on infected wood materials.

Rather few population genetic studies of basidiomycetes have been conducted, but in outcrossing (heterothallic) taxa like *S. lacrymans* panmictic conditions have mainly been observed in natural populations (Kauserud & Schumacher 2003b, a). Rather surprisingly, we observed an excess of heterozygotes in both the European and the Japanese populations, this pattern being especially pronounced in Europe. We speculate in study IV that this peculiar pattern could be due to linkage between (some of the) microsatellite markers and parts of the genome that are influenced by frequency-dependent selection, such as the MAT and *vic* genes. This highly speculative hypothesis could potentially be investigated further using whole genome sequence data.

Distribution of mating types in *Serpula lacrymans*

In study V, the allelic richness and geographic distribution of mating alleles of the MAT A locus was indirectly studied using a tightly linked genetic marker as a proxy. Since the sequence divergence is presumably very high within the MAT regions it may therefore be difficult to study the molecular variation in the MAT alleles themselves. This strategy for indirectly studying MAT alleles was put forward by James et al. (2007).They demonstrated that a conserved gene order (shared synteny) exists between the mating type genes and neighbouring genes in most Agaricomycetes (James 2007). One such locus is the gene encoding mitochondrial intermediate peptidase (*mip*), located close to the MAT A locus in Agaricomycetes investigated (Stankis *et al.* 1992; Casselton *et al.* 1995).

In homobasidiomycetes, multiple alleles exist in the mating type loci as demonstrated by classical mating studies (Whitehouse 1949; Raper 1966). It is thought that inverse negative frequency-dependent selection ('rare allele advantage') promotes maintenance of a high richness of MAT alleles in populations of these fungi (Raper 1966; Murphy & Miller 1997). Such a selection regime may also lead to the occurrence of 'trans-specific polymorphisms' because of extended coalescence times between alleles (Devier et al. 2009).

In study V we detected, as expected, a high allelic richness and molecular variation in the mating type linked marker in populations of *S. lacrymans* as compared to other presumably neutral markers. Comparable amount of genetic variation appeared in the mating type linked marker in *S. lacrymans* populations from nature and buildings, which contrast the pattern observed with neutral genetic markers where natural populations are far more genetically variable. Furthermore, less geographic structuring of the allelic variation in the mating type linked marker appeared than observed with neutral markers. The investigated marker also displayed trans-species polymorphism wherein some alleles from the closely related species *S. himantioides* are more similar to those of *S. lacrymans* than other alleles from *S. himantioides*. All these results are in line with the idea that strong negative frequency-dependent selection maintains high levels of genetic variation in MAT-linked genomic regions, even in the recently bottlenecked populations of *S. lacrymans*. Our study also suggests that DNA regions physically linked to the hypervariable mating type may serve as suitable markers to separate closely related fungal isolates.

In the founder populations of *S. lacrymans* (i.e. Europe and Japan), alleles co-occurring in heterokaryotic individuals were more divergent than expected by chance, which agrees with the expectation for populations where few mating alleles exist. If a high

number of mating alleles occur in a population, we argue that such a pattern would be difficult to observe. This observation support earlier studies that have indicated that a limited amount of mating alleles exist in Europe (Kauserud et al. 2006b).

Conclusions and future perspectives

This thesis includes results that shed new light upon the evolutionary history of the devastating dry rot fungus *S. lacrymans*. We show that *S. lacrymans* belongs to a monophyletic group (Serpulaceae) that also include species forming ectomycorrhiza and that a single transition to ectomycorrhizal growth has happened in Serpulaceae about 60-40 My ago. As in many other basidiomycetes, cryptic species is present both in *S. lacrymans* and its sister *S. himantioides*. We provide solid evidence for that *S. lacrymans* includes one non-aggressive lineage (var. *shastensis*) as well as the aggressive form var. *lacrymans* and that these two forms probably differentiated related to a Beringian vicariance event about 12 (23-4) My ago. From its natural range in East Asia, we describe how var. lacrymans has spread worldwide becoming an invasive demolisher of wood-constructions. By population genetic analyses we characterize the population structure of the invasive populations in Europe and Japan and shows that a higher genetic variation occurs in Japan, which is rather natural since it is more closely related to the source population in mainland Asia. We also demonstrate that sexual dispersal by basidiospores characterizes the invasive populations. The allelic richness and distribution of mating types in *S. lacrymans* have been investigated and all the data indicate that the investigated mating type gene (MAT A) is strongly influenced by frequency-dependent selection.

These days a small revolution in biology is going on related to the introduction of new high throughput sequencing technologies. This makes it possible to conduct genomic analyses that were beyond our reach just a few years ago. A high number of fungal genomes have been sequenced using the new, as well as 'old', technologies, and recently two haploid genomes of *S. lacrymans* have been sequenced. Several other genomes of *S. lacrymans* will be sequences in near future, paving the way for a variety of genomic studies of *S. lacrymans*. One further research topic will certainly be to reveal which genomic changes that have accompanied the transition from a free-living form of *S. lacrymans* to the form residing in buildings. *S. lacrymans* may also serve as a good model to understand the genomic basis for the formation of rhizomorphs. To investigate the genomic basis of *S. lacrymans* high decomposition ability as a brown rotter will certainly also be a hot research topic.

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Ingeborg Bjorvand Engh

I

II

Multiple cryptic species with divergent substrate affinities in the *Serpula himantioides* species complex

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Abstract

Serpula himantioides is a widespread saprotrophic morphospecies mainly colonising coniferous wood in nature, but it appears frequently in buildings as well. From an earlier study, it is known that at least three divergent lineages occur within the *S. himantioides* species complex. In this study, a broader sample of *S. himantioides* isolates has been analysed by multi-locus sequencing, including new isolates from Asia, North and South America. Altogether five phylogenetical species (PS1-5) were detected, all recognised across independent gene phylogenies. A new southern South American phylogenetic species (PS1) was found, representing an early diverging lineage within the *S. himantioides* species complex. The two closely related PS2 and PS3 lineages included isolates from North America only, and PS4 was also dominated by North American isolates. Most of the investigated isolates (76%) clustered into PS5, a lineage that has been found on most continents, including North America. Overall, little phylogeographical structure was found in PS5, indicating frequent and recent long-distance dispersal events within this widespread lineage. Our analyses indicate that South and North America is the centre of divergence for the *S. himantioides* species complex. Some of the lineages seem adapted to various substrates, but PS5 is able to decay a wide array of angiosperms and gymnosperms, which may have facilitated the spread of this lineage throughout the world.

Kew words: *Serpula himantioides*; saprotrophic; phylogenetic species; cryptic species; phylogeography;

Introduction

Serpula himantioides (Fr.) P. Karst. is a saprotrophic morphospecies with a wide geographical distribution, observed on all continents except Antarctica. It is mainly found on dead wood of various coniferous tree species, but do also commonly occur in buildings. It produces thin, resupinate and brownish annual basidiocarps and has a heterothallic tetrapolar mating system, based on Northern Hemisphere specimens (Harmsen 1960; Hwang 1955). In a previous study, three genetically well-differentiated lineages were detected within *S. himantioides* across independent gene phylogenies (Kauserud et al. 2006). These three lineages also showed compatible mating within lineages, and incompatible mating between lineages. Thus, as many other basidiomycetes (Geml et al. 2006; Kauserud et al. 2007a; Nilsson et al. 2003), *S. himantioides* seems to represent a species complex including multiple cryptic lineages. Especially when it comes to morphospecies that produces simple, resupinate fruiting structures, such as *S. himantioides*, it might be problematic to detect species boundaries by morphological means (Taylor et al. 2006). Likewise, multiple cryptic species has also been detected in several *Coniophora* species that also produce simple and resupinate fruiting bodies (Ainsworth and Rayner 1990; Kauserud et al. 2007a).

Biogeographical studies show that fungi have complex histories of vicariance and dispersal in the same way as plants and animals (Matheny et al. 2009; Taylor et al. 2006). Although long distance dispersal events may be rare, it is the best explanation for the present day distribution of many fungal taxa (Hibbett 2001; Hosaka et al. 2008; James et al. 2001; Moncalvo and Buchanan 2008; Zervakis et al. 2004). For some fungi, the natural biogeographical patterns may be blurred by modern spread by man (Brasier and Buck 2001; Coetzee et al. 2001; Kauserud et al. 2007b; Slippers et al. 2001).

In the dry rot fungus *S. lacrymans* that is closely related to *S. himantioides*, there seems to have happened a specialization towards growing in buildings (Kauserud et al. 2007b). *S. himantioides* is also known as a common destroyer of wooden constructions, but whether any adaptations towards this growing habit have happened in any of the *S. himantioides* lineages is not clear.

In this study, a broader sample of *S. himantioides* is included compared to Kauserud et al. (2006), including newly obtained isolates from Asia and both of the American continents. The aims of this study were to (1) analyse whether even more phylogenetic species can be

found within *S. himantioides*, (2) check whether a biogeographical structure of various *S. himantioides* lineages can be observed, and (3) analyse whether any substrate specialization has happened during the diversification of the *S. himantioides* species complex. To illuminate these topics, the isolates where analysed by multi-locus sequencing of three independent DNA regions.

Material and methods

The material included in this study is listed in Table 1. Compared to Kauserud et al. (2006), 40 new isolates of *S. himantioides* were included in this study. DNA was extracted from the new cultures following a 2% CTAB (cetyl trimethylammonium bromide) miniprep method described by Murray & Thompson (1980) with minor modifications: DNA was resuspended in 100 µL distilled sterile H_2O at the final step of extraction. The four DNA markers ITS, LSU, *hsp* and *tub*, were PCR amplified and sequenced as outlined in Kauserud et al. (2006). All sequences have been accessioned in GenBank (for accession nos. see Table 1).

Phylogenetic analyses were conducted using TNT (Goloboff et al. 2008). Heuristic searches were performed with 1000 random addition sequences and TBR branch swapping. Jackknife analyses were performed with 10,000 replicates, 36% removal probability, and absolute frequencies as output. Bayesian analyses were performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with models inferred from MrModeltest 2.3 (Posada and Crandall 1998). Two independent runs with 5 chains (4 heated) were run for 10,000,000 generations and summarised after discarding 25% burn-in. Initially, each DNA region was run separately, but as there was no significant incongruence between datasets, a concatenated dataset were used for all further analyses. For Bayesian analyses, the different regions were analysed with an independent model for each partition (ITS= GTR+I, LSU= GTR, *tub*= GTR+G, and *hsp*=SYM+G).

Genealogical sorting index (GSI) statistics (Cummings et al. 2008) were performed to test for phylogenetic correlation between isolates from different substrates, continents, and habitats. Substrates were categorized as *Pinus*, *Picea*, *Nothofagus*, unknown conifer, and unknown hardwood. Continents were categorised according to geography and habitat categorized as indoor or outdoor.

Results and discussion

In Fig. 1, a multi-locus phylogeny of the 67 analysed isolates of *S. himantioides* is shown, demonstrating that the isolates group into five different lineages with high support. A high

congruence in topology was observed across the four investigated DNA regions when these were analysed separately (Supplementary Information, Fig. S1A-C). The five lineages were named PS1 to PS5 corresponding to phylogenetic species under the phylogenetic species recognition definition (Kroken and Taylor 2001, 2009; Taylor et al. 2000). This, together with earlier mating experiments that has shown that there are compatible matings within the lineages PS3, PS4, and PS5 but incompatible across lineages (Harmsen 1960; Kauserud et al. 2006), indicate that PS1 to PS5 represent different biological species. As there is only one sample in PS2, and no experimental crosses could be performed, it may be premature to conclude with certainty that this is a distinct species. But as the genetic divergence between PS2 and PS3 is comparable to the divergence to the other phylogenetic species within *S. himantioides*, we will treat it as a separate entity here.

A recent dated molecular phylogeny of the family Serpulaceae has dated the oldest divergence of the cryptic lineages of *Serpula himantioides* to approx. 12 million years ago (Engh et al. in prep). A similar time estimate was made for the split between *Serpula lacrymans* var. *lacrymans* and *Serpula lacrymans* var. *shastensis* that are still able to mate *in vitro* (Harmsen 1960).

Lineage PS1 is sister to the other lineages and includes five South American isolates. PS2, PS3, and PS4 include mainly North American isolates. However, the sample size here is too small to conclude that the lineages are mainly restricted to this continent. PS5 is a widely distributed lineage that has been detected on all continents except for South America and Antarctica. The lack of a distinct phylogeographical structure within PS5 presumably indicates recent long-distance dispersal events. It is well-known that many basidiomycetes have been spread by man on infected timber or plants (Coetzee et al. 2001; Gonthier et al. 2004; Linzer et al. 2008; Pringle et al. 2009) and this could also be the case with *S. himantioides*.

Within PS5, a high level of genetic variation is found among the isolates from East Asia and North America. Overall, South and North America seem to have played an important role during the evolution of the *S. himantioides* species complex. The GSI analysis showed that there was a highly significant grouping of the South American samples (GSI 1.00 p<0.00001), but samples from other continents showed no significant grouping. One might speculate that allopatric speciation in South and North America may have happened in the ancestral lineage splitting into PS1 and the ancestral lineage to PS2-PS5. Our analyses indicate furthermore that PS2, PS3, and PS4 have a primary affinity to North America, but with some northern European representatives. The almost cosmopolitan PS5 may have spread

out from North America to Eastern Asia and more recently obtained a world-wide distribution with the help of man. In Fig. 2, the geographical distribution of the North American and European isolates is shown. Although speculative, it may be that PS5 has a more temperate distribution on the North American continent while PS4 has a more boreal distribution. In the European samples PS4 was only found in Norway while the other European samples were all in PS5.

There is no evidence for a specialisation towards growing on wooden constructions, as has apparently happened in *S. lacrymans* var. *lacrymans* (Bagchee 1954; Kauserud et al. 2007b; White et al. 2001). In the phylogenetic tree (Fig. 1), isolates derived from nature and buildings appear both in PS1 and PS5 without any apparent structuring, indicating that a constant influx of spores happens from nature to buildings and possibly vice versa. This is supported by GSI analysis that showed that there were no grouping of outdoor (GSI 0.048 p=0.41) or indoor (GSI 0.051 p=0.13) isolates.

There was however a slightly significant signal in the isolates from *Pinus* substrates (GSI 0.0836 p= 0.047). Isolates from *Pinus* substrates were only found in the S4 (one isolate) and S5 lineages. The two PS1 isolates that were found outdoors were obtained from *Nothofagus*, (GSI=0.239 p=0.011). The substrate from the three indoor isolates in the same clade is unknown, but it is not unlikely that these are also *Nothofagus*, as this is a common source of building material in the region (Martínez Pastur et al. 2000). In isolates from PS2, PS3, and PS4, most isolates were from *Picea*, and none of these were from buildings. In the PS5 lineage a lot of different substrates were found, from *Eucalyptus* and *Alnus* to *Abies* and *Pinus*. This indicates that the putative allopatric speciation event leading to the split between PS1 and the PS2-5 lineages was accompanied with a host preference partition as well as a geographic split. In addition, there seems to have been a switch from a predominantly *Abies* host preference seen in the PS2 and PS4 lineages, to a higher degree of non specific host preference in the PS5 lineage. Although PS5 may initially also have been a host specialist, PS5 has today a wider host range than the other lineages in addition to its much wider geographic distribution. The ability to colonise a wide spectrum of substrates may have facilitated the spread of this lineage throughout the world.

This study underlines the importance of having a broad geographic sample when analysing intraspecies variation and divergence. Undiscovered cryptic species may have a restricted distribution in unsampled areas. We have discovered two new cryptic lineages in this study compared to Kauserud et al. (2006), enabling a better understanding of the biogeography and substrate preferences of the *Serpula himantioides* species complex.

Figure 1. Phylogenetic tree from a Bayesian analysis of a combined dataset of ITS, LSU, *tef*, and *hsp* sequences. Numbers below branches indicate posterior probability values. Numbers above brances indicate parsimony jack-knife support values (only values above 50% is shown). The corresponding parsimony trees were of length 350 with CI=0.763 and RC=0.674.

Figure 2. Approximate geographic distribution of the analysed isolates and cryptic species of *Serpula himantioides* in North America and Europe. Google Maps™ mapping service.

Supplementary figure 1: Parsimony strict consensus trees from A: ITS and LSU sequences, B: *tub* sequences, and C: *hsp* sequences.

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Table 1. Specimens included in this study. Locality information, culture collection, strain number, substrate information, and GenBank numbers are given where they are available.

Code	Locality	Collector	Culture collection[1]	Strain number	Country	Substrate	LSU	ITS	*tub*	*hsp*
SH16	Belgium: Louvain-la-Nueve	G.L. Hennebert	MUCL	30528	BEL	brick wall	AM076531	AM076498	AJ518086	AM076442
SH17	British Columbia, Aleza Lake	N.a.	MUCL	30795	CAN	*Picea glauca*	AM076527	AM076494	AJ557380	AM076438
SH18	British Columbia, Mt Revelstoke	N.a.	WFPL	49B	CAN	*Calliropsis nootkatensis*	AM076532	AM076499	AJ557377	AM076443
SH19	Bedgebury Pinetum	N.a.	WFPL	49C	GBR	inner bark, *Pinus* sp.	AM076533	AM076500	AJ518087	AM076444
SH20	British Columbia, Cowichan Lake	N.a.	WFPL	506A	CAN	*Pseudotsuga menziesii*	AM076526	AM076492	AJ557382	AM076436
SH22	UK	N.a.	MUCL	30855	GBR	log, *Larix* sp.	AM076535	AM076502	AJ518088	AM076446
SH23	Denmark	N.a.	MUCL	30856	DEN	*Pinus* sp. post	AM076536	AM076503	AJ557379	AM076447
SH24	Denmark	N.a.	MUCL	30857	DEN	Pole; decayed	AM076537	AM076504	AJ518089	AM076448
SH25	Antwerpen, Schooten (rue André Ullenslei)	G.L. Hennebert	MUCL	31289	BEL	bathroom in house	AM076538	AM076505	AJ518090	AM076449
SH26	Manicaland, Chimanimani area, Muguzo Forest Research Station area	Pascal S., Decock C.	MUCL	38575	ZIM	dead trunk and litter, *Pinus* sp. and *Pinus* litter	AM076539	AM076506	AJ557373	AM076450
SH27	Manicaland, Chimanimani area, Muguzo Forest Research Station area	Pascal S., Decock C.	MUCL	38576	ZIM	dead trunk and litter, *Pinus* sp. and *Pinus* litter	AM076540	AM076507	AM076430	AM076451
SH28	UK	J. Carrey	MUCL	38935	GBR	Soil	AM076541	AM076508	AM076423	AM076452
SH29	Vosges, Col de la Schlucht, Sentier des roches	Decock C.	MUCL	38979	FRA	dead standing trunk, coniferous	AM076542	AM076509	AM076427	AM076453
SH30	Bruxelles, St. Josse	Decock C.	MUCL	39729	BEL	cellar, house	AM076543	AM076510	AM076425	AM076454
SH31	Akershus, Ås, Slørstadskogen	F. Roll-Hansen	NFRI	82-90/7	NOR	*Picea abies*	AM076528	AM076495	N.a.	AM076439

Code	Locality	Collector	Culture collection[1]	Strain number	Country	Substrate	LSU	ITS	tub	hsp
SH96	Maryland, Beltsville	R.W. Davidson H Butin	FSC CFS-Atlantic	FSC-31	USA	Abies sp.	AM076529	AM076496	AM076825	AM076440
SH99	Bursfelde		CBS	383.82	GER	Picea abies	AM076547	AM076514	AM076432	AM076458
SH100	Wilsede	O. Schmidt	Schmidt	P218	GER	Spruce stump	AM076548	AM076515	AM076434	AM076459
SH101	Putten, Schovenhorst	J.A. Stalpers	CBS	302.82	NED	gymnosperm wood	AM076549	AM076516	AM076416	AM076460
SH103	Schleswig-Holstein, Malente	O. Schmidt	Schmidt	P283	GER	Spruce stump	AM076550	AM076517	AM076424	AM076461
SH104	Schleswig-Holstein, Malente	O. Schmidt	Schmidt	P284	GER	Spruce stump	HM135711	HM135661	HM135575	HM135627
SH105	Schleswig-Holstein, Malente	O. Schmidt	Schmidt	P288	GER	Spruce stump	AM076551	AM076518	AM076418	AM076462
SH112	Ceska Trebova	J. Volc	CCBAS	110	CZE	Bric house cellar. Spruce beam	AM076553	AM076520	AM076419	N.a.
SH113	Hedmark: Engerdal: Kvemskjølen SØ.	H. Kauserud	MUCL	46270	NOR	Pinus sylvestris	AM076530	AM076497	AM076414	AM076441
SH114	126 Paradise Valley Rd.	Edwards	NZFS	1436	NZL	ground	AM076554	AM076521	AM076421	AM076465
SH115	Yunman Province, Chu Xiong, Zi Xi Shan Nature Reserve, 2400 m alt.	Decock C.	MUCL	47005	CHN	base of a living trunk, Pinus sp.	HM135703	HM135653	HM135568	HM135620
SH116	Yunman Province, Chu Xiong, Zi Xi Shan Nature Reserve, 2400 m alt.	Decock C.	MUCL	47007	CHN	base of a living trunk, Pinus sp.	HM135712	HM135662	HM135576	HM135628
SH117	Limal	Decock C.	MUCL	47155	BEL	timber, Pinus sp.	HM135713	HM135663	HM135577	HM135629
SH120	Brabant Wallon, Ottignies-Louvain-la-Neuve	Hennebert G.	MUCL	47228	BEL	dead wood in garden, Chamaecyparis sp.	HM135714	HM135664	HM135578	HM135630
SH125	Normandie, Le Tilleul Othon	Gesquière P.	MUCL	47679	FRA	housetimber, Pinus sp.	HM135715	HM135665	HM135579	N.a.
SH127	Yunman, Chu Xiong, Zi Xi Shan Nature Reserve, 2400 m alt	Decock C.)	MUCL	47928	CHN	dead stump, Pinus sp.	HM135704	HM135654	HM135569	HM135621
SH129	Yunman, Chu Xiong, Zi Xi Shan Nature Reserve, 2400 m alt	Decock C.	MUCL	47930	CHN	dead stump, Pinus sp.	HM135701	HM135651	HM135566	HM135618

Code	Locality	Collector	Culture collection[1]	Strain number	Country	Substrate	LSU	ITS	*tub*	*hsp*
SH130	Yunnan, Chu Xiong, Zi Xi Shan Nature Reserve, 2400 m alt	Decock C.	MUCL	47931	CHN	dead stump, *Pinus* sp.	HM135702	HM135652	HM135567	HM135619
SH133	British columbia, Snooke		WFPL	506B	CAN	*Pseudotsuga menziesii*	AM076544	AM076511	AM076415	N.a.
SH134	Ontario, Lake Nipigon	J.R. Hansbrough	CFMR	Bud-1908	CAN	*Picea glauca*	HM135716	HM135666	HM135580	N.a.
SH135	Washington, Lewis; Centralia; Weyerhaeuser Timber Co., Forestry Research Center	K.R. Shea	CFMR	Sh-17	USA	*Picea sitchensis*, 17 yrs old	HM135717	HM135667	HM135581	HM135631
SH136	Nevada; Nye; G Tunnel, Madison Drift Nuclear test site	R.L. Gilbertson	CFMR	RLG-12941-Sp	USA	*Pseudotsuga menziesii*, white rhizomorph near	HM135718	HM135668	HM135582	HM135632
SH137	Ontario; Ft. William; Lake Nipigon, on Black Sturgion Conc. GLP	G. Englerth	CFMR	Bud-205A	CAN	*Picea glauca*	HM135719	HM135669	HM135583	HM135633
SH140	Vermont; Warren; Clay Brook area, Green Mt. Nat'l Forest	P.V. Mook	CFMR	PVM-52B-R	USA	*Picea rubens*, 293 yrs old	HM135721	HM135671	HM135585	N.a.
SH139	Munchen, Forstbotanisches Institute	F.F. Lombard	CFMR	R-1	GER	*Picea*, brown and red stain study	HM135720	HM135670	HM135584	HM135634
SH141	Colorado, Bears Ears Ranger District, Routt Nat'l Forest	T.E. Hinds	CFMR	Colo-59-1650	USA	*Picea engelmannii*	HM135705	HM135655	HM135570	HM135622
SH142	Oregon	R. Graham	CFMR	MD-110	USA	*Alnus* stake	HM135722	HM135672	HM135586	N.a.
SH144	New York; Warren; Warrensburg	J.H. Ginns	CFMR	JHG-459-Sp	USA	*Tsuga canadensis*	HM135723	HM135673	HM135587	HM135635
SH147	Arizona, Portal, Rustler Park, Coronado Nat'l Forest	J.L. Lowe & R.L. Gilbertson	CFMR	L-9729-Sp	USA	*Pinus*	HM135724	HM135674	HM135588	HM135636
SH148	Arizona, Palisades, Santa Catalina Mts, Coronado Nat'l Forest	R.L. Gilbertson	CFMR	RLG-11350-Sp	USA	*Pinus ponderosa*	HM135725	HM135675	HM135589	HM135637
SH150	Georgia, Athens, Oconee National Forest	W.A. Campbell	CFMR	FP-94342-R	USA	*Pinus echinata*	HM135726	HM135676	HM135590	HM135638

Code	Locality	Collector	Culture collection[1]	Strain number	Country	Substrate	LSU	ITS	*tub*	*hsp*
SH152	Maryland, Beltsville	R.W. Davidson	CFMR	FP-97366-Sp	USA	*Pinus*	HM135727	HM135677	HM135591	N.a.
SH154	Maryland, Beltsville, Plant industrial station	R.W. Davidson	CFMR	FP-97367-R	USA	*Pinus virginiana*	HM135728	HM135678	HM135592	N.a.
SH155	Maryland, Beltsville, Plant industrial station	R.W. Davidson	CFMR	FP-97439-Sp	USA	*Pinus virginiana*	HM135729	HM135679	HM135593	HM135639
SH156	Maine, Kittern	J.D. Diller	CFMR	FP-97448-Sp	USA	Soil	HM135730	HM135680	HM135594	HM135640
SH157	Maryland, Holiday beech	H.H. McKay	CFMR	FP-104042-T	USA	*Eucalyptus* wood stakes	HM135731	HM135681	HM135595	HM135641
SH158	Maryland, Beltsville	J.D. Diller	CFMR	FP-104137-T	USA	*Pinus* leaves, twigs	HM135732	HM135682	HM135596	HM135642
SH159	Maryland, Beltsville	R.W. Davidson	CFMR	FP-104361-Sp	USA	Hardwood	HM135733	HM135683	HM135597	HM135643
SH160	Maryland, Beltsville	R.W. Davidson	CFMR	FP-104397-Sp	USA	Hardwood	HM135734	HM135684	HM135598	N.a.
SH161	Maryland, Beltsville	R.W. Davidson	CFMR	FP-104405-Sp	USA	Hardwood	HM135735	HM135685	HM135599	N.a.
SH162	Maryland, Beltsville	R.W. Davidson	CFMR	FP-104572-Sp	USA	*Pinus*	HM135736	HM135686	HM135600	HM135644
SH163	Oregon, Hungry Horse, Coram Expt Forest	M.J. Larsen	CFMR	FP-134010-Sp	USA	Conifer	HM135737	HM135687	HM135601	HM135645
SH164	Alaska, North side of Kenai Lake, Kenai Peninsula	N.a.	CFMR	HHB-17587-Sp	USA	*Picea glauca*	AF518648	AM076493	AM076412	AM076437
SH167	Yunnan, Chu Xiong, Zi Xi Shan Nature Reserve	Decock C.	MUCL	46914	CHN	dead wood, *Pinus* sp.	HM135738	HM135688	HM135602	HM135646
SH168	Concepción	Goetz Palfner	CONC-F	323-2	CHI	From house	HM135699	HM135649	HM135564	HM135616
SH169	Concepción	Goetz Palfner	CONC-F	324-1	CHI	From house	HM135700	HM135650	HM135565	HM135617

Code	Locality	Collector	Culture collection[1]	Strain number	Country	Substrate	LSU	ITS	*tub*	*hsp*
SH170	Concepción	Goetz Palfner	CONC-F	324-2	CHI	From house	HM135739	HM135689	HM135603	N.a.
SH171	Tierra del Fuego, Depto. Ushaia, El Valdez	A. Greslebin	CIEFAP	388	ARG	Fallen trunk of *Nothofagus pumilio*	HM135740	HM135690	HM135604	N.a.
SH172	Tierra del Fuego, Depto. Ushaia, Estancia Moat	A. Greslebin	CIEFAP	1517	ARG	Fallen trunk of *Nothofagus betuloides* "guindo"	HM135741	HM135691	HM135605	N.a.
SH175	Durango	C. Decock and R. Valenzuela	MUCL	52396	MEX	soil and buried wood	HM159424	HM146135	HM159426	HM159422
SH176	Durango	C. Decock and R. Valenzuela	MUCL	52397	MEX	soil and buried wood	HM159425	HM146136	HM159427	HM159423
SH181	S. Jutland, Froslev Plantage	Hallenberg	Hallenberg	2024PS	DEN	*Picea* stump	AM076555	AM076522	AM076428	AM076466

1 CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, CCBAS = Culture Collection of Basidiomycetes, Pragua, Czech republic, CFMR = Center For Forest Mycology Research, Forest Products Laboratory, USA, CIEFAP = Centro de Investigación y Extensión Forestal Andino Patagónico, Argentina, CONC-F = From G. Palfner, Concepción, Chile, FSC = Fredericton Stock Culture Collection (Now Atlantic Forestry Centre, Fredericton Canada), Hallenberg = From N. Hallenberg, Gothenburg, Sweden, MUCL = Mycothèque de l'Université catholique de Louvain, Belgium, NZFS = New Zealand Forest Research Institute, NFRI = Norwegian Forest and Landscape Institute, Norway, Schmidt = From O. Schmidt, Hamburg Germany, WFPL = Western Forest Products Laboratory (Now Forintec).
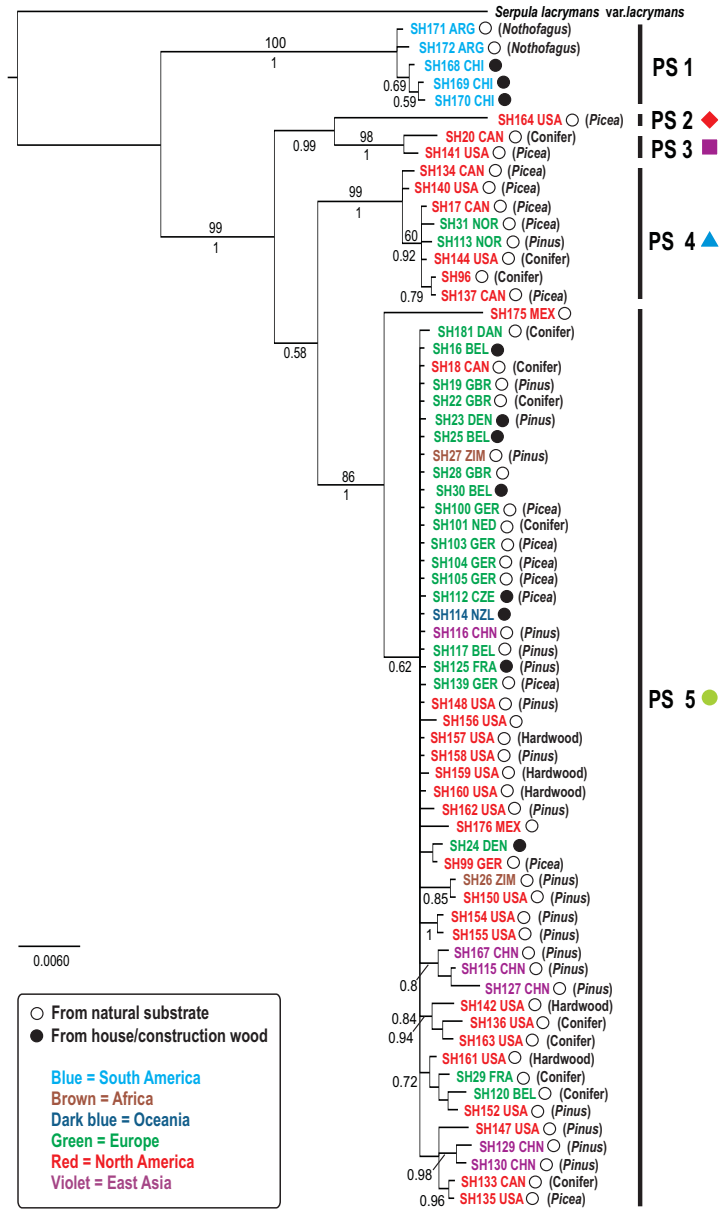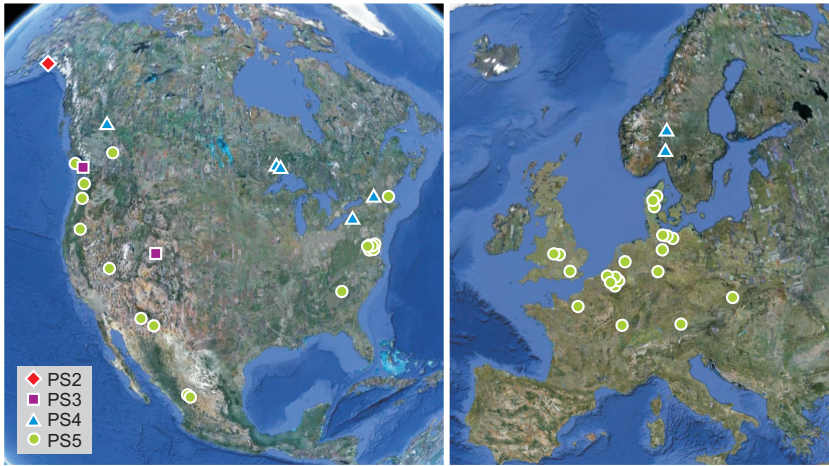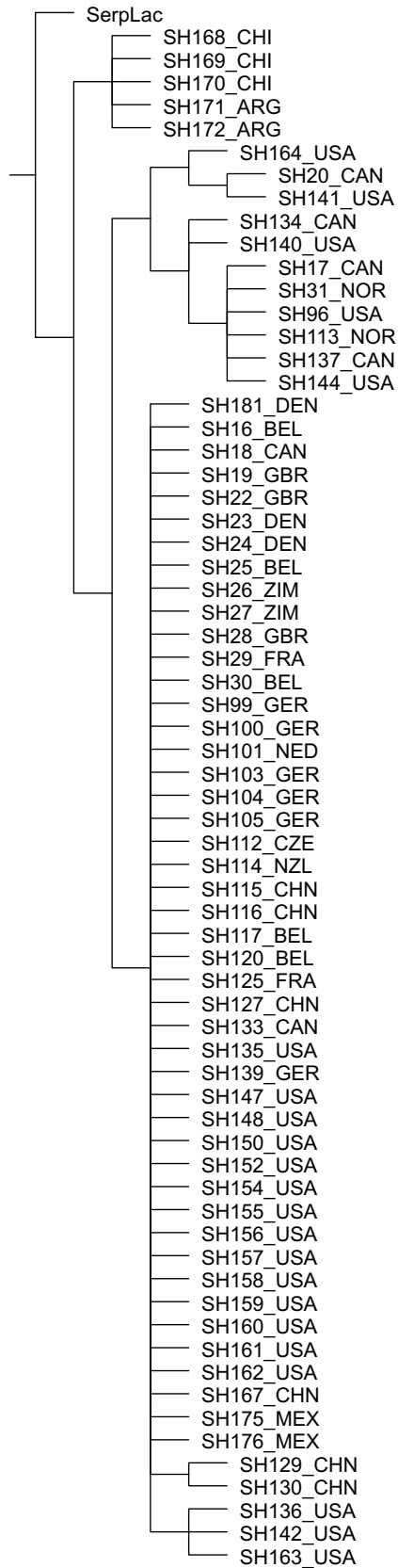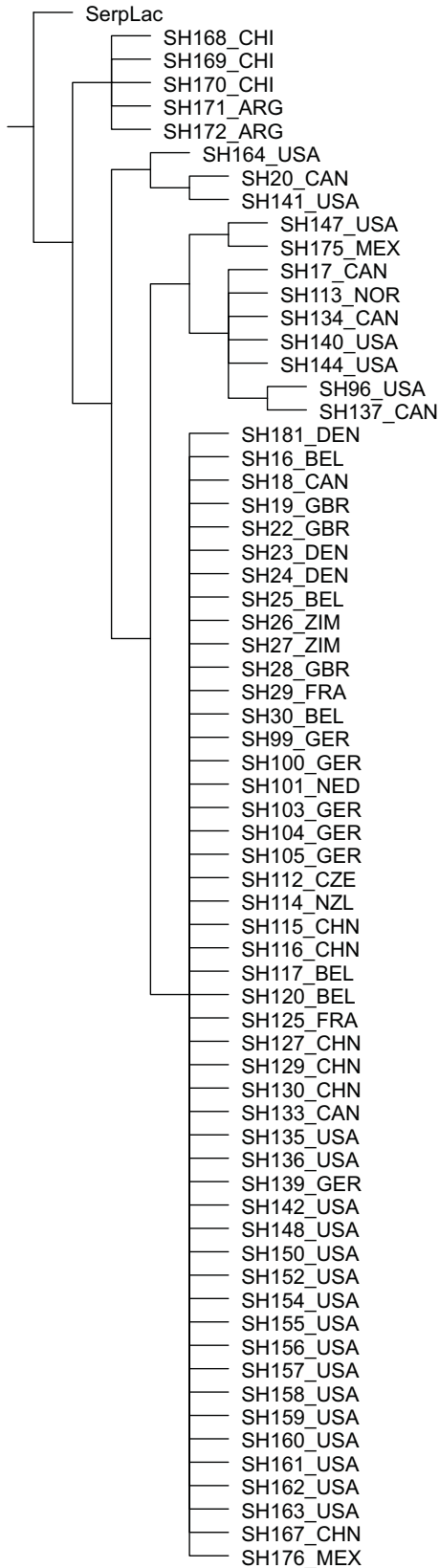
Fig. 1

Fig. 2

Supplementary figure 1A Strict consensus of ribosomal sequences

SerpLac
SH168_CHI
SH169_CHI
SH170_CHI
SH171_ARG
SH172_ARG
SH164_USA
SH20_CAN
SH141_USA
SH134_CAN
SH140_USA
SH17_CAN
SH31_NOR
SH96_USA
SH113_NOR
SH137_CAN
SH144_USA
SH181_DEN
SH16_BEL
SH18_CAN
SH19_GBR
SH22_GBR
SH23_DEN
SH24_DEN
SH25_BEL
SH26_ZIM
SH27_ZIM
SH28_GBR
SH29_FRA
SH30_BEL
SH99_GER
SH100_GER
SH101_NED
SH103_GER
SH104_GER
SH105_GER
SH112_CZE
SH114_NZL
SH115_CHN
SH116_CHN
SH117_BEL
SH120_BEL
SH125_FRA
SH127_CHN
SH133_CAN
SH135_USA
SH139_GER
SH147_USA
SH148_USA
SH150_USA
SH152_USA
SH154_USA
SH155_USA
SH156_USA
SH157_USA
SH158_USA
SH159_USA
SH160_USA
SH161_USA
SH162_USA
SH167_CHN
SH175_MEX
SH176_MEX
SH129_CHN
SH130_CHN
SH136_USA
SH142_USA
SH163_USA

Supplementary figure 1B Strict consensus of *tub* sequences

Supplementary figure 1C Strict consensus of *hsp* sequences

SerpLac
SH168_CHI
SH169_CHI
SH164_USA
SH20_CAN
SH141_USA
SH17_CAN
SH31_NOR
SH96_USA
SH113_NOR
SH137_CAN
SH144_USA
SH181_DEN
SH16_BEL
SH18_CAN
SH19_GBR
SH22_GBR
SH23_DEN
SH24_DEN
SH25_BEL
SH26_ZIM
SH27_ZIM
SH28_GBR
SH29_FRA
SH30_BEL
SH99_GER
SH100_GER
SH101_NED
SH103_GER
SH104_GER
SH105_GER
SH114_NZL
SH115_CHN
SH116_CHN
SH117_BEL
SH120_BEL
SH127_CHN
SH129_CHN
SH130_CHN
SH135_USA
SH136_USA
SH139_GER
SH147_USA
SH148_USA
SH150_USA
SH152_USA
SH155_USA
SH156_USA
SH157_USA
SH158_USA
SH159_USA
SH162_USA
SH163_USA
SH167_CHN
SH175_MEX
SH176_MEX

III

IV

V

BMC
Genetics

RESEARCH ARTICLE

High variability in a mating type linked region in the dry rot fungus *Serpula lacrymans* caused by frequency-dependent selection?

Ingeborg Bjorvand Engh[1], Inger Skrede[1], Glenn-Peter Sætre[2] and Håvard Kauserud*[1]

Abstract

Background: The mating type loci that govern the mating process in fungi are thought to be influenced by negative frequency-dependent selection due to rare allele advantage. In this study we used a mating type linked DNA marker as a proxy to indirectly study the allelic richness and geographic distribution of mating types of one mating type locus (MAT A) in worldwide populations of the dry rot fungus *Serpula lacrymans*. This fungus, which causes serious destruction to wooden constructions in temperate regions worldwide, has recently expanded its geographic range with a concomitant genetic bottleneck.

Results: High allelic richness and molecular variation was detected in the mating type linked marker as compared to other presumably neutral markers. Comparable amounts of genetic variation appeared in the mating type linked marker in populations from nature and buildings, which contrast the pattern observed with neutral genetic markers where natural populations were far more variable. Some geographic structuring of the allelic variation in the mating type linked marker appeared, but far less than that observed with neutral markers. In founder populations of *S. lacrymans*, alleles co-occurring in heterokaryotic individuals were more divergent than expected by chance, which agrees with the expectation for populations where few mating alleles exists. The analyzed DNA marker displays trans-species polymorphism wherein some alleles from the closely related species *S. himantoides* are more similar to those of *S. lacrymans* than other alleles from *S. himantoides*.

Conclusions: Our results support the idea that strong negative frequency-dependent selection maintains high levels of genetic variation in MAT-linked genomic regions, even in recently bottlenecked populations of *S. lacrymans*.

Background

A high allelic richness is maintained in some genetic loci due to negative frequency-dependent selection caused by rare allele advantage, which counteracts the effect of genetic drift (reviewed by Richman 2000). The MHC (Major Histocompatibility Complex) system of animals and the SI (self incompatibility) system in plants are well-known examples [1]. The mating type (MAT) loci of fungi, which governs the mating process, are also thought to be influenced by negative frequency-dependent selection because haploid mycelia possessing rare MAT alleles have higher chances for mating compared to those possessing frequent alleles [2,3].

Different types of mating systems occur in the fungal kingdom. Most basidiomycetes have a tetrapolar mating system where two separate gene complexes, MAT A and MAT B, govern the mating process. MAT A encodes homeodomain transcription factors, MAT-B encodes pheromones and pheromone receptors, and together they control mate recognition, clamp connection formation and pairing of nuclei in the formation of dikaryotic mycelium [4]. For mating to occur in tetrapolar species, different allelic versions must be present at both mating type loci. In homobasidiomycetes (i.e. basidiomycetes with a non-divided basidium), multiple alleles exist in the mating type loci and negative frequency-dependent selection can promote maintenance of a high richness of MAT alleles in populations of these fungi [5,6]. This rare allele advantage may also lead to 'trans-specific polymorphisms' because of extended coalescence times between

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alleles [7]. By classical mating studies it has been shown that a large number of MAT alleles are present in some taxa. In *Schizophyllum commune*, it has been estimated that around 160 A mating types exist in nature [5], while in *Coprinopsis cinerea*, the number of A mating type alleles was estimated to be 100 [8]. Although this high allelic diversity suggests that MAT would be an excellent target for the development of population genetic markers, MAT alleles have so far not been used in population genetics studies of Agaricomycetes (Basidiomycota). This is likely due to the fact that MAT alleles themselves are highly divergent in sequence, making it difficult to design universal primers.

It has been demonstrated that a conserved gene order (shared synteny) exists between the mating type genes and neighbouring genes in most Agaricomycetes [9]. One such locus is the gene encoding mitochondrial intermediate peptidase (*mip*), located close to the MAT A locus (less than 1 kb for *S. commune*, *Coprinopsis cinerea* and *C. scobicola*) in Agaricomycetes investigated [10,11]. Because of physical linkage, we may expect that this gene is indirectly affected by negative frequency-dependent selection acting on MAT, and therefore that high allelic richness will be maintained also in the *mip* region due to linkage disequilibrium. Accordingly, instead of targeting the mating types directly, which poses technical difficulties due to massive molecular divergence, targeting neighbouring 'non-mating type MAT-linked genes', such as *mip*, may yield proxies for analysing the allelic richness of mating types [9] and a source for highly variable markers.

In this study we analyse a genetic marker covering a portion of the *mip* gene, a neighbouring spacer region and the 3'-prime end of the mating type gene, homeodomain 1 (HD1), as a proxy to analyse the richness of mating types (MAT A) within the dry rot fungus *Serpula lacrymans* (Boletales, Basidiomycota). *Serpula lacrymans* is the most damaging destroyer of wood constructions in temperate regions. By using various presumably neutral genetic markers, we have previously shown that the dry rot fungus is divided into two main lineages that probably represent different species; one non-aggressive residing naturally in North America and Asia (var. *shastensis*), and another aggressive lineage appearing on all continents (var. *lacrymans*) [12]. Genetic analyses pinpoint mainland Asia as the origin of the aggressive form var. *lacrymans*, from where it has migrated worldwide to Europe, North- and South America and Oceania followed by local population expansions [12]. This recently spread lineage of var. *lacrymans*, probably spread by man in historic time, is known as 'the Cosmopolitan group' [12]. Little genetic variation occurs in the bottlenecked founder populations of var. *lacrymans* worldwide, while more genetic variation is found in the source population in mainland

Asia, as well as in var. *shastensis* [12,13]. In accordance with this inferred massive recent expansion from a much smaller founder population, only a few (6) vegetative compatibility types (VC-types) have been detected in the European genetically depleted population of var. *lacrymans* [14]. Vegetative compatibility is a self-nonself recognition system used to separate own mycelium from other mycelia. Normally, one VC-type corresponds to one genet, but in genetically depleted populations, like in *S. lacrymans*, different genets can belong to the same VC-type. Higher genetic variation and a correspondingly higher number of VC-types have been detected in the Japanese indoor population, which probably was founded during an independent founder event from the Asian source population [13]. The morphospecies *Serpula himantioides*, which is used as outgroup in our analyses, is the sister taxon to *S. lacrymans* and includes several subgroups that probably represent independent ('cryptic') species [12].

The aims of the present study are to use a non-mating type MAT-linked marker as a proxy to indirectly study the allelic richness of mating type A in a worldwide sample of the genetically deprived *S. lacrymans*, and to investigate whether the level of molecular variation at the MAT-linked loci is consistent with negative frequency-dependent selection. Furthermore, we analyse the allelic richness in a geographic context and investigate whether more variation occurs in natural, outdoor populations of var. *shastensis* and var. *lacrymans* compared to the founder populations of var. *lacrymans* that strictly appears in buildings. Finally, we evaluate whether the mating type linked marker can be used to separate closely related isolates of var. *lacrymans*.

Methods
Material
A total of 83 cultures and dried specimens of *S. lacrymans* and *S. himantioides* were included in this study (see additional file 1: Information about the analyzed material). DNA was extracted following a 2% CTAB (cetyl trimethylammonium bromide) miniprep method described by [15] with minor modifications: DNA was resuspended in 100 μL distilled sterile H_2O at the final step of extraction.

PCR
We first tested the primers MIP1F and MIP1R [16] on different *Serpula* strains. This primer set has successfully been used to amplify a part of the non-mating type MAT-linked *mip* gene in various other fungi [16]. Positive amplicons were obtained from a few isolates only (probably due to primer mismatch). Based on an alignment including partial *mip* sequences from three Boletales species [16], as well as three *Serpula* sequences obtained

using the MIP1 primer set, we designed the new primers mip60F (GGMAAYCAYCACGAAGAYCC) and mip190F (TTCAGCCATCTATTYGGGTACGG). In order to maximize sequence length we employed an uneven PCR approach as described in [17], combining the primers mip60F and mip190F and twelve different RAPD primers [17]. Positive amplicons from different primer combinations were sequenced, and finally the new primers mip55R (GCGGACAAACAAGCAAAGTT) and mip82R (CTGAAGATGCTGGAGGAAGC) were designed based on the resulting alignments and further combined with mip60F and mip190F to amplify the partial *mip* region from all included isolates and specimens (Table 1). PCR amplification with primers mip60F or mip190F in combi-

nation with primers mip55R or mip82R was performed with the proofreading enzyme Dynazyme EXT DNA Polymerase (Finnzymes) with reactions containing 16.5 µl of 100× diluted template DNA, 1.5 µl each of forward and reverse primers (5 µM stocks), 2.5 µl of dNTPs (2 µM stock), 2.5 µl of Dynazyme EXT 10× reaction buffer, 0.5 µl Dynazyme EXT DNA Polymerase (25 µl total reaction volume). PCR reactions with Dynazyme EXT were performed with the following protocol: 2 min at 94°C; followed by 35 cycles of 30 s at 94°C, 45 s at 54°C, and 1 min at 72°C; followed by a 7 min extension at 72°C and an indefinite hold at 4°C. Based on the two genome sequences of *Serpula lacrymans* S7.3 and S7.9 (U.S. Government Department of Energy - Joint Genome Insitute)

Table 1: Information about molecular variation in the four sequenced loci of *Serpula lacrymans*.

Locus	Group	Ecol[1]	#	S	k	π	Theta W	Tajima's D	Fu and Li's D*	Fu and Li's F*
MAT linked marker	*S. lacrymans*	N+B	116	182	19.4	0.048	34.17	-1.74	-3.45*	-3.22*
	var. *lacrymans*	N+B	95	183	23.1	0.049	35.70	-1.48	-3.42*	-3.10*
	var. *shastensis*	N	21	170	34.5	0.051	47.25	-1.37	-1.77	-1.93
	var. *lacr.* Asia	N	16	158	45.09	0.062	47.62	0.43	0.64	0.67
	var. *lacr.* Japan	B	27	132	25.76	0.049	34.25	-1.22	-1.28	-1.49
	var. *lacr.* Cosmo.	B	51	213	38.91	0.057	47.34	-0.9	-2.61*	-2.34
ITS	*S. lacrymans*	N+B	150	11	2.2	0.004	1.97	0.32	0.32	0.67
	var. *lacrymans*	N+B	126	3	0.6	0.001	0.56	0.18	-0.64	-0.44
	var. *shastensis*	N	24	2	0.3	0.0006	0.54	-0.89	0.84	0.42
	var. *lacr.* Asia	N	20	1	0.51	0.0009	0.28	1.43	0.65	0.98
	var. *lacr.* Japan	B	32	1	0.42	0.0007	0.25	1.04	0.59	0.82
	var. *lacr.* Cosmo.	B	74	2	0.05	0.0001	0.41	-1.42	- 2.71*	-2.71*
gpd	*S. lacrymans*	N+B	122	107	5.4	0.007	19.90	-2.36**	2.36*	0.38
	var. *lacrymans*	N+B	98	7	1.5	0.002	1.36	0.21	-0.55	-0.55
	var. *shastensis*	N	24	106	18.1	0.022	28.39	-1.45	1.84*	0.94
	var. *lacr.* Asia	N	8	5	1.61	0.0020	1.93	-0.76	-0.49	-0.61
	var. *lacr.* Japan	B	32	6	1.38	0.0017	1.49	-0.21	-1.15	-1.01
	var. *lacr.* Cosmo.	B	58	0	-	-	-	-	-	-
tub	*S. lacrymans*	N+B	146	30	7.07	0.018	5.40	0.90	1.25	1.34
	var. *lacrymans*	N+B	122	1	0.14	0.0004	0.19	-0.29	0.48	0.29
	var. *shastensis*	N	24	6	1.30	0.003	1.61	-0.57	-0.24	-0.39
	var. *lacr.* Asia	N	18	1	0.21	0.0005	0.29	-0.53	0.67	0.40
	var. *lacr.* Japan	B	32	1	0.35	0.0008	0.25	0.64	0.59	0.69
	var. *lacr.* Cosmo.	B	72	0	-	-	-	-	-	-

The information about ecology, molecular variation and deviations from neutral evolution in the four analyzed sequence loci are given for different sub-groups of *Serpula lacrymans*.
[1]N = nature, B = building, # indicates number of investigated sequences, S = number of segregating sites, k = average number of nucleotide differences, π = nucleotide diversity.

a new primer (Mip_ins3R; ACTCCGCTGAAGTCCAC-CTGC) was designed in an insert occurring in some allelic versions of the mating type linked marker. Mip_ins3R was combined with mip190F to reveal the presence of this insert (see below) using the same PCR conditions as above. We sequenced nine amplicons directly to assess the homology of the insert.

Cloning and sequencing

We used a cloning procedure to separate different *mip* alleles co-amplified from the heterokaryotic isolates and specimens. Fragments were cloned with the TOPO TA Cloning kit (Invitrogen) using blue/white screening according to the manufacturer's manual. Positive colonies were subjected to direct PCR with the M13R/T7 primers with the same PCR conditions as described above. The resulting amplicons were sequenced using an ABI 3730 DNA analyser (Applied Biosystems, Foster City). We aligned the cloned sequences from each isolate/specimen manually in separate alignments using BioEdit 7 [18] (from three to twenty, see additional file 1: Information about the analyzed material), and two divergent alleles (when present) were separated. It is well known that artificial mutations and chimeric sequences can be obtained when using a clone-based approach. When more than two copies of each allele were present in the alignment, we considered polymorphisms occurring only within one single sequence (being 'autapomorphic') as mutations generated *in vitro* during PCR and discarded these. When only two copies of each allele were present we assumed heterozygosity at the sites in which the two copies differed. When only one allelic copy was present this version was accepted as is. Sequences from the internal transcribed spacer (ITS) nrDNA region and parts of the beta tubulin (*tub*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) genes have previously been obtained from the isolates/specimens as specified in [12]. These sequences were used as comparisons with the *mip*-sequences analysed here. All sequences have been deposited in GenBank under the accession numbers given in additional file 1: Information about the analyzed material.

Phylogenetic and statistical analyses

We established sequence alignments for the mating type linked marker *mip*, as well as ITS, *gpd* and *tub* and ran phylogenetic analyses on the two first datasets. 'Best-fit' evolutionary models were estimated for all analyses using the Akaike information criterion (AIC) as implemented in MrModeltest 2.3 [19]. The SYM+I+G model was specified as prior for the MAT linked data set while the HKY+I model for the ITS data set. Posterior probabilities were determined by MrBayes [20,21] twice by running one cold and four heated chains for 10×10^6 generations in parallel mode, saving trees every 1000th generation. A

50% majority rule consensus tree was used to calculate posterior probabilities including the proportion of trees gathered after the convergence of likelihood scores was reached. We run parsimony analyses using TNT [22], where Jackknife support values [23] were obtained using 1000 replicates. Jackknife support values above 50 were superimposed on the consensus tree from MrBayes.

Due to the dikaryotic stage of the isolates/specimens, heterozygous sites (with two nucleotides in same position) appeared in many of the *gpd*, ITS and *tub* sequences. In order to use the information in the heterozygous sequence sites and calculate more accurate estimates of molecular variation for these regions, haplotype datasets were constructed for the different DNA regions. For example, in a DNA sequence ('genotype') containing an 'Y' (=C/T) the two resulting sequence haplotypes will include either a 'C' or a 'T'. In sequences with more than one heterozygous site, the heterozygous phase was inferred using a procedure yielding the minimum number of alleles. In short, sequences homozygous at all sites or heterozygous at only one site (known putative haplotypes) are used as templates for inferring phase of sequences with multiple heterozygous sites. Hence, sequences with two or more heterozygous sites are, whenever possible, assigned to known putative haplotypes found in the sample of sequences. Alternatively, haplotypes requiring a minimum number of mutational steps are inferred. The procedure may underestimate the number of haplotypes if recombinant genotypes occur. Notably, descriptive statistics of level of polymorphism (such as π and k), as well as tests of neutrality (Tajima's D and Fu & Li's F and D tests) will not be affected since these statistics are based on polymorphic sites per se and not haplotypes. In ITS no more than one heterozygous site appeared per sequence. Nucleotide diversity π (average number of nucleotide differences per site), *k* (average number of nucleotide differences per sequence per site) and estimates of the population mutation parameter theta *θ* were calculated for each of the four sequenced loci using the program DnaSP version 4.50.2 [24]. To test for deviation from neutral evolution, we performed Fu and Li's D, Fu and Li's F and Tajima's D tests using DnaSP. The HKA test [25] was performed with the program HKA for all four loci (published by Jody Hey; http://lifesci.rutgers.edu/~heylab/ProgramsandData/Programs/HKA/HKA_Documentation.htm) with 1000 simulations.

To test whether the two alleles co-amplified from a single individual were more divergent than expected by chance, we calculated pairwise similarities of all alleles using Bioedit. This matrix was then used to calculate the mean similarity of alleles occurring within individuals, which was compared to the mean similarity of alleles across individuals, by a two-sample unequal variance t test using the software R [26]. This test was done inde-

pendently for different groups of isolates; all isolates of *S. lacrymans* var. *lacrymans*, the cosmopolitan (Europe + North America + Oceania) group of var. *lacrymans*, European isolates of var. *lacrymans*, Japanese isolates of var. *lacrymans*, and all isolates of *S. lacrymans* var. *shastensis*.

Results and discussion

The MAT-linked marker

The sequences we obtained from the MAT-linked marker ranged from 718 to 810 bp. In the sequence alignment (860 bp), the first 285 positions constituted the 3' end of the *mip* gene, positions 286-832 a spacer region, and 833-860 the 3' end of the HD1 gene of MAT A. A total of 126 sequences of the MAT-linked marker were obtained from the 83 analysed dikaryotic isolates/specimens. In a dikaryotic basidiomycete it is expected that two different MAT A alleles occur in each isolate/specimen, but we obtained two alleles from only 53% of the analysed dikaryons. This could be due to (i) failure to detect both alleles due to limited number of cloned fragments (6-20) from each isolate, (ii) primer mismatch (or other reasons for PCR amplification failure), or (iii) that different MAT alleles share the same mating type linked marker (e.g. due to recombination and/or that the linked marker is more conserved than the adjacent MAT alleles). Notably, a comparison of the recently analysed genome sequences of *Serpula lacrymans* var. *lacrymans* (i.e. the two sequenced homokaryons S7.3 and S7.9, obtained from the same spore family), revealed that a large insertion (~18 kbp) occurred between *mip* and the mating type locus in one of the genomes (S7.9). Thus, a plausible explanation for why only one allele was amplified in a large proportion of the isolates is that this insertion occurs in many of the analysed isolates. Indeed, PCR amplification with one primer located within and one outside the insert gave positive amplicons from isolates where only one allele originally was obtained. Surprisingly, from two of the isolates we obtained three and four different alleles (see below).

Molecular and allelic variation

We found higher molecular variation in the mating type linked marker compared to the other sequenced loci (Table 1). Fig. 1 shows higher allelic variation in the mating type linked marker than in the ITS region. In the phylogenetic tree obtained from the mating type linked marker, the samples of var. *lacrymans* from Europe, North-America and Oceania (i.e. the 'Cosmopolitan group' [12], and the Japanese population appeared to a large extent intermixed on several branches. This is in contrast to the ITS tree (Fig. 1), as well as trees based on other analysed markers [12] where the Japanese and Cosmopolitan samples appear in two quite distinct groups.

Furthermore, a comparable amount of genetic variation appeared in the mating type linked marker in populations of *S. lacrymans* from nature and buildings. This is in stark contrast to the pattern observed using neutral genetic markers, where natural populations were far more genetically variable than those from buildings (Table 1). The high level of genetic variation at the mating type linked marker compared to the presumably neutrally evolving markers may be explained by negative frequency-dependent selection at the MAT A locus maintaining genetic variation also in the vicinity of the locus under selection. In accordance with this hypothesis the results from the HKA test showed that there was significant deviation from neutral evolution when we compared the mating type linked marker with the presumably neutral markers ITS, *tub* or *gpd*. This test was based on intra- and inter-specific divergence within and between *S. lacrymans* var. *shastensis* and var. *lacrymans*. Balancing selection, such as negative frequency-dependent selection, will often tend to keep alleles at intermediate frequencies. Accordingly, rare alleles will tend to be underrepresented compared to neutral expectation. Thus neutrality tests, such as Tajima's D will often yield positive test statistics at such loci. In contrast, the test statistics of Tajima's D, Fu and Li's D (Table 1) were negative at the mating type linked marker in the populations investigated here. In a previous study we found strong signals for a recent population expansion in these fungi [12], a demographic process that usually would affect the test-statistics of these neutrality tests in the opposite direction compared to balancing selection. Hence, we suggest that demographic effects may override the expected signal from balancing selection in this case.

Negative frequency-dependent selection counteracts the sorting effect of genetic drift, and may maintain a high level of molecular and allelic variation also in neighbouring linked regions. Previous studies has shown that tight genetic linkage to loci subjected to strong negative frequency-dependent selection will maintain high levels of polymorphism also at otherwise neutrally evolving loci [27,28]. Thus, we find it likely that the high level of genetic variation at our mating type linked marker is due to associated effects of negative frequency-dependent selection at MAT A, and not because it itself is subject to negative frequency-dependent selection itself.

Species phylogeny

Although we find high levels of molecular variation at the mating type linked marker, the alleles largely group in accordance with known species delimitations [12]. All alleles obtained from the *S. himantioides* morphospecies cluster together (Fig. 1a) and all alleles from var. *lacrymans*, except a few trans-species polymorphisms (see below) occur in one group. However, alleles from the var.
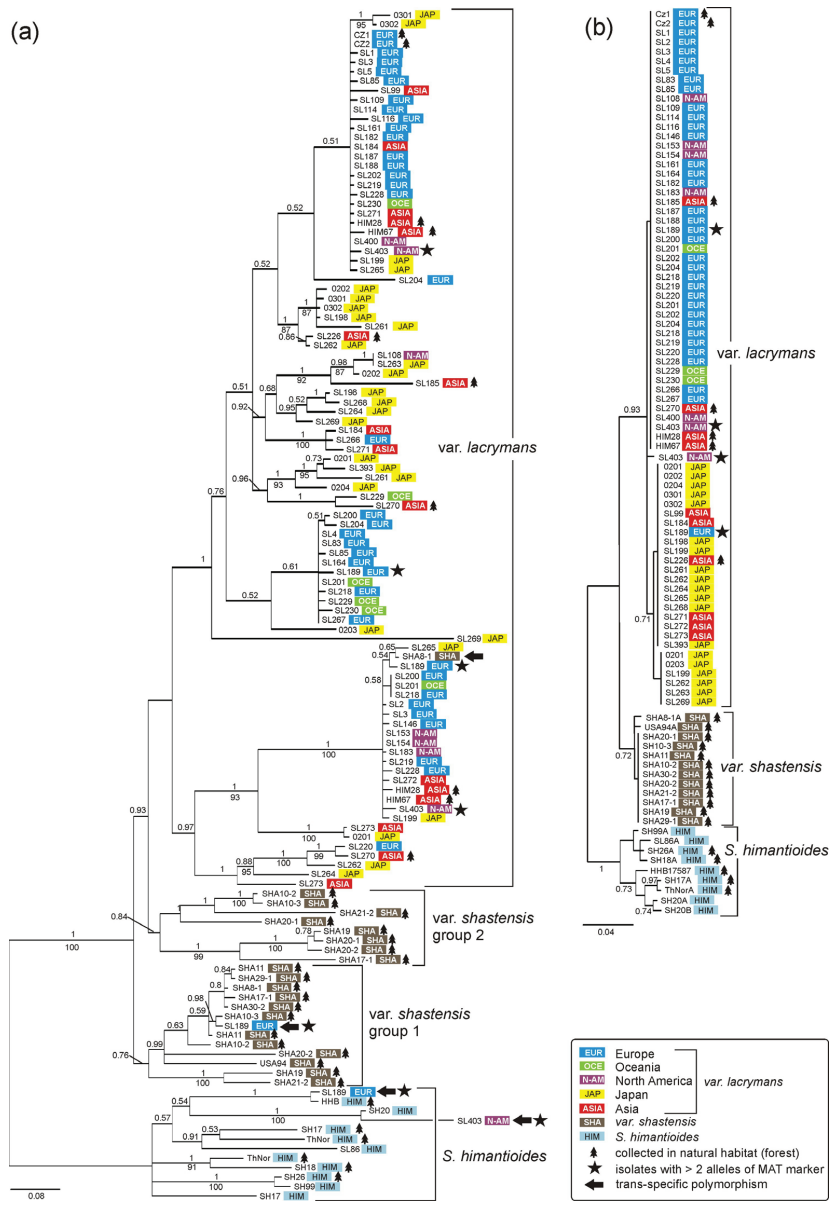
Figure 1 Phylogenies of the MAT A linked marker and ITS. 50% majority rule consensus trees obtained from Bayesian analyses of (a) the MAT A linked marker data sets, and (b) the ITS region. In both datasets DNA sequences were obtained from 83 isolates representing *S. lacrymans* and *S. himantioides*. Bayesian posterior probabilities above 0.50 are given above branches and Jackknife support values (1000 replicates) above 50 are given below branches. The tree symbols indicate isolates derived from a natural habitat (forest), otherwise the isolates were obtained from buildings. The star symbols indicate isolates with more than two alleles of the mating type linked marker while the arrows pinpoints trans-specific polymorphisms.

shastensis group separate into two different sister groups. Four trans-specific polymorphisms are observed, indicated by arrows in Fig. 1a. One sequence derived from a var. *shastensis* isolate (SHA8-1) clusters within the var. *lacrymans* group. On the opposite, one sequence obtained from a European var. *lacrymans* isolate (SL189) clusters within var. *shastensis* and another sequence obtained from the same isolate (SL189) clusters within *S. himantioides*. Lastly, one North American isolate of var. *lacrymans* (SL403) groups within *S. himantioides*. Notably, from SL189 and SL403, four and three different alleles were obtained (indicated by stars in Fig. 1a). The very same isolates are heterozygous at ITS and include two divergent haplotypes (Fig. 1b), of which one has a divergent placement according to geographic origin. One SL189 ITS allele clusters with Asian isolates and SL403 has a unique ITS haplotype. These peculiar observations can be explained in various ways. Ancient gene duplications events might be maintained in the genome of some or all isolates. If so, it should be expected that the trans-specific alleles cluster as more early diverging lineages in the respective groups. Alternatively, SL189 and SL403 could represent multiple heterokaryotic hybrids, including more than two karyotypes, which may account for the high number of alleles and divergent ITS haplotypes. It has been documented that the mycelia of other basidiomycetes can include more than two different nuclei [29]. However, it seems rather unlikely that the German isolate SL189 has acquired a copy from var. *shastensis* that mainly lives in California. More research is definitively needed to investigate possible scenarios for these puzzling observations.

Geography

We would expect relatively little correspondence between geographic and genetic distances in a DNA region linked to a locus under strong selection [30]. However, some geographic structure can be observed in Fig. 1a. The European sequences clustered mainly into three main groups that might correspond to three MAT A alleles (see discussion below). Notably, within these three groups the sequences were either identical or very similar, only including some autapomorphic mutations. The distribution of European alleles corresponds well with other results indicating that the European population has recently expanded [13]. Several strictly Japanese subgroups appeared as well. Notably, the Japanese alleles were in general more divergent than the European ones. This also corresponds well with the observation that higher genetic variation occurs in Japan in microsatellites, AFLPs and four sequenced loci [12,13]. Interestingly, alleles from the Asian mainland population, where var. *lacrymans* also has a natural distribution in forests, were distributed throughout the var. *lacrymans* part of

the tree. In [12] it was observed that the natural Asian population included most of the genetic variation observed in the indoor founder populations, and this seems also to be the case for the MAT A-linked alleles.

Dikaryons

In cases where two alleles of the mating type linked marker were amplified from the same dikaryon, these invariably turned out to be divergent, which is to be expected due to the linkage to MAT A. We found the two alleles of dikaryons to be significantly more divergent than expected by chance in the European population (p-value = 0.003), in the Cosmopolitan group (p-value = 0.010) and in all samples of var. *lacrymans* taken together (p-value = 0.017). However, we found no significant elevation of divergence in the Japanese population when analysed alone (p-value = 0.905), nor in var. *shastensis* (p-value = 0.678). These differences between the populations may be due to different number of mating type alleles present in the various groups. When a high number of mating types occur in a genetically variable population, which is likely to be the case for the Japanese population and var. *shastensis*, most primary mycelia will be able to form a dikaryon, resembling panmictic conditions. However, in the European population and the Cosmopolitan group as a whole, a limited number of mating types is likely to occur (see [31]), which means that only a portion of the primary mycelia will be able to mate. Selection is likely to favour individuals with different mating types in a genetically bottlenecked population, whereas random association of mating types is more likely in a genetically variable population. Alternatively, because linkage to mating type loci under strong balancing selection can create a scenario in which degeneration of the linked genes occurs [28], the preponderance of dissimilar *mip* alleles within dikaryons of var. *lacrymans* could be due to the phenomenon of associative overdominance due to recessive deleterious alleles at MAT linked loci [32,33]. This could be reinforced if the number of MAT alleles in the populations is small and the alleles are maintained in the populations for longer periods [33]. However, *S. lacrymans* has a free-living haploid stagen and genetic load due to exposure of recessive mutations would thus be reduced. Tentatively, therefore, we suggest that the former hypothesis of different strengths of selection for divergent alleles in populations with different levels of genetic variation seems more plausible than that of associative overdominance.

Conclusions

In this study we use a mating type linked marker as a proxy to infer variation of mating types in MAT A in *S. lacrymans*. We present evidence consistent with the MAT A region being influenced by frequency-dependent

selection, favouring rare alleles. Although analysis of a locus linked to a gene of interest may provide important information about the target gene, as argued here, one problem is that mutations at the analysed marker may muddle correct assignment of alleles at the gene of interest. For instance, we observed three main groups of sequences in the European population that may correspond to three MAT A alleles, but with some sequence variation within the groups, possibly caused by novel mutations at the analysed marker. To reveal the actual connection between the sequenced alleles and the mating types segregation analyses of spore families with mating types known from crossing experiments should be conducted.

Additional material

Additional file 1 Analyzed material. Information about GenBank accession numbers, geographic origin and ecology (from building or nature) of the analyzed specimens.

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