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**Ecological impacts of *Phlebiopsis gigantea* biocontrol treatment against *Heterobasidion* spp. as revealed by fungal community profiling and population analyses**

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Academic dissertation

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## ABSTRACT

Wood decay fungi belonging to the species complex *Heterobasidion annosum sensu lato* are among the most common and economically important species causing root rot and stem decay in conifers of the northern temperate regions. New infections by these pathogens can be suppressed by tree stump treatments using chemical or biological control agents. In Finland, the corticiaceous fungus *Phlebiopsis gigantea* has been formulated into a commercial biocontrol agent called Rotstop (Verdera Ltd.).

This thesis addresses the ecological impacts of Rotstop biocontrol treatment on the mycoflora of conifer stumps. Locally, fungal communities within Rotstop-treated and untreated stumps were analyzed using a novel method based on DGGE profiling of small subunit ribosomal DNA fragments amplified directly from wood samples. Population analyses for *P. gigantea* and *H. annosum s.l.* were conducted to evaluate possible risks associated with local and/or global distribution of the Rotstop strain.

Based on molecular community profiling by DGGE, we detected a few individual wood-inhabiting fungal species (OTUs) that seemed to have suffered or benefited from the Rotstop biocontrol treatment. The DGGE analyses also revealed fungal diversity not retrieved by cultivation and some fungal sequence types untypical for decomposing conifer wood. However, statistical analysis of DGGE community profiles obtained from Rotstop-treated and untreated conifer stumps revealed that the Rotstop treatment had not caused a statistically significant reduction in the species diversity of wood-inhabiting fungi within our experimental forest plots.

Locally, ISSR genotyping of cultured *P. gigantea* strains showed that the Rotstop biocontrol strain was capable of surviving up to six years within treated Norway spruce stumps, while in Scots pine stumps it was sooner replaced by successor fungal species. In addition, the spread of resident *P. gigantea* strains into Rotstop-treated forest stands seemed effective in preventing the formation of genetically monomorphic populations in the short run. On a global scale, we detected a considerable level of genetic differentiation between the interfertile European and North American populations of *P. gigantea*. These results strongly suggest that local biocontrol strains should be used in order to prevent global spread of *P. gigantea* and hybrid formation between geographically isolated populations.

The population analysis for *H. annosum s.l.* revealed a collection of Chinese fungal strains that showed a high degree of laboratory fertility with three different allopatric *H. annosum s.l.* taxa. However, based on the molecular markers, the Chinese strains could be clearly affiliated with the *H. parviporum* taxonomical cluster, which thus appears to have a continuous distribution range from Europe through southern Siberia to northern China.

**Keywords:** Rotstop, wood decay, DGGE, ISSR fingerprinting, ribosomal DNA

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*Espoo, May 2008*



## LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, which in the text will be referred to by their Roman numerals (I-V).

- I. Dai, Y.-C., **Vainio, E. J.**, Hantula, J., Niemelä, T. & Korhonen, K. (2003). Investigations on *Heterobasidion annosum s.lat.* in central and eastern Asia with the aid of mating tests and DNA fingerprinting. *Forest Pathology* 33: 269-286.
- II. **Vainio, E. J.** & Hantula, J. (2000). Genetic differentiation between European and North American populations of *Phlebiopsis gigantea*. *Mycologia* 92: 436-446.
- III. **Vainio, E. J.**, Lipponen, K. & Hantula, J. (2001). Persistence of a biocontrol strain of *Phlebiopsis gigantea* in conifer stumps and its effects on within-species genetic diversity. *Forest Pathology* 31: 285-295.
- IV. **Vainio, E. J.** & Hantula, J. (2000). Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycological Research* 104: 927-936.
- V. **Vainio, E. J.**, Hallaksela, A.-M., Lipponen, K & Hantula, J. (2005). Direct analysis of ribosomal DNA in denaturing gradients: application on the effects of *Phlebiopsis gigantea* treatment on fungal communities of conifer stumps. *Mycological Research* 109: 103-114.

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## INTRODUCTION

### The fungal individual, population and species

The life cycle of a fungal individual begins with a single meiotic spore containing one haploid nucleus. In homothallic species capable of self fertilization, the formation of fruiting structures can be accomplished by a single haploid individual, while in heterothallic species two different nuclei are required for sexual reproduction. In many fungal taxa, including most ascomycetes (phylum Ascomycota) and zygomycetes (phylum Zygomycota), a vegetative individual usually prevails in haploid nuclear condition, and the formation of heterokaryotic hyphae *via* cellular fusion between sexually compatible strains is shortly followed by the generation of fruiting structures (Bos 1996, Worrall 1997). In contrast, vegetative individuals of most basidiomycete fungi (phylum Basidiomycota) are dikaryotic (thus, haploid strains readily mate to form dikaryotic hyphae, but after cellular fusion the two parental nuclei are maintained separately, and fusion of the nuclei (karyogamy) does not occur until immediately prior to meiosis), (Anderson and Kohn 1995).

During its life cycle, a haploid (or homokaryotic, when several copies of a haploid nucleus exist within single cells) fungal mycelium may encounter many other fungal individuals, and the resulting interactions are regulated by three different incompatibility systems. The sexual incompatibility system controls mating between two haploid mycelia belonging to the same species and delimits inbreeding between siblings. Thus, the generation of a heterokaryotic (secondary) mycelium calls for different alleles at specific mating-type loci in the parental strains (Worrall 1997). In basidiomycete fungi, the mating type genes are encoded by either one locus (bipolar, unifactorial mating system) or two loci (bifactorial, tetrapolar mating system) with numerous alleles (Korhonen 1978a, Korhonen 1978b, Korhonen and Kauppila 1988).

In turn, when a secondary heterokaryotic mycelium is established, it typically shows antagonistic reactions (nonself rejection) towards genetically different individuals from the same species. This kind of response is called somatic (vegetative) incompatibility, and is regarded as a means of maintaining the physiological, ecological and genetic integrity of fungal individuals (Rayner 1991, Worrall 1997, Lind et al. 2007a). However, hyphal fusion is possible between heterokaryotic mycelia provided that they are homoallelic at all somatic incompatibility loci, which usually requires them to be very closely related (Hansen et al. 1993, Worrall 1997, Lind et al. 2007a). In some cases, a heterokaryotic mycelium can also function as a nuclear donor by fertilizing a haploid individual of the same species in a process termed the Buller phenomenon (Korhonen 1978a, Korhonen and Kauppila 1988). In practice, testing for somatic incompatibility reactions between heterokaryotic isolates has been successfully used for the identification of genetically distinct fungal individuals of both *Heterobasidion annosum* (Fr.) Bref. *sensu lato* (Basidiomycota, *Bondarzewiaceae*) and *Phlebiopsis gigantea* (Fr.) Jülich (Basidiomycota, *Corticaceae*), the study organisms of the present thesis (Stenlid 1985, Piri 1996, Holmer and Stenlid 1997, Roy et al. 1997, Swedjemark and Stenlid 2001, Petterson et al. 2003, Roy et al. 2003, Vasiliauskas et al. 2004, 2005a, Sánchez et al. 2007).

Classically, a population can be determined as a group of organisms of the same species living within a sufficiently restricted geographical area so that any member can potentially mate with any other sexually compatible individual (Hartl and Clark 2007). Among fungi, this definition can be sometimes complicated by vast differences in the size of individual strains and their mode of reproduction. Thus, while fungal meiospores fulfill the characteristics of typical micro-organisms, some genetically distinct fungal individuals (genets) may form massive and long-lived vegetative entities producing numerous sexual fruiting bodies along with asexual propagules and/or foraging structures, all representing descendants (ramets) of the same genet (Smith et al. 1992, Taylor et al. 2006).

As for all sexually reproducing organisms, fungal populations are shaped by the evolutionary forces of mutation, recombination, selection and gene flow (or restrained gene flow, caused by e.g. inbreeding, population subdivision or periods of small population size). Many species of fungi maintain a highly outcrossing mating system resulting in efficient recombination and the generation of enormous amounts of sexual spores (Stenlid and Gustafsson 2001 and references therein). Furthermore, fungal meiospores are very small and sometimes capable of long-range migration over marine areas devoid of suitable habitats (Kallio 1970, James et al. 2001, Stenlid and Gustafsson 2001). In turn, some fungal species show global distribution ranges due to man-mediated transfer (Gonthier et al. 2004, Zhou et al. 2007).

However, in the absence of gene flow, separate populations begin to diverge genetically through the accumulation of mutations or events like polyploidization or gene duplication (Olson and Stenlid 2001, Kohn 2005). By definition, genetically isolated populations can be either sympatric (spatially connected with overlapping geographical ranges) or allopatric (separate with discrete ranges). Moreover, reproductive isolation barriers between genetically isolated populations can be either prezygotic (lack of sexual reproduction) or postzygotic (normal formation of sexual fruiting structures but reduced meiospore viability), and can be defined as intrinsic (failure of hybrids or hybrid progeny) or extrinsic (inability to mate or reduced fitness of hybrid progeny), see Taylor et al. (2006), Kohn et al. (2005). In fact, most fungal species with global distribution ranges show population subdivision or the presence of “cryptic” species only recognizable by genetic marker molecules (Taylor 2006). Accordingly, fungal species can be recognized based on phenotypic characters (morphological species recognition, MSR), reproductive isolation (biological species recognition, BSR) or by genetic isolation revealed by multiple gene genealogies (phylogenetic species recognition, PSR), see Taylor et al. 2000, 2006.

Genetically, species barriers between fungi are maintained by incompatibility reactions resulting in intersterility. Thus, the generation of a secondary hybrid mycelium fails unless the parental strains share identical alleles for specific intersterility genes (see e.g. Lind et al. 2005 for references). In sympatry, reproductive isolation can be mediated by the absence of gene flow between populations adapted on different host plants (Kohn 2005). However, some closely related phylogenetic species of fungi are not fully intersterile as determined by laboratory pairing experiments (this is also the case with several *Heterobasidion* species, see below). Also in several cases, allopatric populations show a higher degree of interfertility compared to sympatric ones, which is considered to indicate that in sympatry, prezygotic reproductive isolation is enhanced by natural selection (reinforcement), (Anderson et al. 1980, Capretti et al. 1990, Korhonen et al. 1992, Taylor 2006, Garbelotto et al. 2007).

## Ecology of wood decay fungi

The continuity of forest ecosystems is largely dependent on fungi, as these organisms are the most important agents releasing carbon and nutrients from woody tissues. Fungi have adopted various ecological roles towards their tree hosts, ranging from clearly symbiotic interactions (e.g. mycorrhizal associations) or effectively neutral relationships (e.g. leaf endophytes) to parasitic or pathogenic relationships. As an example, an undamaged, apparently healthy Norway spruce of the age of 61 years has been shown to harbor in its above ground parts nearly a hundred fungal taxonomical groups, mostly needle epiphytes (Müller and Hallaksela 2000). In turn, necrotrophic fungal pathogens (like *H. annosum s.l.*) are capable of attacking intact plant tissues and tolerating this high-stress environment that contains various antifungal substances (Rayner and Boddy 1988, Asiegbu et al. 1998). Finally, saprophytic fungal species like *P. gigantea* infect or become active within dead or moribund plant tissues.

In turn, even saproxylic fungi (species dependent upon dead or dying wood during some part of their life cycle) show a wide range of life strategies. Thus, some species mostly utilize easily assimilated compounds like simple sugars, starch and proteins and take advantage of freshly exposed woody substrates (e.g. the blue stain fungi *Ophiostoma/Ceratocystis* spp.) or dead mycelia of primary decay species (many species of microfungal ascomycetes), while fungal species mainly responsible for wood decay are capable of degrading cellulose and hemicelluloses (brown rot fungi) or both cellulose compounds and lignin (white rot fungi), (Rayner and Boddy 1988). Taxonomically, typical wood-decay fungi belong to basidiomycetes, especially polypores (bracket fungi with a porous hymenial surface, including *Heterobasidion* spp.), corticiaceous fungi (characterized by soft, irregularly shaped fruitbodies, including *P. gigantea*) or agarics (gilled mushrooms like *Hypholoma* spp. or *Armillaria* spp.).

Moreover, some wood decay fungi (e.g. pathogenic species like *H. annosum s.l.* or early saprotrophs like *P. gigantea* or *Stereum sanguinolentum*) typically infect freshly exposed woody tissues, while others use a competitive strategy (secondary resource capture) in colonizing already infected wood (Niemelä et al. 1995, Holmer et al. 1997, Toljander et al. 2006). The establishment of competitive species typically results in high fungal species richness in the intermediate stages of the wood decay process (Niemelä et al. 1995, Renvall 1995). However, as the decomposition advances, nutrients become limited and only the most stress-tolerant species prevail. This species succession can be non-selective regarding to the pioneer decomposer species, but some fungal species (e.g. *Antrodiella* spp.) selectively prefer wood initially decayed by specific primary decay fungi (Niemelä et al. 1995).

As decaying wood is inherently a discontinuous nutritional resource and habitat patch for saproxylic fungi, they must have a means of arrival to a new substrate. Fungal species that can only migrate by spores can be described as resource-unit-restricted, as individual genets are constrained within discrete habitat patches, while fungal species capable of vegetative foraging using rhizomorphs (e.g. *Armillaria* spp.) or tree root contacts (e.g. *Heterobasidion* spp.) are described as non-unit-restricted (Rayner 1991). On a landscape level, pathogenic wood decay fungi affect the species composition of forest stands by selectively killing certain tree species and creating suitable habitats for many saproxylic plant and animal species or fungal successors (Niemelä 1995, Filip and Morrison 1998, Siitonen 2001, Stubblefield et al. 2005). They also contribute to the formation of tree mortality centers and canopy gaps that are considered important factors in maintaining the

structural, functional and species diversity of boreal forests (Kuuluvainen 1994, Bendel et al. 2006).

However, generally commercially managed forests tend to be rather uniform in their tree species composition, size and spacing. Indeed, boreal forest landscape has been subjected to great changes during the last hundred years due to suppression of forest fires and fragmentation of old-growth forests (Östlund et al. 1997, Kouki et al. 2001). Current forest management practices have also drastically decreased the amount of dead wood (especially coarse woody debris, CWD, which has been decreased as much as 90-98% in managed compared to old-growth forests, Siitonen 2001). These developments have caused strong qualitative and quantitative changes in the fungal species composition of managed compared to natural forests (Bader et al. 1995, Sippola et al. 2001, Penttilä et al. 2004), which is especially evident for many threatened polypore species (Högberg and Stenlid 1999, Stenlid and Gustafsson 2001). However, even in managed forests, woody baits or decaying stumps have been shown to harbor a vast variety of wood-inhabiting fungi (Käärik and Rennerfelt 1957, Hallaksela 1977, Lindhe et al. 2004, Vasiliauskas et al. 2004, 2005a, 2005b). Furthermore, the occurrence of wood-decay fungi appears not to be limited by their dispersal capacity, but instead seems to be determined by substrate availability and ability to establish (Nordén and Larsson 2000, Stenlid and Gustafsson 2001, Rolstad et al. 2004).

### ***Heterobasidion annosum* species complex**

Decay fungi belonging to the species complex *Heterobasidion annosum* (Fr.) Bref. *sensu lato* (s.l.) (Basidiomycotina, *Bondarzewiaceae*, syn. *Fomes annosus*, see Niemelä and Korhonen 1998 for the nomenclature) are among the most common and economically important species causing root rot and stem decay in conifers of the northern temperate regions (see reviews; Woodward et al. 1998, Asiegbu et al. 2005a). *H. annosum* s.l. species are capable of attacking living host tree tissues and causing white-rot wood decay by secreting various extracellular enzymes (Asiegbu et al. 1998, 2005a). Recently, the interaction of *H. annosum* s.l. species with their conifer hosts has been elucidated by several studies using real-time (quantitative) PCR profiling (Hietala et al. 2003, 2004, Karlsson et al. 2007) or expressed sequence tag (EST) analysis (Karlsson et al. 2003, Asiegbu et al. 2005b, Adomas and Asiegbu 2006, Adomas et al. 2007, Koutaniemi et al. 2007, Yakovlev et al. 2008).

The primary mode of infection for *H. annosum* s.l. is by airborne basidiospores (Redfern and Stenlid 1998). Sporocarps of *Heterobasidion* species occur on old conifer stumps and logs and can also typically be found from the root systems of windthrown trees. Although most of the basidiospores fall within a range of a few meters from the fruitbody, in some cases they have been shown capable of traveling hundreds of kilometers (Rishbeth 1959a, Kallio 1970, Gonthier et al. 2001). Moreover, vegetative mycelial spread of *H. annosum* s.l. via root contacts from infected stumps into neighboring healthy trees sometimes results in heavy infestation of managed forest stands (Rishbeth 1950, Stenlid and Redfern 1998, Piri and Korhonen 2001). Consequently, current forest management practices have considerably increased the occurrence of *H. annosum* s.l., while in virgin forests these species are relatively rare (Penttilä et al. 2004). In addition, individual genets of *H. annosum* s.l. have been shown capable of surviving several decades in conifer stumps, which thereby serve as infection sources during more than one tree generations (Greig and

Pratt 1976, Piri et al. 1990, Piri 1996, 2003, Lygis et al. 2004a, Piri and Korhonen 2007). Some *H. annosum s.l.* individuals are also able to colonize tens of tree stumps (Piri 1996), or spreading tens of meters by lateral growth (Stenlid and Redfern 1998, Lygis et al. 2004a, Sánchez et al. 2007). On the other hand, single conifer stumps are sometimes occupied by numerous genetically different strains of *H. annosum s.l.* (Swedjemark and Stenlid 2001). *H. annosum s.l.* species also produce asexual conidial spores that have been suggested to be mediated by insect vectors (Kadlec et al. 1992) or liberated into the air by wind gusts associated with high humidity or mist (Möykkynen 1997).

The mating system of *Heterobasidion annosum s.l.* is bipolar (unifactorial) heterothallic (Korhonen 1978a, Holt et al. 1983). Thus, one multiallelic locus has been attributed to the determination of a range of a hundred different mating type alleles (Chase and Ullrich 1983, Stenlid 1985, Korhonen and Stenlid 1998). In turn, the somatic incompatibility system of *H. annosum s.l.* has recently been shown to be controlled by four loci (Lind et al. 2007a).

The *Heterobasidion annosum s.l.* species complex was long regarded as a single cosmopolitan species with a wide host range, but mating experiments have revealed the existence of five separate *Heterobasidion* species showing differences in their host tree preferences (Korhonen 1978a, Chase and Ullrich 1988, Capretti et al. 1990, Korhonen et al. 1992, Niemelä and Korhonen 1998). Differentiation between these biological species has been confirmed using numerous different molecular approaches including isoenzyme analysis (Karlsson and Stenlid 1991, Orosina et al. 1993, Maijala et al. 1995, Goggioli et al. 1998), fatty acid and sterol profiles (Müller et al. 1995), and various genetic marker molecules (Fabritius and Karjalainen 1993, Kasuga et al. 1993, Stenlid et al. 1994, La Porta et al. 1997, Garbelotto et al. 1998, Goggioli et al. 1998, Vainio et al. 1999, Kasuga and Mitchelson 2000, Johannesson and Stenlid 2003, Maijala et al. 2003).

In Europe, three *Heterobasidion* taxa have been recently recognized as separate species (Niemelä and Korhonen 1998). *H. annosum* (Fr.) Bref. *sensu stricto* (= European P intersterility group) is typically associated with butt rot and mortality in pine trees (*Pinus* spp.), but it also frequently infects other conifers and some deciduous tree species, including birch (*Betula* spp.), (Korhonen 1978a, Korhonen and Piri 1994, Korhonen and Dai 2005). It occurs commonly in the Nordic countries ranging also to Southern Europe, while its eastern distribution seems to be limited to the Altai region in southern Siberia (Korhonen 1978a, Capretti et al. 1990, LaPorta et al. 1997, Korhonen and Dai 2005, study I).

*H. parviporum* Niemelä & Korhonen (= European S intersterility group) characteristically causes butt and heart decay in Norway spruce, but also attacks other conifers, and is highly infectious to *Abies sibirica* in Eastern Europe (Korhonen 1978a, Korhonen et al. 1997, Korhonen and Dai 2005, Dai et al. 2006). The *H. parviporum* taxonomical cluster (see Study I, Dai et al. 2006) appears to have a continuous distribution range from Europe through southern Siberia to northern China, and related isolates have recently been found also in Japan (Ota et al. 2006). In turn, *H. abietinum* Niemelä & Korhonen (= European F intersterility group) has a relatively restricted distribution ranging from southern and central Europe to western Turkey and Russian Caucasus, and mainly occurs on *Abies* spp. host trees (Capretti et al. 1990, Tsopelas and Korhonen 1996, Korhonen and Dai 2005, Doğmuş-Lehtijärvi et al. 2006, Sánchez et al. 2007, Zamponi et al. 2007). In the Alps, all of the three European species exist in sympatry (Gonthier et al. 2001, Gonthier et al. 2005).

Two species of *H. annosum s.l.* have also been identified from North America, designated as the North American P and S intersterility groups (Chase and Ullrich 1988, Harrington et al. 1989, Chase and Ullrich 1990a, Otrrosina et al. 1993). Briefly, the North American S group infects several coniferous tree genera (e.g. *Abies* and *Tsuga*) while the P group prefers pines (Filip and Morrison 1998, Korhonen and Stenlid 1998). Recently, *Heterobasidion* strains belonging to the North American P group have been discovered from the Italian peninsula, supposedly having been introduced into Europe *via* transport of woody material used by the North American military troops during the Second World War (Gonthier et al. 2004, D'Amico et al. 2007, Gonthier et al. 2007).

Interfertility among the *H. annosum s.l.* species has been shown to be controlled by five genetic loci (Chase and Ullrich 1990b, Lind et al. 2005). Thus, fungal strains need to be homoallelic for dominant (+) alleles at one or more loci in order to mate. In practice, interspecies crosses can be relatively easily generated by laboratory mating experiments (Korhonen 1978a, Chase and Ullrich 1988, Harrington et al. 1989, Chase and Ullrich 1990a, Stenlid and Karlsson 1991), especially between the closely related taxa *H. abietinum*, *H. parviporum* and the North American S group or between the *H. annosum s.s.* and the North American P group (Chase and Ullrich 1988, Stenlid and Karlsson 1991). In addition, *H. abietinum* isolates show a considerably higher interfertility with North European *H. parviporum* isolates compared to Central European *H. parviporum* strains (Capretti et al. 1990, Korhonen et al. 1992, 1997), which is consistent of these populations having been shaped by selective reinforcement in sympatry.

### ***Phlebiopsis gigantea***

*P. gigantea* (Fr.) Jülich (Basidiomycota, *Corticaceae*; syn. *Peniophora gigantea*, *Phanerochaete gigantea*, *Phlebia gigantea*) is one of the most characteristic fungal species found in stumps, fallen trunks and other remains of coniferous wood in the boreal forest region (Käärrik and Rennerfelt 1957, Meredith 1959, Kallio 1965, Greig 1976, Petäistö 1978, Eriksson et al. 1981, Rönnerberg et al. 2006b). As *P. gigantea* is not very common in primeval forests (Eriksson and Strid 1969, Renvall 1995), it seems to have greatly benefited from current forest management practices. This white-rot species is not pathogenic to living tree tissues, but it can cause considerable damages in stored timber. Consequently, felled tree trunks susceptible to *P. gigantea* infections during biocontrol treatments should not be stored for longer than four weeks in order to prevent decay damages (Mäkelä and Korhonen 1998). Laboratory experiments have also shown that when high oidiospore inoculums are used, *P. gigantea* is able to colonize non-suberized spruce seedling roots and thus could potentially act as a facultative (although weak) necrotrophic pathogen (Asiegbu et al. 1996). In turn, a recent study by Vasiliauskas et al. (2007) describes *P. gigantea* capable of forming structures resembling a mycorrhizal mantle when inoculated on spruce seedling roots.

The fruitbodies of *P. gigantea* are annual and its basidiospores have been shown to travel distances of hundreds of kilometers (Rishbeth 1959a). The basidiospore infection of this species is very effective, and one year after tree felling several genetically different individuals of *P. gigantea* can usually be found in a single pine stump (Annesi et al. 2005, Study III). Thus, its population size can be regarded as very large. However, unlike *H. annosum s.l.*, *P. gigantea* has not been shown capable of vegetative spread *via* plant root contacts, and therefore can be considered unit-restricted. In addition, the life span of *P.*

*gigantea* infections is relatively short as it seldom survives in pine stumps more than 3-5 years (Rishbeth 1963, study III). In addition to meiospores, *P. gigantea* produces asexual oidial spores (arthroconidia). Although these spores are not considered able to travel long distances, in North America they have been isolated from bark beetle galleries, suggesting that they might be involved in insect-mediated transfer of *P. gigantea* (Hunt and Cobb 1982, Hsiau and Harrington 2003).

The mating system of *P. gigantea* has been shown to be heterothallic bipolar, but in laboratory pure cultures this fungus is also capable of homokaryotic fruiting (Korhonen and Kauppila 1988). Hyphal cells of both homokaryotic and heterokaryotic strains of *P. gigantea* are multinucleate, and the asexual oidial spores can be either homokaryotic or heterokaryotic in their nuclear condition (Korhonen and Kauppila 1988). Based on morphological characters, *P. gigantea* is regarded as a single species throughout its geographical range, which covers the boreal forest regions of the Northern Hemisphere as well as parts of South Africa, Australia and New Zealand (Vaartaja 1968, Eriksson et al. 1981, Lundquist 1986, Korhonen and Kauppila 1988, Korhonen et al. 1997, Roy et al. 1997, Hood et al. 2002, Grillo et al. 2005). Laboratory mating experiments have also shown that the European and North American populations of *P. gigantea* are highly interfertile and no putative intersterility groups have been detected so far (Grillo et al. 2005). However, during the present thesis (study II), clear differentiation was detected between North American and European populations of *P. gigantea* as revealed by multilocus DNA fingerprinting.

## **Control of *Heterobasidion* infections**

### *Silvicultural and chemical methods*

Forest stands that have already been infested with *H. annosum s.l.* are likely to transmit the disease to the next tree generation (Piri and Korhonen 2001, Lygis et al. 2004a and the references therein). Logging has been found to increase the rate of spread of *H. parviporum* in the root systems of infected spruce trees and therefore delay of thinning is usually recommended for diseased stands (Rishbeth 1952, Korhonen et al. 1998, Pettersson et al. 2003). Alternatively, infected forest stands can be regenerated using a more resistant tree species (Piri 2003) or an admixture of tree species instead of a pure conifer stand (Piri et al. 1990, Piri 1996, Korhonen et al. 1998, Lygis et al. 2004a, 2004b, Piri 2003). More resistant tree cultivars and candidate genes for molecular breeding programmes are also continuously searched for (Swedjemark and Karlsson 2004).

In turn, further infections can be suppressed by preventing spore-mediated transmission of *H. annosum s.l.* into newly felled stumps. The period of stump susceptibility to *Heterobasidion* infection is limited to a few weeks after felling, depending on the conifer host species (Woods 2000 and references therein). In practice, stump infections of *H. annosum s.l.* can be reduced using wintertime loggings (Thor and Stenlid 2005), stump removal (Rishbeth 1952, Stenlid 1987, Greig et al. 2001) or stump treatments with chemical or biological pesticides.

Numerous chemical substances have been tested for stump treatment against *H. annosum s.l.* (see Pratt et al. 1998 for a review). Basically, the chemicals used can be categorized as either fungitoxic or beneficial to fungi that compete with *H. annosum s.l.* The most effective chemicals against *H. annosum s.l.* are urea (Rishbeth 1959b, Hallaksela

and Nevalainen 1981, Varese et al. 2003, Thor and Stenlid 2005, Nicolotti and Gonthier 2005) and boron compounds (Rishbeth 1959b, Greig 1976, Varese et al. 2003, Nicolotti and Gonthier 2005, Thor and Stenlid 2005). However, trials have been conducted using a wide variety of substances ranging from tar or paints to creosote, sodium nitrite, ammonium sulphamate, propiconazole or copper oxychloride (Rishbeth 1952, 1959b, Kallio 1965, Greig 1976, Nicolotti et al. 1999, Varese et al. 1999, 2003, Nicolotti and Gonthier 2005).

### *Biological control*

Antagonism and competition between vegetative fungal mycelia can often be readily observed in decaying stumps or logs, where different fungal individuals typically occupy distinct discoloration regions, sometimes clearly separated from each other by dark demarcation zone lines (Rayner and Boddy 1988). Competitive replacement between different species of decay fungi has been investigated by several laboratory studies (Holmer and Stenlid 1996, Toljander et al. 2006). During the last 50 decades or so, numerous fungi have also been tested for antagonism and competitiveness against *H. annosum s.l.* in the search for biological pesticides (Holdenrieder and Greig 1998 and the references therein). However, currently *P. gigantea* is the only fungus used in commercial scale.

Besides *P. gigantea*, the tested species include several common wood decomposing basidiomycetes like *Bjerkandera adusta*, *Fomitopsis pinicola*, *Gloeophyllum* spp., *Hypholoma* spp., *Resinicium bicolor*, *Trametes versicolor* and *Sistotrema brinkmannii* (Kallio 1971, Holdenrieder 1984, Capretti and Mugnai 1989, Holmer and Stenlid 1996, 1997, Nicolotti and Varese 1996, Nicolotti et al. 1999, Varese et al. 1999, 2003). Due to being widely used as biocontrol agents, *Trichoderma* spp. are by far the most extensively tested ascomycetous fungi against *Heterobasidion* spp. decay (Rishbeth 1963, Kallio 1971, Kallio and Hallaksela 1979, Johansson and Marklund 1980, Holdenrieder 1984, Capretti and Mugnai 1989, Nicolotti and Varese 1996, Nicolotti et al. 1999, Varese et al. 1999, 2003, Berglund et al. 2005). Other tested ascomycetes include e.g. *Botrytis cinerea*, *Ceratocystis* spp., *Nectria fuckeliana*, *Penicillium* spp. and *Scytalidium lignicola* (Rishbeth 1963, Kallio and Hallaksela 1979, Holdenrieder 1984, Capretti and Mugnai 1989, Nicolotti and Varese 1996).

As different forest ecosystems and host tree species require different means of protection, the search for effective biocontrol agents against *H. annosum s.l.* is an ongoing process (Nicolotti et al. 1999, Varese et al. 1999, Roy et al. 2001, 2003, Varese et al. 2003, Hettich et al. 2007). Promising results have been obtained with e.g. *Phanerochaete velutina* and *Verticillium bulbillosum* (Nicolotti et al. 1999). In turn, trials with bacterial isolates have shown relatively weak antagonistic reactions against *H. annosum s.l.* (see Johansson and Marklund 1980, Holdenrieder and Greig 1998, Murray and Woodward 2003).

As for *P. gigantea*, its ability to replace *H. annosum s.l.* was noticed as early as during the 1950's by John Rishbeth (Rishbeth 1951, 1952). During the following decades, *P. gigantea* has repeatedly been confirmed to be an effective control agent against *Heterobasidion* infections (Rishbeth 1963, Greig 1976, Kallio and Hallaksela 1979, Jokinen 1984, Korhonen et al. 1994, Pratt et al. 2000, Sierota 2003, Annesi et al. 2005, Berglund et al. 2005, Nicolotti and Gonthier 2005). Currently, several commercially available *P. gigantea* products are in use within Europe. The earliest commercial formulations were developed in the United Kingdom (Greig 1976), where several different *P. gigantea* isolates have been used for the preparation of a biocontrol product called PG suspension (Holdenrieder and Greig 1998, Pratt et al. 2000). Similarly, local strains are continuously



screened during the production of a *P. gigantea* formulation called PG IBL in Poland (Pratt et al. 2000, Sierota 2003 and the references therein).

In the Nordic countries, the commercially available products have been developed using single isolates of *P. gigantea*. In Finland, a *P. gigantea* strain isolated from spruce wood in 1987 has been formulated into a biological pesticide called Rotstop (Verdera Ltd.), (Korhonen et al. 1994). The use of Rotstop allows practically complete prevention of *H. annosum* infections in Scots pine stumps (Korhonen et al. 1994), while in Norway spruce the competitiveness of this strain is somewhat lower (Korhonen et al. 1994, Nicolotti et al. 1999, Berglund and Rönnerberg 2004). During recent years, Rotstop treatments have been widely used in the Nordic countries (Thor 2003). However, in Sweden this preparation has been newly replaced by a native *P. gigantea* strain called Rotstop S (Berglund et al. 2005, Rönnerberg et al. 2006a).

Several modes of action have been identified for biocontrol fungi (Cook 1993, Mathre et al. 1999, Butt and Copping 2000, Avis et al. 2001, Brimner and Boland 2003). Thus, suppression of the target pathogen can be accomplished through e.g. direct attack (mycoparasitism) or by secretion of antibiotic metabolites by the biocontrol agent. In addition, the use of biocontrol fungi can restrain the pathogen's growth *via* resource competition or by mutualistic interactions with the host plant. Although *P. gigantea* has been for a long time known as a strong competitor against *H. annosum*, the precise mode of interaction between these fungi remained unclear until recently. However, an expressed sequence tag analysis by Adomas et al. (2006) confirms resource competition to be the main mode of interaction between *H. parviporum* and *P. gigantea* (a diverse range of proteins important for nutrient acquisition were shown to be preferentially expressed during the interaction of these fungi).

Generally, biological pesticides are considered environment-friendly and less probable for resistance development compared to chemical treatments or fungicides (Mathre et al. 1999, Brimner and Boland 2003). However, possible hazards associated with biocontrol fungi include negative effects on the host plant due to toxicity, pathogenicity or the induction of plant defense mechanisms. Also, the use of biocontrol treatments could cause competitive displacement of a beneficial microorganism (like a mycorrhizal symbiont of the host plant). Similarly, toxigenic, pathogenic or competitive impacts could be directed towards other non-target organisms (including e.g. important crop plants) present in the same habitat (Avis et al. 2001, Brimner and Boland 2003).

Recent studies comparing chemical and biological stump treatments have indicated that the environmental impacts of *P. gigantea* seem to be less severe compared to urea for both ground vegetation bryophytes and vascular plants (Westlund and Nohrstedt 2000) and for fungal communities inhabiting Norway spruce stumps (Vasiliauskas et al. 2004). Similarly, Varese et al. (2003) found that the most drastic effects on stump fungal communities were caused by the application of borate, while among the biological treatments *P. gigantea* and *Trichoderma harzianum* had the greatest impacts. In the current thesis, molecular markers were used for assessing whether Rotstop biocontrol treatment had caused changes in the fungal community composition within spruce and pine stumps (study IV) or influenced the population structure of *P. gigantea* in the test plots (study III).

## Molecular markers for phylogenetic analysis

### *Dispersed repetitive DNA elements*

The term microsatellite (also called SSR for simple sequence repeats) refers to noncoding DNA sequences composed of tandem repeat arrays of short nucleotide motifs typically 1-6 bases in length (Shapiro and Sternberg 2005, Selkoe and Toonen 2006). Genetic markers based on microsatellites are usually considered selectively neutral (Selkoe and Toonen 2006). However, microsatellites have been associated with functional roles in e.g. chromatin organization, recombination, replication and regulation of gene expression (Li et al. 2002, Shapiro and Sternberg 2005), and they seem to be concentrated within noncoding genomic regions. Due to their repetitive character, microsatellites are prone to mutational events like replication slippage and unequal crossing over that cause changes in motif repeat numbers (Li et al. 2002). As microsatellites are both abundant and highly variable in most organisms, they can be used for detailed population analyses addressing topics like population size and geographical differentiation, migration, clonality and progeny analysis (Selkoe and Toonen 2006). However, microsatellite markers are usually species-specific, which delimits their applicability for higher-level phylogenetic analyses.

Polymorphic genetic markers based on microsatellite repeats can be generated by two basically different approaches: (i) multilocus fingerprinting by random amplification of any (arbitrary) DNA stretches located between two repeated sequence motifs or (ii) amplification of locus-specific repetitive DNA elements with primers annealing to their flanking regions. Multilocus SSR fingerprints can be produced simply by using primers based on the repeat motifs themselves (Garbelotto et al. 1993, Garbelotto et al. 1998). In turn, the ISSR (or RAMS) multilocus fingerprinting technique (Zietkiewicz et al. 1994, Hantula et al. 1996) uses primers that contain an anchor sequence which prevents their hybridization into multiple positions within a single microsatellite array, and also enables the detection of codominant length alleles for part of the marker loci. However, part of ISSR markers show only on/off type of variation and therefore do not allow the detection of heterozygotes among diploid fungal strains. ISSR fingerprinting has been used for population studies or progeny analysis for several wood-associated fungi (Hantula et al. 1997, 1998, Grillo et al. 2000, Dai et al. 2002, Kauserud and Schumacher 2003a, 2003b), including *H. annosum s.l.* (Vainio and Hantula 1999, study I) and *P. gigantea* (Vainio et al. 1998, Grillo et al. 2005, Annesi et al. 2005, studies II and III).

The generation of locus-specific codominant microsatellite markers traditionally includes sequence analysis of the SSR flanking regions using a genomic clone library and a hybridization assay. Specific microsatellite markers have been recently described for various fungal biocontrol agents (Enkerli et al. 2004, Dalleau-Clouet et al. 2005, Harvey 2006), and also for *H. annosum s.l.* (Johannesson and Stenlid 2004). Alternatively, locus-specific primers can be produced using the SCAR (sequence characterized amplified region) approach. SCAR primers are designed based on sequence analysis of a single marker band produced by an arbitrary primer, and these primers target sequence regions located between two separate repetitive arrays. Besides population studies, microsatellite flanking SCAR primers have also been developed for the differentiation between *H. annosum s.l.* isolates belonging to different intersterility groups (Hantula and Vainio 2003, D'Amico et al. 2007).

### *Other repeated DNA motifs*

Some PCR-based techniques like the RAPD method (Williams et al. 1990) arbitrarily primed (AP) PCR (Welsh and McClelland 1990) and universally primed (UP) PCR (Bulat et al. 2000) utilize completely random primer sequences for the generation of multilocus genetic fingerprints. Thus, the primers are expected to anneal to genome regions that show random sequence matches for the arbitrary primers (usually allowing also some mismatched bases). RAPD primers have been widely used in fungal population genetics, including several studies for *H. annosum s.l.* (Garbelotto et al. 1993, Fabritius and Karjalainen 1993, La Porta et al. 1997, Goggioli et al. 1998) and also for the identification of *P. gigantea* genotypes (Roy et al. 1997). RAPD markers have also been commonly used for the design of SCAR primers for the monitoring of fungal biocontrol agents (Abbasi et al. 1999, Bulat et al. 2000, Massart et al. 2005).

For AP-PCR, one of the most widely used primers has been derived from the bacteriophage M13 core sequence (Jeffreys et al. 1985, Ryskov 1988). Fingerprinting with the M13 primer has been shown applicable for many saproxylic fungal species, including *H. annosum s.l.* (Stenlid et al. 1994, Garbelotto et al. 1998, 1999, Zamponi et al. 2007). This approach was also used in the current thesis for both *H. annosum s. l.* (study I) and *P. gigantea* (study III).

Other multilocus fingerprinting tools used for the identification of fungal biocontrol strains include the REP and ERIC PCR protocols (Versalovic et al. 1991; see Atkins et al. 2003, Grosch et al. 2006 for fungal applications). In addition, some novel multilocus PCR techniques have been designed to target mobile genetic elements with a “copy and paste” (class I type) mode of retrotransposition (Kalendar et al. 1999). Recent studies describe the use of retroelement markers for the rice blast fungal pathogen (Chadha and Gopalakrishna 2005) and the edible fungus *Tricholoma matsutake* (Murata et al. 2005). In turn, the AFLP technique (Vos et al. 1995) uses multiple steps (restriction digestion, ligation of adapters and PCR amplification) for the generation of complex multilocus fingerprints. AFLP markers have been recently used for the identification of industrial and biocontrol fungal strains (Lima et al. 2003, Hynes et al. 2006) and for the generation of a genetic linkage map for *H. annosum s.l.* (Lind et al. 2005).

### *The ribosomal RNA gene cluster*

The ribosomal RNA (rRNA) gene cluster encodes for the universal RNA molecules that form the structural and catalytical backbones of ribosomes. The nuclear rRNA operon is repeated as tandem arrays within the genome, and each operon contains both coding and intronic sequences. In fungi, the coding regions include genes for the small subunit RNA (SSU or 18S rDNA), large subunit RNA (LSU or 28S rDNA) and 5.8S rDNA (some fungal species also have a gene for a 5S rRNA), while the noncoding regions consist of the internally transcribed spacers (ITS1 and ITS2) and the intergenic spacer (IGS1 and IGS2) regions (Mitchell et al. 1995).

The rRNA operon copy number varies greatly between organisms ranging from about forty copies to over twenty thousand repeats in animals and plants (Prokopowich et al. 2003). In fungi, the number of rDNA arrays is typically in the range of 50-200 repeats (see James et al. 2001, Ganley and Kobayashi 2007). Due to selective pressure, the coding sequences are believed to be subjected to concerted evolution which causes the rDNA operon to behave like a single copy region. Thus, unequal recombination continuously

sweeps over one rRNA repeat variant to fixation within the array, resulting in extremely low level of variation between the rDNA repeats (Ganley and Kobayashi 2007). For the intronic spacer regions, a higher level of polymorphism is tolerated, and neutral sequence variants can be easily spread by homogenization.

The usefulness of rRNA molecules as evolutionary chronometers was first noticed by Woese and colleagues (Woese and Fox 1977, Woese 1987), and the following studies challenged the traditional phylogenetic classifications of many micro-organisms including the oomycetes and myxomycetes traditionally included within the fungal kingdom (Bruns et al. 1991, Mitchell et al. 1995). Being the most conserved sequence within the rDNA cluster, the SSU rDNA allows taxonomical comparisons between highly dissimilar species and the construction of universal evolutionary trees (Sogin et al. 1986, Woese 1987, Bruns et al. 1991, Mitchell et al. 1995). In turn, the LSU rDNA shows a higher degree of variation and usually allows species-level differentiation between fungal taxa. Currently, fungal phylogenies are usually constructed by multilocus approaches using sequences of the SSU and LSU rDNAs as well as mitochondrial markers and/or selected nuclear low-copy genes (James et al. 2006, Lutzoni et al. 2004, Matheny et al. 2007, Hibbett et al. 2007). This kind of multigene analysis can also be combined with phylogenetic character mapping, where taxonomical distributions of morphological characters are plotted along molecule-inferred phylogenies (Hibbett and Binder 2002, Binder et al. 2005).

The intronic ITS and IGS spacers are usually highly polymorphic between closely related species, and often show also intraspecific sequence variation. Based on this, the ITS and/or IGS molecules have been used for phylogenetic and population studies among many wood-associated fungal species (Hallenberg et al. 1996, Harrington et al. 1998, Kasuga and Mitchelson 2000, James et al. 2001, Kauserud and Schumacher 2003a, 2003b). The level of intraspecific sequence polymorphism varies greatly between fungal taxa, which is also evident for *P. gigantea* and *H. annosum s.l.* (see studies I and II, and discussion of the present thesis). Additional (secondary) insertion sequences resulting in considerable ITS length polymorphisms have also been found within some fungal taxa like *Cantharellus* spp. (Feibelman et al. 1994) and *Xylaria* spp. (Platas et al. 2004). The short 5.8S rDNA is usually included in ITS analyses because it is located in between the ITS1 and ITS2 regions and does not alone possess enough variable characters for phylogenetic classifications.

The overall level of horizontal (lateral) gene transfer between distantly related species is considered to be very low for SSU rDNA molecules (Choi and Kim 2007). However, horizontal transfer has been observed in certain group I intron sequences occurring within the SSU rDNA genes of some homobasidiomycete fungi (Hibbett et al. 1996) and zygomycetes (Tanabe and Yokota 2002).

#### *Nuclear low copy genes and mitochondrial markers*

The single copy or low-copy genes used for phylogenetic analyses usually encode for elementary structural proteins or housekeeping genes participating in basic cellular functions like transcription, translation or cell metabolism (Baldauf et al. 2000, James et al. 2006). Selected housekeeping genes have also been used for resolving phylogenetic relationships among the phylum Basidiomycota (Matheny et al. 2007) and for phylogeographical investigations of *Heterobasidion* species (Johannesson and Stenlid 2003, Ota et al. 2006). Although not selectively neutral, nuclear genes with ecologically essential functions can sometimes be used in resolving taxonomical questions. For *H. annosum s.l.*,

genes encoding for lignin degradation enzymes (manganese peroxidases and laccases) have been used for phylogenetic analysis (Maijala 2003, Asiegbu et al. 2004).

Mitochondrial genomes are highly variable between species in their size and gene content, and evolve with different rates between the eukaryotic lineages (Gray et al. 1999, Bullerwell and Lang 2005). The mitochondrial (mt) SSU and LSU rDNA molecules evolve more rapidly than their nuclear counterparts, and are similarly useful in resolving phylogenetic relationships among fungi (Lutzoni et al. 2004, Hong et al. 2002). Mitochondrial sequences have also been used for the development of taxon-specific primers for the identification of *Heterobasidion* species (Garbelotto et al. 1998, Gonthier et al. 2001, 2007, D'Amico et al. 2007). The European *H. parviporum* and *H. abietinum* can be differentiated using taxon-specific primers directed to the mt-LSU rDNA (Garbelotto et al. 1998).

## **Fungal community profiling**

### *Sporocarp inventories and culture-based methods*

The occurrence, geographical distribution and ecological role of saproxylic fungal species has been traditionally investigated using sporocarp inventories, which are especially useful for rare, endangered species (e.g. Renvall et al. 1995, Sippola et al. 2001, Lindhe et al. 2004, Penttilä et al. 2004). Since, however, fruitbody development is affected by environmental factors and many microfungi form inconspicuous sporocarps difficult to identify, fruitbody distribution may present a limited view of the fungal diversity existing as vegetative mycelia. The discrepancy between fruitbody occurrence and vegetative mycelial diversity has been observed within both mycorrhizal (Gardes and Bruns 1996, Jonsson et al. 1999) and wood decay fungal communities (Allmér et al. 2006).

In turn, all culture-based methods are limited by their selectivity, mainly due to the lack of suitable culturing media for some species and the tendency of fast-growing species to overgrow slower-growing ones in mixed cultures. Recent studies have also shown a clearly different fungal species composition as revealed by cultivation or molecular analysis from decayed conifer roots (Menkis et al. 2006), spruce branch debris (Allmér et al. 2006) or conifer bark beetles (Lim et al. 2005). This suggests that a full picture of a fungal community can only be obtained using a combined analysis.

### *PCR amplification with group-specific primers*

The use of PCR primers with a wide range of target organisms (so called universal primers) allows the simultaneous amplification of many different DNA templates present in a single environmental sample. The SSU rDNA molecule contains several highly conserved sequence regions, which have been utilized for the design of various universal primers. On the other hand, more variable rDNA regions can be used for the design of taxon-specific primers. A pioneering study by White et al. (1990) described a collection of primers for the amplification of fungal ITS, SSU and mitochondrial rDNA molecules, and many of these primers are still extensively used. Several other universal fungal primer sets have also been described for SSU rDNA amplification (Kappe et al. 1996, Smit et al. 1999, Borneman and Hartin 2000, May et al. 2001, Lord et al. 2002, Vandenkoornhuysen et al. 2002, Nikolcheva

et al. 2003, Nieguitsila et al. 2007). In the current thesis, universal fungal SSU rDNA primers were designed for DGGE community fingerprinting purposes (study IV).

In turn, the polymorphic ITS molecule has been used as a target for several taxon-specific primers for wood decay fungi (Brown et al. 1993, Kim et al. 1999), mycorrhizal basidiomycetes (Gardes and Bruns 1993), conifer root pathogens (Hamelin et al. 1996) or indoor rot fungi (Moreth and Smith 2000). A recent study by Guglielmo et al. 2007 describe a selection of taxon-specific primers for the identification of eleven important wood decay fungal taxa using ITS, nuclear and mitochondrial LSU rDNA target molecules.

Besides detecting the presence of a target organism by conventional PCR, group-specific primers can be used for real-time PCR applications. In real-time PCR, the amplification of target templates is monitored by using a DNA-binding dye or a fluorescently labeled sequence-specific probe, which allows the quantification of microbes within host plant tissues (Schna et al. 2004). This approach has been used for example in the monitoring of *H. parviporum* colonization within Norway spruce tissues (Hietala et al. 2003) and for the detection of *Candida* sp. biocontrol strains in apples (Massart et al. 2005).

In turn, the techniques called length heterogeneity PCR (LH-PCR, Suzuki et al. 1998) and ARISA (automated ribosomal intergenic spacer analysis) allow the separation between different rDNA sequence variants produced using a universal primer pair. These methods detect minor rDNA length polymorphisms using an automated sequencer, and have been used for fungal community profiling from e.g. soil or compost samples (Ranjard et al. 2003, Hansgate et al. 2005).

Sometimes universal rDNA primers are also used for producing complementary DNA by reverse transcription of expressed RNA molecules prior to conventional PCR. This approach enables the selective analysis of metabolically active microbes from environmental samples, and has been used for community fingerprinting for soil fungi (Girvan et al. 2004, Pennanen et al. 2004).

### *Denaturing gel electrophoresis*

Denaturing gradient gel electrophoresis (DGGE) was originally designed for the detection of point mutations (Fischer and Lerman 1983, Sheffield et al. 1989). In denaturing conditions, double-stranded DNA fragments become partially single-stranded and show different migration rates within the gradient gels according to their melting behavior. Because DGGE allows the simultaneous analysis of multiple sequence variants, it has also been shown applicable for the analysis of environmental samples containing several microbial species (Muyzer et al. 1993).

For fungi, the pioneering DGGE community analysis was conducted by Kowalchuk et al. (1997) in order to reveal pathogenic fungi from grass root samples. Several applications for various fungal communities and substrata have followed, mostly using SSU rDNA target molecules (May et al. 2001, Schabereiter-Gurtner et al. 2001, Kowalchuk et al. 2002, Nikolcheva et al. 2003, Ma et al. 2005). During the present thesis, a DGGE protocol was designed for the analysis of saproxylic fungi directly from wood samples (studies IV, V). However, a particular DGGE protocol is usually applicable for many different sample types (see Appendix 2). Alternative target molecules used for DGGE community fingerprinting include e.g. the ITS rDNA (Anderson et al. 2003a, Korkama et al. 2006) and LSU rDNA (Marshall et al. 2003, Diouf et al. 2005). In the present thesis, DGGE was also used for the screening of ITS sequence variants for phylogenetic analyses (studies I and II).

The Temperature gradient gel electrophoresis (TGGE) method uses denaturing gradients generated by increasing temperature during the electrophoresis, and is similarly applicable for fungal community analyses (Smit et al. 1999). TGGE profiling of ITS molecules has also been used for the analysis of wood decomposer fungal communities from beech wood samples (Kulhánková et al. 2006). Some applications also utilize SSCP (Single Strand Conformational Polymorphisms) gels for the generation of fungal community fingerprints (Grosch et al. 2006).

#### *Restriction analysis*

The RFLP technique (Restriction Fragment Length Polymorphisms) can be used for the detection of DNA sequence polymorphisms that cause changes in restriction enzyme recognition sites. Conventional RFLP has been widely used for fungal species identification from relatively simple environmental samples like mycorrhizal rootlets (Gardes and Bruns 1996), living spruce trees (Johannesson and Stenlid 1999) or artificially inoculated wood blocks or chips (Jasalavich et al. 2000, Adair et al. 2002). However, the analysis of environmental samples with multiple species usually requires a cloning or culturing step before the RFLP analysis.

In contrast, terminal RFLP (T-RFLP, Liu et al. 1997) allows the analysis of multiple species from a single sample because only the terminal restriction fragment from each sequence variant is included in the analysis. T-RFLP has been recently used as an identification tool for artificially inoculated wood decay fungal strains (Nikolcheva et al. 2003) and also for fungal community profiling from spruce branches (Allmér et al. 2006) or pine wood blocks (Råberg et al. 2007).

#### *Hybridization assays*

Hybridization of specific probe oligonucleotides with target DNA or RNA can be used for the detection of microbes even without PCR amplification. In FISH (fluorescent *in situ* hybridization, DeLong et al. 1989), the hybridization of fluorescent probes with rRNA molecules is monitored by direct microscopical investigation (see Baker et al. 2004 for fungal applications).

Alternatively, hybridizations can be conducted using membrane-bound probe arrays (also called macroarrays or reverse dot blot arrays). Membrane hybridization arrays have been recently designed for a set of wood decay fungi (Oh et al. 2003) and also for community profiling of soil fungi (Valinsky et al. 2002). It should also be mentioned, that commercial microarrays (microchips) are currently available for selected fungal species like the rice blast pathogenic fungus (*Magnaporthe grisea*), and can be used for the monitoring of gene expression levels for thousands of different genes (Xu et al. 2006). While these technologies open up new potential for microbial community profiling, it must be kept in mind that array-based methods do not reveal unexpected species, and therefore are most suitable for the detection of community changes or indicator species in relatively well-known habitats.

#### *Cloning and sequence-based analysis*

The observation that a vast majority of bacterial species could not be cultivated under laboratory conditions resulted in the adoption of molecular approaches in microbial

ecology. The first bacterial community studies were conducted before the PCR era by rRNA sequence analysis (Pace et al. 1985). However, the invention of PCR (Saiki et al. 1988) allowed the generation of large clone libraries containing PCR fragments amplified directly from environmental samples (Olsen 1990). Cloning is a powerful approach in revealing rare molecular variants or unculturable species, and has been widely used for fungal community studies (Smit et al. 1999, Borneman and Hartin 2000, Vandenkoornhuyse et al. 2002, Jumpponen 2007). Initial screening of large clone libraries is often conducted using DGGE and/or RFLP analysis (Gomes et al. 2003, Lim et al. 2005, Costa et al. 2006). During the current thesis, a few indicative DGGE bands (SSU rDNA fragments) were cloned and sequenced for identification purposes (study V). Alternatively, wood-associated fungal communities have been recently profiled by direct sequencing of ITS rDNA amplicons without a cloning step (see Menkis et al. 2006 for the analysis of root decay samples, and Zaremski et al. 2005 for commercial timber samples).

However, all PCR-based methods are inevitably affected by the selectivity of the primers used. Some recent large-scale cloning investigations have circumvented this bias by the analysis of unamplified environmental DNA fragments (Venter et al. 2004, DeLong et al. 2006). This approach, called metagenomics, requires extensive amounts of sequence data because (unlike PCR) they target any DNA molecules and are not enriched for a specific sequence type.



## AIMS OF THE THESIS

Along with wide-scale *Phlebiopsis gigantea* biocontrol treatments the assessment of possible risks is necessary due to both economical and environmental reasons. Globally, knowledge about the level of genetic polymorphism and geographical differentiation within *P. gigantea* is essential for evaluating whether local biocontrol strains should be used in order to prevent the spread of exotic genetic material into new geographical regions. Locally, large-scale distribution of a single biocontrol genotype could lead to diminishment of genetic variation in *P. gigantea*, which could affect the ability of this species to compete against *H. annosum s.l.* In turn, selective pressures generated by the Rotstop treatments could change the pathogenicity of the *H. annosum s.l.* target pathogens in the long run. The level of genetic polymorphism and differentiation among *Heterobasidion* spp. also determines their potential to resistance development against *P. gigantea*. On the other hand, Rotstop biocontrol applications could affect the overall mycodiversity within the treated forest plots, causing some species to suffer or benefit from the treatment. This could have also economical consequences if some species of forest pathogenic wood-decay fungi would benefit from the treatment.

Specifically, the objective of the current thesis was to shed light on the following questions:

- What is the taxonomical status of *H. annosum s.l.* strains isolated from China?
- Are the North American and European populations of *P. gigantea* genetically differentiated?
- How do Rotstop biocontrol treatments affect the local population structure of *P. gigantea* and how long does the Rotstop genotype prevail in treated stumps?
- What is the applicability of DGGE methodology in the analysis of wood decay mycoflora directly from environmental samples?
- Does Rotstop treatment cause major changes in the community structure of wood decomposing fungi within treated conifer stumps?

## MATERIALS AND METHODS

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### Materials for DNA samples

### Studies

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#### *Cultured fungal isolates*

<i>H. annosum</i> s.l. culture collection (Europe, China; YCD, KK)	I
<i>P. gigantea</i> culture collection (Europe, North America; KK, GB)	II
<i>P. gigantea</i> stump isolates (Finland; KL, EV, JH)	III
Reference collection of wood decay fungi (AMH)	IV

#### *Stump wood samples*

Norway spruce, untreated (Finland; JH, EV)	IV
Norway spruce, untreated, 1 or 6-year-old (Finland; KL, JH, EV)	V
Norway spruce, Rotstop-treated, 6-year-old (Finland; KL, JH, EV)	V
Scots pine, untreated, 1 or 6-year-old (Finland; KL, JH, EV)	V
Scots pine, Rotstop-treated, 6-year-old (Finland; KL, JH, EV)	V

YCD =Yu-Cheng Dai, KK =Kari Korhonen, GB =Guy Bussières, EV = Eeva Vainio, JH =Jarkko Hantula, AMH = Anna-Maija Hallaksela, KL = Katriina Lipponen

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### Methods

### Studies

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#### *Polymerase chain reaction (PCR) amplification*

Multilocus DNA fingerprinting with ISSR (RAMS) primers	I, II, III, IV
Arbitrarily-primed (AP) PCR with the M13 core sequence	I, III
ITS rDNA fragment amplification (ITS1-5.8S rDNA-ITS2)	I, II
SSU rDNA amplification (fragments of 390 bp and 1650 bp)	IV, V

#### *Electrophoresis*

Synergel agarose gels for multilocus DNA fingerprint pattern analysis	I, II, III, IV
Denaturing gradient gel electrophoresis (DGGE) for ITS molecules	I, II
DGGE for 390 bp SSU rDNA fragments	IV, V
DGGE for 1650 bp SSU rDNA fragments	IV, V

#### *Molecular cloning*

Cloning of PCR-amplified ITS rDNA fragments into <i>E. coli</i> DH5 $\alpha$	I, II
Cloning of PCR-amplified SSU rDNA fragments into <i>E. coli</i> DH5 $\alpha$	V

*Bioinformatics and phylogenetic analysis*

Analysis of molecular variance (AMOVA)	I, II
Sequence alignment (by Mega or GCG software)	I, II, IV
Blast (FastA) search in the NCBI GenBank database	I, V
Primer design	IV
Dendrogram construction by NJ clustering	I, II
Dendrogram construction by UPGMA clustering	I

*Statistical testing*

Fisher's exact test	V
Shannon-Weaver heterogeneity index	V
Gini heterogeneity index	V

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## RESULTS AND DISCUSSION

### Methodological considerations

#### *RAMS and AP-PCR multilocus markers (I, II, III, IV)*

In this thesis, multilocus DNA fingerprinting was used for several applications including (i) taxonomical affiliation of Chinese isolates of *H. annosum s.l.* (I), (ii) assessing the level of geographical differentiation between the North American and European *P. gigantea* populations (II), (iii) identification of the Rotstop biocontrol strain and differentiation between indigenous *P. gigantea* isolates (III), and (iv) strain identification of unknown wood decay fungal isolates (IV).

During study I, the taxonomical status of Chinese *H. annosum s.l.* strains that showed equal laboratory fertility with both *H. parviporum* and *H. abietinum* was assessed using multilocus fingerprinting with the M13 AP-PCR primer as well as two RAMS primers (CGA and GAG). In previous studies, M13 fingerprinting had shown a clear differentiation between *H. parviporum* and *H. annosum s.s.* in Europe (Stenlid et al. 1994), while our RAMS analysis (Vainio and Hantula 1999) had revealed several group-specific markers for *H. annosum s.s.*, *H. parviporum* or *H. abietinum*. In study I, the multilocus fingerprinting allowed affiliation of the Chinese strains with the *H. parviporum* cluster, and also revealed fixed indicative markers for both *H. abietinum* and the North American S group.

As for *P. gigantea*, only two different RAMS primers (CGA and TCG) have been described applicable for multilocus fingerprinting (see studies II and III, Vainio et al. 1998, Annesi et al. 2005, Grillo et al. 2005; note that the CGA and TCG primer sequences are palindromic and target trinucleotide repeat motifs that do not cause a frameshift if located within coding gene regions). Based on experimental testing during our studies (data not shown), several other RAMS primers (e.g. AGA, GAAA, CCA, CT, GT, TCC) had shown either insufficient amplification or simple, monomorphic fingerprints for *P. gigantea*. However, the CGA primer generates fingerprint patterns with several variable characters suitable for population analysis (Vainio et al. 1998). During study II, CGA pattern analysis allowed the identification of several markers occurring only within European or North American populations of *P. gigantea*. In addition, a set of markers also occurred among all the isolates, thus generating a characteristic marker pattern for this species.

During study III, RAMS and M13 primers were also used for the identification of diagnostic markers that would allow differentiation between the Rotstop biocontrol strain and wild-type *P. gigantea* strains isolated from biocontrol-treated and untreated control forest plots. All the *P. gigantea* strains isolated from 6-year-old treated spruce stumps were identical in their fingerprint pattern with the Rotstop-strain, whereas all isolates from 1-year-old untreated spruce and pine stumps differed from it (III). The random occurrence of the Rotstop marker combination was also evaluated by multiplication over the marker frequencies of eleven informative fingerprint bands. Thus, we found one Rotstop band (CGA-640) that was rare among the indigenous strains isolated from our sample plots (III), and ten markers that were only found among the wild-type strains. The obtained probability value is based on the assumption that the markers show a random Mendelian segregation as described for ISSR markers based on human pedigree analysis (Zietkiewicz 1994, see also

Czembor and Arseniuk 2000 for progeny analysis of *Phaeosphaeria* fungi by microsatellite-primed SSR markers), and assumes there is no linkage disequilibrium between the markers. (Actually, the observed marker frequencies correspond to genotype frequencies and hence the occurrence of a marker represents the dominant genotype including both homozygotic and heterozygotic strains). In this sense, the two most informative markers alone (CGA-640 and M13-450) would have allowed the separation of the Rotstop strain from most isolates (approximately 1% of strains would have the Rotstop marker combination by chance in the current sample population). Other studies have shown that the CGA-640 marker is rare also among other European populations (Study II, Annesi et al. 2005), and thus might be suitable for the development of specific SCAR primers for the Rotstop isolate.

Generally, arbitrary multilocus fingerprinting techniques are straightforward to use as no prior knowledge of the target sequences is needed, and several primers can be simply tested. These methods typically produce characteristic marker patterns that allow separation between different fungal species. In study IV, RAMS fingerprinting was used as an identification tool for cultured fungal strains isolated from environmental wood samples.

However, it must be noted that arbitrary multilocus fingerprinting methods have often been associated with reproducibility problems, which can be caused by e.g. sensitivity of the PCR amplification for DNA template concentration or impurities present in the sample. Indeed, most studies conducted by arbitrary fingerprinting primers describe omitting part of the markers due to unreproducibility. Similarly, also during the current thesis, some unreliable fingerprinting markers were observed (see e.g. study II), and consequently excluded from the analysis (thus, if a marker was found instable in a single fungal strain, it was not scored from the remaining isolates either).

Another problem with arbitrary multilocus primers is the difficulty of comparing results obtained by different research groups due to the subjective nature of the marker selection and scoring, resulting in poorly connectable data. Thus, while Stenlid et al. (1994) describe scoring a total of 23 M13 markers from *H. annosum* group S isolates, we only selected twelve M13 markers for the population analysis of *H. parviporum* (study I). Similarly, Annesi et al. 2005 use a slightly different marker selection for their RAMS fingerprinting analysis for *P. gigantea* compared to our studies (II, III).

Despite these limitations, it is evident that different multilocus fingerprinting techniques like ISSR (RAMS), RAPD or AP-PCR have been quite efficient in differentiating between the *H. annosum s.l.* intersterility groups, often generating diagnostic markers for these closely related taxa (see Garbelotto et al. 1998 for diagnostic group-specific AP-PCR markers, Vainio et al. 1999 and study I for group-specific RAMS markers and Fabritius and Karjalainen 1993, Garbelotto et al. 1993 and Goggioli et al. 1998 for diagnostic RAPD markers for *H. annosum s.l.*). These kind of fixed group-specific markers are very informative, and it has been stated that even a single fixed group-specific marker provides evidence of genetic isolation for the corresponding phylogenetic group (Taylor et al. 2000). Nevertheless, in the future it would be highly useful to generate additional markers (e.g. SCARs, SSR or AFLP markers) for *P. gigantea* population studies and biocontrol strain monitoring purposes. For *H. annosum s.l.*, a wide selection of polymorphic microsatellite and AFLP markers have been recently developed (Johannesson and Stenlid 2004, Lind et al. 2005a).

*ITS rDNA markers (I, II)*

ITS rDNA analysis was used in the present thesis for (i) resolving the taxonomical status of the Chinese *H. annosum s.l.* strains (ii) assessing the level of geographical differentiation between North American and European *P. gigantea* strains. Methodologically, the analyses were conducted using DGGE and sequence analysis.

The level of ITS sequence variation has been shown to be relatively low within the species of *Heterobasidion*. Thus, no ITS polymorphisms have been observed within the European *H. annosum s.s.* and *H. abietinum*, while a relatively low degree of variation has been found within the European *H. parviporum* and North American S and P intersterility groups (Kasuga et al. 1993, Harrington et al. 1998), as well as within *H. araucariae* (Harrington et al. 1998) and *H. insulare* (Dai et al. 2002). During study I, the ITS alleles of Chinese *H. annosum s.l.* isolates were putatively identified using DGGE, and sequences were determined for the representative ITS ribotypes. Consistent with the multilocus fingerprinting analysis, the Chinese strains showed a higher level of intraspecific variation in their ITS sequences compared to the European *H. parviporum* clade. In addition, the ITS analysis revealed a 5.8S rDNA insertion diagnostic for the Chinese isolates (which separated these strains from both the European *H. parviporum* isolates and the North American S isolates).

Most *Heterobasidion* species can be identified based on polymorphic characters in their ITS sequences. Thus, the European *H. parviporum* and *H. annosum s.s.* and the North American P and S groups as well as *H. insulare* and *H. araucariae* can all be differentiated from each other by their ITS sequences (Kasuga et al. 1993, Garbelotto et al. 1996, Harrington et al. 1998). As an exception, some European isolates of *H. parviporum* are indistinguishable from *H. abietinum* by both their ITS and IGS-1 region (Kasuga et al. 1993, Harrington 1998, Kasuga and Mitchelson 2000), although based on housekeeping gene analysis these species have been separated for a considerable evolutionary time (Johannesson and Stenlid 2003).

For *P. gigantea*, the use of DGGE and sequence analysis resulted in the identification of altogether five different ITS allele types (study II) that showed approximately 98% of sequence similarity to each other. However, the ITS polymorphisms observed did not allow the differentiation between European and North American populations, as one of the alleles was identical in sequence between the continents. In turn, updated information acquired from the NCBI GenBank database (Benson et al. 2007; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) reveal a set of recently submitted *P. gigantea* ITS sequences that show an even higher level of variation among the European strains of this fungus. Using the NCBI Blast program (Altschul et al. 1990; [www.ncbi.nlm.nih.gov/blast/Blast.cgi](http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)), certain *P. gigantea* sequences yield as low as 96% sequence similarities to our submissions. An updated sequence alignment of the publicly available sequence variants for *P. gigantea* is provided in Appendix 1.

The high number of ITS sequence variants observed for *P. gigantea* might suggest the presence of rare alleles that could be used as indicative markers for certain geographical populations or even fungal strains. Unfortunately, by NCBI Blast analysis the ITS sequence of the Rotstop isolate (accession DQ320133 by Vasiliauskas et al. 2007) shows 100% sequence similarity with seven other *P. gigantea* sequences (see Appendix 3), and therefore does not contain rare diagnostic polymorphisms suitable for strain identification for this particular biocontrol isolate. However, it should be noted that according to our DGGE analysis (study II), the Rotstop strain is heterozygous for two ITS alleles (designated as A and C in study II). By Blast comparisons, the newly submitted Rotstop sequence seems to

correspond to the shorter ITS allele (C), while the longer allele variant remains uncharacterized for this biocontrol strain.

The accumulation of environmental sequence data within the public databases is also obvious for both *P. gigantea* and *H. annosum*. Several putatively identified ITS sequences with high similarities to *P. gigantea* have been recently submitted to the GenBank database. These sequences have been retrieved from airborne single spores (Kausserud et al. 2005), mycorrhizal root tips (Menkis et al. 2005) or soil samples (Belbahri et al. 2006, unpublished results, see NCBI accessions EF174449, EF174447, EF174441 and EF174440), and show between 98-100% of sequence similarity to the Rotstop sequence. These percentage similarities fall well within the intraspecies variation range for this fungus (according to Blast comparisons, *P. gigantea* shows ITS sequence similarity of 93% to the most closely matching related species, namely *Phlebiopsis flavidoalba*, GenBank accession EU118662).

Similarly, several environmental sequences with high similarities (in the range of within-species variation) to *H. annosum s.l.* have been recently described from e.g. nasal mucus samples (Buzina et al. 2003), airborne fungal spores (Kausserud et al. 2005), on the surface of bark beetles (Kim et al. 2005, Lim et al. 2005) or Norway spruce woody remains (Allmér et al. 2006). These environmental sequences reflect the common usage of ITS sequences for identification purposes and community fingerprinting among fungi.

#### *SSU rDNA markers (IV, V)*

In the present thesis, SSU rDNA was used as a target molecule for fungal community fingerprinting by DGGE (studies IV, V). In general, the main properties of successful PCR primers for multitemplate amplifications are (i) specificity (successful amplification from target templates and selectivity against other DNAs), (ii) sensitivity (efficient annealing to target molecules), and (iii) lack of amplification bias among the various target organisms. In studies IV and V, the main objective for the primers was to select against host tree DNA that might compete with the sometimes scarce fungal templates present in wood samples. Thus, a fungus-selective primer (FR1) was designed and experimentally tested in combination with four other primers to evaluate their specificity for fungi. PCR experiments using fungal pure cultures and axenic plant tissues revealed a highly enhanced fungal selectivity for the primer pairs even in the presence of a thousand-fold excess of plant DNA from pine, spruce or birch (IV). The fungal specificity of the primers was also evaluated by sequence comparisons to the GenBank database, which revealed matching sequences to primer FR1 among a high variety of fungal species, but also occasional isolates of Echinodermata, Choanoflagellida, Mollusca, Arthropoda and Magnoliophyta. This is consistent with a recently conducted bioinformatics analysis (Hagn et al. 2003) reporting matches for primer pair FR1/FF390 with all fungal rDNAs, but also cross-reactivity with some plant (Slopalinida), red algal and invertebrate (Porifera and Cnidaria) sequences.

In practice, recent fungal community analyses using the primer pairs described in study IV have sometimes reported amplification from non-target organisms including *Amoeba* (Malosso et al. 2006), Hydrozoa, Arthropoda and/or Annelida (Zuccaro et al. 2003, Sekiguchi et al. 2008), Cercozoa (Green et al. 2004), Oomycota (Hoshino and Matsumoto 2007) or even *Spinacia* sp. plant templates (Hoshino and Matsumoto 2007). However, it must be noted that these studies have been conducted using very different sample types (soil, rhizosphere or algal tissue samples), and also the PCR protocols have been quite highly modified in part of the studies (see Appendix 2). In study V, a selection of eight

indicative SSU rDNA ribotypes (DGGE bands) was subjected to sequence analysis, and all the resulting sequences revealed to be most closely related with either basidiomycetous or ascomycetous fungal taxa. Updated NCBI Blast search results for these sequences are reported in Appendix 3. In addition, several recent community profiling studies describe obtaining only fungal sequences when using our primers (Gomes et al. 2003, Kowalchuk et al. 2003, Lilja et al. 2005, Chung et al. 2006, Götz et al. 2006, Van der Wal et al. 2006, Blackwood et al. 2007, Sekiguchi et al. 2007, see Appendix 2).

Similarly to the primers designed in study IV, several widely used fungus-specific primer sets for SSU rDNA (White et al. 1990, Kappe et al. 1996, Smit et al. 1999, Borneman and Hartin 2000) have been shown to amplify some non-target templates (Anderson et al. 2003b, Hagn et al. 2003, Anderson and Cairney 2004, O'Brien et al. 2005, Malosso et al. 2006). Therefore, the presence of possible plant or animal contamination sources should be carefully evaluated if profiling protocols are adopted for new microbial communities or sample types.

The amplification efficacy (sensitivity) of a primer pair can be influenced by template-related reasons as well as sample-related reasons like the presence of large amounts of predominating non-target DNA (e.g. plant or animal host DNA) or impurities that might inhibit the DNA polymerase. The sensitivity of the primer pairs designed in study IV was initially tested using a culture collection of 46 wood-inhabiting fungal species, only one of which failed in PCR amplification (see also Appendix 2 for studies by Zuccaro et al. 2003 and Green et al. 2004 for testing the primers using fungal culture collections). During study V, on average 86% of the environmental wood samples successfully yielded amplification products. Although not directly comparable to culture-based studies (due to e.g. differences in sample sizes), this percentage seems quite high (see Petäistö et al. 1978 and Vasiliauskas et al. 2005a, where fungal cultures were obtained from approximately 68% and 65% of Scots pine and Norway spruce stump wood samples, respectively). In addition, on average two (and up to nine), separate DGGE bands were detected from individual wood samples during study V. Therefore, our PCR protocol seemed adequate for revealing a diverse fungal community from decomposing stump wood samples. However, the suitability of the primer sets for completely different sample types or fungal assemblages might not always be optimal, as insufficient amplification has been observed using the primers for certain ectomycorrhizal communities (Pennanen et al. 2005) or some agricultural soil samples (Hagn et al. 2003, Oros-Sichler et al. 2006).

During multitemplate amplifications, preferential amplification of certain target molecules can be caused by several reasons. Thus, in addition to actual nucleotide mismatches, amplification bias may result from differences between the target organisms in their rRNA operon copy number (Farrelly et al. 1995, Crosby and Criddle 2003) or the accessibility of the rDNA repeats within their genomes (the efficacy of strand separation is affected by e.g. template secondary structure and overall guanine-plus-cytosine content, see Polz and Cavanaugh 1998, Ishii and Fukui 2001). Furthermore, self-annealing of the most abundant templates sometimes causes a bias towards 1:1 mixtures of different amplicons regardless of the initial ratio of the templates (Suzuki and Giovannoni 1996). In study IV, the possibility of amplification bias was evaluated based on PCR testing using artificial template mixtures from a set of common wood-inhabiting fungi (IV), and each species could be detected by DGGE even when its template concentration constituted only 10% of the total DNA used for amplification. Artificial community fingerprints were also successfully generated from PCR mixtures containing equal amounts of DNA from eight different wood-inhabiting tester species (IV).



On the other hand, the evaluation of species diversity using community profile patterns is based on the assumption that each species is represented by a single molecular marker. This relationship could be skewed due to within-species variation in the target molecule. During study IV, no within-species variation was observed between different tester strains representing a single fungal species as revealed by DGGE. However, multiple SSU rDNA fragments were detected from two individual strains from our culture collection (*Cylindrobasidium evolvens* and *Phellinus schweinitzii*), but the reasons behind this phenomenon were not investigated further by sequence analysis (contamination of the reference cultures was not ruled out).

In general, the level of intraspecific variation is considered to be very low for SSU rDNA. However, insertion sequences within the SSU rDNA have been often shown to be hotspots for intraspecific variation (DePriest and Been 1992, Perotto and Bonfante 1998, Lickey et al. 2003), and they might increase the possibility of producing several DGGE bands from one fungal species or isolate. In turn, should introns occur in the primer annealing region, they could result in lack of amplification from the corresponding species. During study IV, unusually long PCR-products were obtained from some fungal pure cultures (*Rhinochadiella* sp., *Gliocladium* sp., *Chondrostereum purpureum*, *Neobulgaria premnophila* and one unidentified mitosporic species) and also from part of the wood samples analyzed during studies IV and V. Therefore, SSU rDNA insertions seem to occur relatively commonly in saproxylic fungi. The Comparative RNA Web (CRW) database (Cannone et al. 2002, [www.rna.icmb.utexas.edu](http://www.rna.icmb.utexas.edu)) lists the occurrence of SSU rDNA introns e.g. in the wood-associated fungal taxa *Exophiala*, *Phialocephala*, *Bionectria*, *Neonectria*, *Graphium*, *Lentinellus*, *Chondrostereum*, *Schizophyllum*, *Panellus* and *Ganoderma*. However, as SSU rDNA insertions were detected only in minority (11%) of our fungal reference species, they were not considered to cause major overestimation of species diversity.

During studies IV and V, the suitability of the designed primers was evaluated only for wood-inhabiting fungal communities. However, several recent studies report successful usage of these primer systems for fungal community fingerprinting from e.g. various soil types (Liiri et al. 2001, Liiri et al. 2002, Perkiömäki and Fritze 2003, Perkiömäki et al. 2003, Björklöf et al. 2003, Girvan et al. 2004, Pennanen et al. 2004, Rantalainen et al. 2004, Hiddink et al. 2005, Rantalainen et al. 2005, Bezemer et al. 2006, Davis et al. 2006, Garbeva et al. 2006, Kang and Mills 2006, Kowalchuk et al. 2006, Drigo et al. 2007, Kardol et al. 2007, Lagomarsino et al. 2007, Yergeau et al. 2007, Sekiguchi et al. 2008) or plant tissues (Pennanen et al. 2001), termite nests (Diouf et al. 2005), and even human gut microflora (Kühbacher et al. 2006) or atherosclerotic plaques (Ott et al. 2007). The primer sets have also been used to analyze cultivated isolates from diseased yam (Abang et al. 2005) and Siberian Larch needles (Kauhanen et al. 2006) and compost (Granit et al. 2007), while some studies report using the primers for nested PCR protocols (see Zuccaro et al. 2003, Green et al. 2004, Costa et al. 2006, Oros-Sichler et al. 2006, Hoshino and Matsumoto 2007). One of the primer pairs (FR1/NS1) has also been shown applicable for the generation of T-RFLP profiles from soil samples (Schwarzenbach et al. 2007).

#### *Sequence-based identification (V)*

The main benefit of sequence-based analyses is that the results can be directly connected across studies. However, when sequences are retrieved from environmental samples by PCR amplification, they are subjected to various forms of PCR artifacts, including errors

generated by the DNA polymerase, the formation of chimeric molecules by template switching of an incompletely extended primer, and heteroduplex formation by the cross-hybridization of heterologous sequences (Speksnijder et al. 2001, Acinas et al. 2005). In practice, the formation of heteroduplex molecules may result in additional bands in community profiles produced by methods like DGGE or RFLP, or even the generation of artificial sequence polymorphisms due to cloned heteroduplexes being subjected to mismatch repair mechanisms of the bacterial host (Speksnijder et al. 2001, Thompson et al. 2002). In turn, chimeric sequences often show low similarities to publicly available reference sequences (Jumpponen 2007). (Naturally, low sequence similarities can also indicate the presence of novel, unidentified phyla, see Vandenkoornhuysse et al. 2002). In study V, the sequences showed relatively high (97 to 100%, see Appendix 3) similarities to publicly available sequences, and therefore we found no indications of novel deeply branching taxonomical groups. In addition, according to a newly conducted data analysis using the Chimera check computer program of the Ribosomal Database Project (Cole et al. 2003; Appendix 3), none of the sequences were suggested as chimeric.

Species identification based on SSU rDNA sequence data is getting more precise due to large-scale sequencing efforts for the fungi (e.g. the AFTOL project, see Lutzoni et al. 2004, Hibbett et al. 2007). However, to this day, the sequence databases cannot be considered comprehensive and even many well-known fungal taxa lack reference sequences. Consequently, a relatively large portion of environmental sequences still show similarities allowing only genus-level affiliation to known taxa (for examples of species identification based on SSU rDNA see Appendix 2, and for recent ITS-based identifications see Kauserud et al. 2005, Kulhánková et al. 2006, Råberg et al. 2007). It should also be noted that the conservative nature of the SSU rDNA molecule sometimes causes difficulties in the affiliation of sequences to a specific species or genus. This was observed during study V especially in the case of two sequence types which showed relatively high sequence similarities to various *Agaricales* species (see Appendix 3). Thus, parallel culturing analysis or comparisons of band types/sequences/RFLP patterns to a relevant culture collection is in many cases still essential for exact species identification. Fortunately, the use of our fungal culture collection allowed the identification of part of the SSU rDNA ribotypes detected by DGGE (V).

Finally, when environmental sequences are compared to publicly available databases, it should be kept in mind that a considerable portion (up to 20%) of published ITS and SSU rDNA sequences might be unreliable (due to being chimeric, incorrectly named, or containing high numbers of unidentified bases, see Bridge et al. 2003, Jumpponen et al. 2007). However, identification of environmental sequences can be considered reliable in cases where the unknown sequence shows a high similarity to a number of independent sequence submissions all representing the same taxonomical group.

#### *Denaturing gradient gel electrophoresis (I, II, IV, V)*

During study IV, four different primer combinations were experimentally tested for their DGGE resolution capacity. Two of these primer pairs, producing either long SSU rDNA fragments of *ca* 1650 bp (FR1-gc/NS1) or short fragments of *ca* 390 bp (FR1-gc/FF390), showed an adequate separation potential among our reference fungi, and were consequently chosen for the analysis of environmental samples (IV, V).

During the analysis of a total of 228 stump wood samples (V), use of the shorter SSU fragment type revealed a higher number of different DGGE band mobility types (in total

48) compared to the longer one (38 different band types). This discrepancy could result from either a lower DGGE resolution capacity or a lower PCR amplification efficacy for the longer fragment type when used for the current stump mycoflora. Testing with our reference fungal collection had shown that the majority of the species could be separated from each other by DGGE (IV). However, in practice it can be expected that part of the sequence variations remain undetected by DGGE resulting in two or more species sharing the same ribotype (Crosby and Criddle 2003). This is especially relevant for fragments longer than 1000 bp (Sheffield et al. 1989). Furthermore, the migration rate of the molecules is not related to their taxonomical status, and among our reference fungi, identical band types were in some cases found between ascomycetous and basidiomycetous taxa (IV).

In study V, the use of two separate primer pairs for each sample allowed species identification of some DGGE band mobility groups (operational taxonomical units, OTUs) based on identical migration with certain tester species (*P. gigantea*, *H. annosum*, *H. parviporum*, *Resinicium bicolor*, *Stereum sanguinolentum* or *Hypholoma capnoides*). However, identification was not attempted for the majority of the band types due to the high number of amplification products in single samples, which made it difficult to reliably connect band types produced by the separate primer sets (differences in the amplification efficacies of the primers also resulted in somewhat different profiles in part of the samples).

In many ecological applications, DGGE profiling is used for revealing changes in mycoflora as a result of a certain experimental treatment (see Appendix 2 for examples). Thus, species identification of the band types is not attempted and the profile patterns are simply compared to each other by statistical analysis. In this context, the possible underestimation of species richness using DGGE profiling should not present a major problem, as it most likely affects both data sets in a similar manner. However, during the current thesis some indicative species affected by the Rotstop treatment might have escaped detection due to being grouped together with an unrelated species.

On the other hand, the formation of heteroduplex molecules could lead to additional bands in community profiles obtained by DGGE. This phenomenon was clearly seen during our ITS analysis of *P. gigantea* for taxonomical purposes (II). Thus, single strains of *P. gigantea* sometimes produced three distinct DGGE bands (corresponding to two ITS alleles and a heteroduplex band), and different strains of this fungus showed clearly distinguishable ribotypes. In community fingerprinting, this kind of intraspecific variation would lead to detection of artificial species diversity. Similarly, *in silico* analyses by Crosby and Criddle (2003) have suggested that ARISA tends to overestimate species richness due to multiple signals from single organisms. In contrast, no additional DGGE bands indicative for heteroduplex formation were observed using artificial multitemplate PCR amplifications of SSU rDNA fragments during studies IV and V.

#### *DNA extraction from environmental wood samples (IV, V)*

In studies IV and V, the total DNA yield from most wood samples was relatively low, and therefore undiluted extracts were used for multitemplate PCR amplifications. However, as the vast majority of wood samples of ca. 0.5 ml yielded amplification products sufficient for DGGE analysis (IV, V), the DNA extraction method described in study IV was considered adequate for our samples. Thus far, the optimal sample size for fungal community profiling has not been thoroughly evaluated for decomposing wood. Allmér et al. (2006) consider that the quantity of 0.1g of pulverized wood from spruce branches was

insufficient for their ITS/T-RFLP analysis, while Menkis et al. (2006) successfully amplified ITS rDNA for sequence analysis using root segments ca. 5 mm in length. In turn, Ranjard et al. (2003) observed strong variations in the fungal composition of small (<1 g) replicate soil samples, although they suggested that the smallest samples could be more adequate for the detection of minor fungal populations. As for decaying wood, which is a highly heterogeneous substrate, pooling of the samples from individual stumps (or branches etc.) could sometimes be a successful sampling strategy if total ribotype scores were used for assessing fungal diversity. It must also be emphasized, that a comprehensive analysis requires that the sampling is not confined only to the heavily decayed wood regions. On the other hand, the detection of several SSU rDNA variants from samples of 0.5 ml (V) shows that cultivation samples should be very small in order to prevent obtaining mixed cultures.

### Ecological remarks

#### *Heterobasidion annosum s.l. (I)*

Wide-scale biocontrol treatments against *H. annosum s.l.* root rot could create selection pressures influencing the virulence of these pathogens in the long run. Thus, although biocontrol usage has been shown to decrease the occurrence of *H. annosum s.l.*, it might also enhance the generation of *H. annosum s.l.* genotypes that are more capable of competing with the biocontrol agent. This is especially significant if the biocontrol treatment is formulated using a single genotype of the biocontrol organism, as in the case of Rotstop. Novel results suggest that there is variation between *Heterobasidion* strains in resistance towards the Rotstop isolate, and by quantitative trait locus (QTL) mapping there seems to be a heritability factor involved (Samils et al. 2007). Interestingly, in some cases oidial treatments with *P. gigantea* have been shown to improve the germination of *H. annosum s.l.* in Norway spruce stumps (Thor and Stenlid 1998). In turn, Gerlagh et al. 1999 found that the usage of *Trichoderma* strains in the control of *Sclerotinia sclerotiorum* increased the number of reproductive structures formed by the pathogen compared to untreated controls. Should a similar situation exist for *H. annosum s.l.*, the application of *P. gigantea* into stumps already infected with the pathogen could actually enhance the generation of pathogen-borne long-range propagules.

Currently, the pathogenicity of *H. parviporum* appears to be lower in the natural forests of China compared to Europe (Dai et al. 2006, study I). This phenomenon might be partly caused by the fact that the most extensive decay damages are found in forest stands with a long history of management (which is the case at many European locations). Interestingly, during study I, the molecular markers used showed a higher level of polymorphism among the Chinese populations of *H. parviporum* compared to the European ones. This observation is consistent with the hypothesis that the center of evolution for the genus *Heterobasidion* would be located in south-eastern Asia, where also other related saprophytic taxa (e.g. *Heterobasidion insulare sensu lato*) can be found, and where the *H. annosum s.l.* species complex is currently relatively poorly known (Niemelä and Korhonen 1998, Dai et al. 2002, study I). It is also likely that even more species of *Heterobasidion* occur in South-Eastern Asia (particularly China or India). In Japan, a new species of *Heterobasidion* has been recently discovered (Ota et al. 2006).

There are several case examples of fungal or oomycetous plant pathogens that have gained increased virulence or a wider host range as a result of genetic exchange between

another pathogen species (Brasier 2000, Olson and Stenlid 2002, Schardl and Craven 2003). As for *H. annosum s.l.*, the high interfertility observed between allopatric populations increases the possibility of similar events. Novel results also suggest that the North American P group would have acquired a mitochondrial rDNA insertion from the sympatric *H. annosum* S group in the Western USA (Linzer et al. 2007).

During study I, the Chinese *H. annosum s.l.* strains revealed to be sexually compatible with European strains of *H. parviporum*, but were also highly interfertile with *H. abietinum* and the North American S group. This was especially notable for isolates representing the eastern Himalayan (South-West Chinese) population that showed a mating frequency of *ca* 96% and 98% with *H. parviporum* and *H. abietinum*, respectively. A similar situation has been previously observed with several other allopatric populations of *H. annosum s.l.*, which makes species identification with the aid of mating tests difficult. However, based on Neighbor Joining dendrogram analysis of DNA fingerprint patterns (I), the Chinese strains clearly clustered together with the European *H. parviporum* (a weak additional subcluster was formed by part of the Chinese S isolates), while *H. abietinum* and the North American S group formed separate clusters. Moreover, according to AMOVA analysis, the Chinese isolates showed significant differentiation from the European *H. parviporum* (27-36%), while their differentiation from *H. abietinum* was distinctly higher (64-67%). In turn, the two Chinese populations (from South-West or North-East China) showed to be closely related to each other (by AMOVA, only 6.6% of the variation was attributable to differentiation between the populations,  $P=0.0964$ ).

During the recent decades, human activity has caused great changes in the natural habitats of *Heterobasidion* species, generating new opportunities for them to prosper, spread and hybridize. Thus, locally current silvicultural practices have greatly increased the occurrence of these fungi, while globally man-mediated transfer has resulted in the intercontinental spread of *Heterobasidion* strains (Gonthier et al. 2004, D'Amico et al. 2007, Gonthier et al. 2007). Both of these factors also increase the probability of more than one *Heterobasidion* species to meet and hybridize when occupying the same niche (Gonthier 2001, Garbelotto et al. 2004, 2007). Gonthier et al. (2007) describe that in Italy the North American P taxon has successfully invaded a habitat in which the indigenous European *H. annosum s.s.* is only marginally present, and the introduced species also seems to be competitive with the native taxon even where both species coexist. Notably, the introduced strains have hybridized with the local *H. annosum s.s.* population in the wild (D'Amico et al. 2007, Gonthier et al. 2007).

In sympatry, natural interspecies *Heterobasidion* spp. hybrids have been considered extremely rare (Gonthier et al. 2001), and only one natural long-lived hybrid strain (a North American S/P hybrid) has been thoroughly described so far (Garbelotto et al. 1996, Garbelotto et al. 2004). A recent study by Garbelotto et al. (2007) also demonstrated that North American S/P hybrids generated in the laboratory were less competitive than the parental genotypes when inoculated on host tree species preferred by one of the parents. Notably, however, in substrates favorable to both parents these hybrids were shown to be equally fit with the parental strains, suggesting that hybrid strains could thrive by shifting their host plant. Moreover, Lind et al. (2007b) found that North American S/P hybrid progeny isolates showed a higher virulence compared to the parental isolates when inoculated on winter-hardened spruce seedlings. It should also be noted that artificially generated North American S/P hybrids have been used for demonstrating that mitochondria play an important role in the virulence of *H. annosum s.l.* (Olson and Stenlid 2001).

*Phlebiopsis gigantea* (II, III, V)

## Global population structure

When used in their native habitat, there are no indications that *P. gigantea* biocontrol strains would develop pathogenic properties towards their tree hosts. However, global spread of exotic *P. gigantea* strains to non-target environments and/or hybridization events between geographically isolated populations could potentially cause changes in the ecological role of this saprophyte, leading to undesirable effects like gaining of pathogenic properties or a broader host range. In this context it should be noted that only four species of conifers (*Pinus sylvestris*, *Picea abies*, *Juniperus communis* and *Taxus baccata*) occur naturally in Finland, while the potential host tree range within North America is considerably wider.

During study II, a considerable level of genetic differentiation was observed between the European and North American populations of *P. gigantea* by AMOVA analysis based on multilocus genetic fingerprints. However, according to laboratory mating experiments (Grillo et al. 2005) this differentiation is not accompanied by reproductive isolation, but seems to represent geographical differentiation at the population level. This agrees with several case examples showing that genetic isolation often precedes reproductive isolation in fungi with global distribution ranges (it has even been argued that phylogenetic species isolated only by geography but not by sexual incompatibility will inevitably evolve reproductive isolation barriers if the geographic isolation persists), (Taylor et al. 2006).

In turn, based on our ITS rDNA sequence analysis it was not possible to identify a diagnostic marker sequence differentiating between all the European and North American strains (II). In addition, ITS alleles with and without a specific insertion sequence were observed in both of these geographically separated populations. The persistence of this unique evolutionary signature sequence in both of the populations agrees with *P. gigantea* having a large population size with free mating. Moreover, the large number of *P. gigantea* ITS sequence variants recently described from Europe (Appendix 1) suggests that many more sequence types would also exist in North America, and our analysis cannot be considered comprehensive. Nevertheless, based on our results the use of local biocontrol strains is highly recommended as hybridization events between the geographical races of this fungus could lead to the formation of novel genotypes.

Currently, risks associated with introduced organisms are commonly recognized, and local *P. gigantea* strains are increasingly being tested for biocontrol purposes (Annesi et al. 2005, Covert and Higgins 2007). Besides this, *P. gigantea* strains are continuously screened because some of the commercially manufactured biocontrol products seem to diminish their competitive activity after being used for approximately two years (Pratt et al. 2000). In Finland, the efficiency of Rotstop is continually monitored by simulated stump treatment experiments carried out every year since the introduction of this biocontrol agent in 1993, and the results indicate that the strain has retained its efficacy over the course of ten years (Korhonen et al. 2003).

## Spread of Rotstop within local populations

A viable heterokaryotic *P. gigantea* strain like the Rotstop biocontrol isolate is expected to produce fruitbodies similarly to any naturally occurring mycelium. Thus, it can also be presumed that Rotstop-derived basidiospores would mate freely with the resident *P.*

*gigantea* population, which would potentially lead to decrease in the wild-type genetic marker pool within the local population. In study III, we addressed this topic by analyzing natural *P. gigantea* infections appearing on fresh stumps that were cut from the experimental forest plots five years after they had been treated with Rotstop. Three different plots were used: (i) plots treated with Rotstop 5 years before the second felling (ii) adjacent plots that had been left untreated (iii) separate control plots nearby the stump treatment experiment.

Based on eleven informative multilocus fingerprinting markers we could separate between several (up to six) different *P. gigantea* strains within single conifer stumps, and to identify 56 strains that differed in their haplotype from the Rotstop isolate. Only three of these strains lacked wild-type indicative markers, and could have therefore represented Rotstop progeny strains. However, the remaining (53) strains contained non-Rotstop markers indicative for wild-type strains with at least one partner from the resident population. In addition, we found no statistically significant differences in the marker frequencies between the treated or untreated plots, although slightly less non-Rotstop markers were found from the treated pine plot. However, our sampling effort was small containing only a few stumps from each plot. Nevertheless, wild-type strains seemed to account for the vast majority of the infections even in the previously treated plots, and therefore the spread of *P. gigantea* into the treated stands seems effective in preventing the formation of genetically monomorphic populations in the short run.

Besides RAMS analysis (Study III, Annesi et al. 2005), the survival of *P. gigantea* biocontrol strains within treated conifer stumps has also been assessed using vegetative compatibility testing (Roy et al. 2003, Vasiliauskas et al. 2004, 2005a) or RAPD fingerprinting (Roy et al. 1997). These studies have indicated that the applied biocontrol strains have persisted as the sole *P. gigantea* genotype found within treated Norway spruce stumps after seven weeks (Vasiliauskas et al. 2004), two months (Roy et al. 2003), four years (Vasiliauskas et al. 2005a) or six years (Study III, Vasiliauskas et al. 2005a) after the treatment. In contrast, based on our results (III, V) within pine stumps *P. gigantea* had been completely replaced by other species six years after the treatment. This discrepancy most likely resulted from the higher decay resistance (hence slower decomposition rate) of spruce wood compared to pine sapwood (Henningsson 1962). It must also be noted that novel results from Poland indicate that *P. gigantea* is able to persist at a relatively low frequency within six-year-old Scots pine stumps (Łakomy et al. 2007).

The ability of *P. gigantea* to spread vegetatively from one stump to another has been considered very limited. The results from study III support this view, as the Rotstop genotype was not retrieved from any stumps cut from the experimental plots when five years had passed from the treatment experiment. However, we did find the Rotstop genotype in two of the untreated 6-year-old spruce stumps cut during the initial treatment experiment. In fact, Rotstop was the only *P. gigantea* genotype found from these stumps, and it was also found from most of the treated spruce stumps. This most probably indicates that the biocontrol agent spreads to some extent into the neighboring woody substrates *via* airborne oidia during the treatment procedure (insect-mediated transfer would be another possibility). However, as the *P. gigantea* ribotype was not detected from the treated stumps by direct DGGE analysis, the biocontrol strain did not seem to cover large volumes of wood (but it should be noted that our sampling was limited to a few centimeters below the stump surface and therefore the biocontrol strain could have still persisted in the deeper wood layers or within the stump roots). Similarly to our results, Vasiliauskas et al. 2005a found that six years after the Rotstop-treatment, *P. gigantea* ceased to be dominant even in

the treated spruce stumps and it was not detected from any of the six-year-old control stumps. In turn, Varese et al. 2003 found that during two years' time from a stump treatment experiment, *P. gigantea* was able to spread into nearby untreated stumps even where this fungal species was quite rare naturally.

According to our observations (III, V) the Rotstop treatment had not increased the overall occurrence of *P. gigantea* within the next stump generation. In fact, based on the molecular community fingerprinting (V), *P. gigantea* revealed to be extremely common within one-year-old untreated pine stumps (occurring in 43-65% of all samples), and it was also very common in one-year-old untreated spruce stumps (found from 17-33% of all DNA samples). Similarly, high natural colonization of Scots pine stumps by *P. gigantea* has been reported in previous studies by Petäistö 1978 and by Holdenrieder and Greig 1998 (the former study reports finding *P. gigantea* within 29% of all cultivation samples, while in the latter study this fungus had infected 5.5-65% of the surface area of pine stumps). The somewhat lower occurrence of *P. gigantea* within spruce stumps also agrees with previous studies (Käärik and Rennerfelt 1957, Kallio 1965, Petäistö 1978). At some forest sites, untreated spruce stumps have even been shown to be colonized by *P. gigantea* at a similar frequency as biocontrol treated stumps (Rönnerberg et al. 2006a).

#### *Wood decay mycoflora* (IV, V)

During study IV, part of the environmental wood samples showed a completely different fungal species composition as revealed by direct molecular analysis (DGGE) or isolation of fungal cultures. Based on morphological characters, the culturable strains in these cases could be identified as *Trichoderma* sp. or *Phialemonium* sp. (IV). The genus *Trichoderma* has been recognized as one of the typical microfungi species that tends to overshadow other species in culture-based analyses, while being more rarely detected by direct molecular methods (Allmér et al. 2006, Van der Wal et al. 2006). On the other hand, Lilja et al. (2005), Zuccaro et al. (2003), Van der Wal et al. (2006, see Appendix 2) and Menkis et al. (2006) report of fungal taxa that were dominant in environmental plant or soil samples as revealed by molecular markers, but could not be recovered as cultures.

As the species composition of the primary wood decomposing mycoflora affects the fungal community developing at later stages of wood decomposition (Rayner and Boddy 1988, Niemelä 1995, Renvall 1995), biocontrol treatments can be expected to cause changes in the stump fungal community structure. During study V, we used statistical testing to analyze whether Rotstop treatment had caused quantitative or qualitative changes in the stump-inhabiting fungal communities within our experimental forest plots. Quantitative changes (impacts on the overall species diversity) were assessed using the Shannon-Weaver and Gini heterogeneity indexes (these heterogeneity tests take into account both species richness and species evenness in a sample, see Peet 1974). In turn, qualitative changes (effects on the species composition) were tested using the Fisher's exact test, which is appropriate for calculating exact probabilities even for small sample sizes (in our case for rarely occurring OTU's, see Ranta 1992).

Our primary observation was that the Rotstop treatment had not caused a statistically significant reduction of the overall species diversity within the treated plots. However, the analysis revealed two different phenomena causing statistical differences between the species compositions of different sample plots. First, part of the one-year-old control stumps showed a low overall species diversity, which caused a statistically significant differentiation when compared to both the untreated or pretreated plots (pretreated stumps



meaning untreated stumps within plots that had been treated six years prior to the sampling). In turn, as the pretreated and untreated plots revealed to be similar in their overall fungal diversity, it can be concluded that the observed differentiation seems to reflect sample plot heterogeneity rather than impacts of the biocontrol treatment.

Secondly, some sample plots were similar in their overall fungal diversity, but showed differences in their species composition. This qualitative impact was revealed by the Fisher's exact test, which indicated a significant differentiation between the old treated and untreated pine plots as well as between all of the old spruce plots (treated, untreated or control). Thus, it is obvious that great variation existed between different test plots regardless of their treatment type.

In a similar manner, Vasiliauskas et al. 2005b found that the fungal communities appearing on spruce woody baits showed a low qualitative similarity between two forest plots separated by only a hundred meters. Locally, formation of the wood decay mycoflora seems to be strongly influenced by spore deposition from indigenous dispersal sources (Edman et al. 2004) as well as site-specific factors (e.g. soil pH, humidity, microclimate; see Snäll and Jonsson 2001, Mattila and Nuutinen 2007) or substrate qualities (nutrient content, pH, moisture, resin content etc.), causing also large within-site variations. Accordingly, the average incidence of *Heterobasidion* infection has been showed to vary greatly between different forest sites (Berglund and Rönnberg 2004). Moreover, the efficacy of Rotstop treatments varies between different forest stands (Berglund and Rönnberg 2004, Berglund et al. 2005, Thor and Stenlid 2005). Consequently, a rather extensive sampling effort would be required for a comprehensive wood decay fungal community analysis. It must also be noted that spread of the Rotstop strain into part of the untreated stumps could have resulted in underestimation of the Rotstop impacts during the present thesis.

Other recent studies have described *P. gigantea* to cause moderate to marked effects on the species richness of treated stumps. Thus, Vasiliauskas et al. (2004, 2005a) found that Rotstop treatment reduced the overall fungal species richness of seven-week-old spruce stumps by about 15%, while after four and six years the decrease in mycodiversity was more pronounced (32% and 46%, respectively). Similarly, Varese et al. (2003) describe that *P. gigantea* had a clear effect on the mycoflora of treated spruce stumps in the Italian Alps, and the impacts seemed to increase over the time.

As for qualitative effects, Kallio and Hallaksela (1979) found that the application of *P. gigantea* did not significantly change the spruce stump mycoflora, although it changed the frequency of the different species present on the stumps. In turn, Vasiliauskas 2005a found that Rotstop treatment had a significant impact on the occurrence of only less than 10% of all isolated species. Accordingly, our primary observation was that most of the fungal species did not appear to be influenced by the treatment (V).

However, previous cultivation-based analyses have revealed a set of species indicated to benefit or suffer from *P. gigantea* treatments. Thus, *H. capnoides* and *Marasmius androsaceus* have been indicated to increase due to *P. gigantea* treatments (Vasiliauskas 2005a), while possible sufferers (besides the *Heterobasidion* target organisms) include *S. sanguinolentum* (Kallio 1971, Kallio and Hallaksela 1979), *Peniophora pithya* and *Sistotrema brinkmannii* (Kallio 1971, Kallio and Hallaksela 1979), as well as *Ascocoryne sarcoides*, *Phialophora fastigiata* and *P. malorum* (Vasiliauskas 2005a). Although not directly comparable to our analysis, Rotstop treatments were shown to significantly reduce the occurrence of *Cladosporium cladosporioides*, *Epicoccum nigrum* and *Phoma putaminum* in spruce stumps in the Italian Alps (Varese et al. 2003). In Canada, treatments

with a local *P. gigantea* strain have been showed to favor stump colonization by *Penicillium* spp. and *Trichoderma* spp (Roy et al. 2003).

According to our DGGE analysis (V), two of the major band types occurring in more than 10% of the samples and six less prominent OTUs appeared to be affected by the treatment (see Appendix 3). Among these eight indicative OTUs, three showed a high sequence similarity to well-known basidiomycetous wood-decay fungi. Thus, one of the sequences was most similar (>99%) to *Tyromyces chioneus* (*Polyporaceae*), which is a white-rot bracket fungus typically growing on decaying wood (Ryman and Holmåsén 1984, Ryvarden and Gilbertson 1994), while another sequence was affiliated with (>99%) species of *Botrybasidium* (*Botrybasidiaceae*). The genus *Botrybasidium* represents saproxylic corticiaceous species commonly found from coniferous woody debris (Eriksson and Ryvarden 1973, see also Renvall 1995, Allmér et al. 2006). The affiliation of one of our sequences with 100% identity to *Helicogloea lagerheimii* (*Atractiellales*) seemed somewhat more enigmatic. According to the USDA (United States Department of Agriculture) fungal databases (see <http://nt.ars-grin.gov/fungaldatabases/index.cfm>) this fungus has not been reported from coniferous wood, although it typically occurs within hardwood species both in North America and Europe (the species seems to be relatively common on broadleaved trees in Norway, see <http://artskart.artsdatabanken.no/FaneKart.aspx>).

In turn, two of the sequences were highly similar to each other, both showing similarities of >98% to various *Agaricales* (*Basidiomycota*) sequences, including e.g. *Resupinatus alboniger*, *Panaeolus foenicicii*, *P. sphinctrinus* and *Agaricus bisporus*. Therefore, identification of these sequence types turned out impossible at this point in time. Similarly, one sequence type was related to various jelly fungi (mitosporic *Tremellales*, *Basidiomycota*), showing relatively low (>97%) sequence similarities to *Cryptococcus* sp., *Fellomyces lichenicola* and *Kockovaella schimae*.

The remaining two sequences were most closely related to ascomycetous taxa. One OTU was most closely (98.6%) related to *Scytalidium lignicola* (mitosporic *Ascomycota*), which is a common saprobe associated with decaying coniferous wood (Menkis et al. 2006, Vasiliauskas et al. 2005a), and also showed 98% similarity to *Chalara strobilina* (mitosporic *Helotiales*). *Chalara* species are common inhabitants of Norway spruce woody debris (Vasiliauskas et al. 2004, 2005a, 2005b, Allmér et al. 2006), and also seem to represent a species group that can be more readily detected by direct DNA analysis than culturing (Menkis et al. 2006). *Chalara* spp. have also been recently identified as the cause of crown dieback of *Fraxinus excelsior* (Kowalski 2006). Note that our sequence type contained an intronic sequence of 469 bp, which was not found from the most closely related sequences, and showed a very low similarity to any GenBank submissions (see Appendix 3).

Finally, one OTU showed quite high (99.3-99.4%) similarities to several endophytic dothideomycetous (*Pezizomycotina*, *Dothideomycetes*) fungal strains isolated by our research group from Siberian larch needles (Kauhanen et al. 2006), and also to *Helicoma isiola* (99.4%, *Pezizomycotina*, *Dothideomycetes*, *Tubeufiaceae*) and *Lophium mytilinum* (99.5%, *Pezizomycotina*, *Dothideomycetes*, *Mytiliniaceae*). According to the USDA fungal databases, *L. mytilinum* is associated with conifer wood, including *Pinus* spp. However, dothideomycetes do not belong to the characteristic fungal species retrieved by culturing from decomposing conifer stumps.

Several recent fungal community studies describe finding untypical fungal taxa from woody substrates. Thus, Lygis et al. (2004a, b) report finding *Phellinus ferrugineofuscus* or *Mycena galopus* from living birch or pine stems although these species have been

considered strict saprobes. Similarly, Vasiliauskas et al. 2005b found fungal taxa traditionally considered litter and well-decayed wood decomposers (*Mycena* and *Collybia*) in freshly cut spruce woody baits exposed for only seven weeks, while Lumley et al. (2000) describe the common occurrence of *Microascaceae* in decaying wood, although these taxa typically colonize dung, litter or soil. Moreover, Vasiliauskas et al. (2004) found *Peniophora cinerea* mycelia from spruce stumps although fruitbodies of this fungus have never been reported on conifers. These examples challenge traditional thinking about the ecological roles of fungal species considered as endophytes, saprobes or pathogens, and also suggest that some species occupy a wider ecological niche than was previously thought, possibly prevailing only as vegetative mycelia within these untypical substrates.

On the other hand, most of the fungal species reported to suffer or benefit from Rotstop treatments represent fungi typical for managed forests. However, it would be important to analyze whether *P. gigantea* treatments cause a threat to the fungal diversity of nearby natural forests possibly containing rare or threatened fungal species. In future studies, it would also be interesting to use molecular markers for the analysis of Rotstop-derived basidiospore isolates from fruitbodies appearing on treated stumps, and to conduct spore dispersal studies for this biocontrol strain (see Nordén and Larsson 2000, Gonthier 2001, Gonthier et al. 2005, Kauserud et al. 2005 for spore trapping techniques used for *H. annosum s.l.* and other wood decay fungi). Moreover, as the biocontrol product inevitably spreads beside the treated stumps during the application procedure, it could have effects on the mycorrhizal fungal community (especially if *P. gigantea* would be capable of colonizing tree seedling roots in the wild, see Vasiliauskas et al. 2007).

## FINAL CONCLUSIONS

In the present thesis, the spread of resident *P. gigantea* strains into Rotstop- treated forest stands seemed effective in preventing the formation of genetically monomorphic populations in the short run. However, as the Rotstop biocontrol agent was shown capable of surviving up to six years within treated Norway spruce stumps, this fungal strain has ample opportunity to produce fruitbodies and spread *via* basidiospores into neighboring forest stands.

On a global scale, we detected a considerable level of genetic differentiation between the interfertile European and North American populations of *P. gigantea*. These results strongly suggest that local biocontrol strains should be used in order to prevent global spread of *P. gigantea* strains and hybrid formation between geographically isolated populations.

Based on molecular community profiling by DGGE, we detected a few individual wood-inhabiting fungal species (OTUs) that seemed to have suffered or benefited from the Rotstop biocontrol treatment. The DGGE analyses also revealed fungal diversity not retrieved by cultivation, and some fungal sequence types untypical for decomposing conifer wood. However, based on the DGGE profiling, the Rotstop treatment had not caused a statistically significant reduction in the species diversity of wood-inhabiting fungi within our experimental forest plots.

The population analysis for *H. annosum s.l.* revealed a collection of Chinese fungal strains that could be clearly affiliated with the *H. parviporum* taxonomical cluster based on ISSR fingerprinting (a moderate level of geographical differentiation from the European *H.*

*parviporum* cluster was detected). Moreover, the Chinese strains showed a high degree of laboratory fertility with three different allopatric *H. annosum s.l.* taxa. These results are consistent with the observations that in the event of gained sympatry this fungal species complex has a broad potential for hybridization even between genetically differentiated taxa.

## FUTURE PROSPECTS

On a global scale, the level of genetic differentiation within *P. gigantea* should be further clarified using isolates from a wider geographical region and additional molecular markers. Currently, the taxonomical status of *P. gigantea* in its southern distribution range is based on morphological characters only, and the degree of geographical differentiation cannot be reliably estimated. Similarly, the possibility that the Rotstop strain would show pathogenic properties against exotic plant species cannot be convincingly ruled out despite *P. gigantea* as a species is known to be saprophytic all over its distribution range. This topic could be elucidated using pathogenicity testing or transcript profiling using plant tissues infected with *P. gigantea*.

In the future, long-term studies are needed to assess whether the genetic polymorphism of *P. gigantea* populations diminishes due to biocontrol usage over several successive logging operations. Moreover, spore-dispersal studies should be conducted to reveal whether the spread of Rotstop-derived basidiospores affects the fungal diversity of nearby natural forests. As for treated conifer stumps, the impacts of Rotstop application on the successional development of the stump mycoflora could be analyzed by a follow-up study using the same group of stumps sampled at closer time intervals.

For *H. annosum*, the possibility of resistance development against *P. gigantea* should be evaluated by experimental testing. On the other hand, unpublished work on *P. gigantea* (H. Sun, personal communication) indicates that more efficient biocontrol strains can be generated by breeding programs based on within-species variation in spore production, growth rate and antagonism against *H. annosum s.l.*

Due to global warming, the snowfree season is expected to become longer in the Nordic countries. This also increases the need for stump biocontrol treatments as the opportunities for spore-mediated *H. annosum s.l.* infections increase. In addition, the distribution limit for *H. annosum* is expected to shift further to north. Therefore the geographical range of this pathogen species complex should be continually monitored. Finally, as *P. gigantea* treatments are not effective against *Heterobasidion* infections already present in the cut stumps, fundamentally novel biocontrol methods are required for disease management within infested forest stands in the future.

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# APPENDIX 1

Sequence alignment based on GenBank submissions for *P. gigantea* containing eleven different sequence variants. The 5.8S rDNA gene is shown in bold.

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DQ320133_Rotstop_Finland_Vasiliauskas^... -CCGGTTTTG AACGGGTGTG AGCTGGCCTC ATACTGTGGC ATGTGCACGC CTGTCTTCAT CCACTCTTCA ACCTCTGTGC
AF087485_C-FP-105126-Sp_USA_Vainio..... T.GA.....
EF174437_UASWS0297_Poland_Belbahri..... T.GA.....
DQ320131_NS234A1_Lithuania_Vasiliauskas... ---.....
AF087484_B-P160_Canada_Vainio..... T.GA.....
AF087487_A-P011_Canada/Finland_Vainio.... T.GA.....
EF174444_UASWS0305_Poland_Belbahri..... T.GA.....
EF174436_UASWS0296_Poland_Belbahri..... T.GA.....
EF174446_UASWS0307_Poland_Belbahri..... T.GA.....
EF174439_UASWS0299_Poland_Belbahri..... T.GA.....
AF087486_C-93318/1_Finland_Vainio..... T.GA.....

DQ320133_Rotstop_Finland_Vasiliauskas.... ACTTATTGTA GGCTGGTGAA GGGTCGCATA GTAA--TGTC CGGCTTGAAG CCTGGTCTAC GTTTTACTAC AAACGCTTCA
AF087485_C-FP-105126-Sp_USA_Vainio..... T.GA.....
EF174437_UASWS0297_Poland_Belbahri..... T.GA.....
DQ320131_NS234A1_Lithuania_Vasiliauskas... ---.....
AF087484_B-P160_Canada_Vainio..... T.GA.....
AF087487_A-P011_Canada/Finland_Vainio.... T.GA.....
EF174444_UASWS0305_Poland_Belbahri..... T.GA.....
EF174436_UASWS0296_Poland_Belbahri..... T.GA.....
EF174446_UASWS0307_Poland_Belbahri..... T.GA.....
EF174439_UASWS0299_Poland_Belbahri..... T.GA.....
AF087486_C-93318/1_Finland_Vainio..... T.GA.....

DQ320133_Rotstop_Finland_Vasiliauskas.... GTTATAGAAT GTCTATCTGC GTATAACGCA TTTAAT---- -ATACAACTT TCAGCAACGG ATCTCTTGGC TCTCGCATCG
AF087485_C-FP-105126-Sp_USA_Vainio..... T.GA.....
EF174437_UASWS0297_Poland_Belbahri..... T.GA.....
DQ320131_NS234A1_Lithuania_Vasiliauskas... ---.....
AF087484_B-P160_Canada_Vainio..... T.GA.....
AF087487_A-P011_Canada/Finland_Vainio.... T.GA.....
EF174444_UASWS0305_Poland_Belbahri..... T.GA.....
EF174436_UASWS0296_Poland_Belbahri..... T.GA.....
EF174446_UASWS0307_Poland_Belbahri..... T.GA.....
EF174439_UASWS0299_Poland_Belbahri..... T.GA.....
AF087486_C-93318/1_Finland_Vainio..... T.GA.....

DQ320133_Rotstop_Finland_Vasiliauskas.... ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACCTTGCG
AF087485_C-FP-105126-Sp_USA_Vainio..... T.GA.....
EF174437_UASWS0297_Poland_Belbahri..... T.GA.....
DQ320131_NS234A1_Lithuania_Vasiliauskas... ---.....
AF087484_B-P160_Canada_Vainio..... T.GA.....
AF087487_A-P011_Canada/Finland_Vainio.... T.GA.....
EF174444_UASWS0305_Poland_Belbahri..... T.GA.....
EF174436_UASWS0296_Poland_Belbahri..... T.GA.....
EF174446_UASWS0307_Poland_Belbahri..... T.GA.....
EF174439_UASWS0299_Poland_Belbahri..... T.GA.....
AF087486_C-93318/1_Finland_Vainio..... T.GA.....

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DQ320133_Rotstop_Finland_Vasiliauskas... CTCCCTGGTA TTCCGGGGAG CATGCCTGTT TGAGTGTGTCAT GGAATTCTCA ACTTCTAATA CTTTTTGTGA TCAGAAGCTT
AF087485_C-FP-105126-Sp_USA_Vainio.....
EF174437_UASWS0297_Poland_Belbahri.....
DQ320131_NS234A1_Lithuania_Vasiliauskas..
AF087484_B-P160_Canada_Vainio.....
AF087487_A-P011_Canada/Finland_Vainio....
EF174444_UASWS0305_Poland_Belbahri..... G.
EF174436_UASWS0296_Poland_Belbahri..... G.
EF174446_UASWS0307_Poland_Belbahri..... G. A
EF174439_UASWS0299_Poland_Belbahri..... G. C. G. A
AF087486_C-93318/1_Finland_Vainio..... G.

DQ320133_Rotstop_Finland_Vasiliauskas... GGATTGGAG GCTCGTGTCT GCTCTCTCGT TAGAGTCGGC TCCTCTTAA TGAATTAGCG TGAATCACTA TGGATCGCTT
AF087485_C-FP-105126-Sp_USA_Vainio.....
EF174437_UASWS0297_Poland_Belbahri.....
DQ320131_NS234A1_Lithuania_Vasiliauskas..
AF087484_B-P160_Canada_Vainio.....
AF087487_A-P011_Canada/Finland_Vainio.... G. T.
EF174444_UASWS0305_Poland_Belbahri..... T. A.
EF174436_UASWS0296_Poland_Belbahri..... T. A.
EF174446_UASWS0307_Poland_Belbahri..... TC. A.
EF174439_UASWS0299_Poland_Belbahri..... TCTC. A.
AF087486_C-93318/1_Finland_Vainio..... TC.

DQ320133_Rotstop_Finland_Vasiliauskas... CGGTGTGATA ATTATCTGCG CCGTAGTCGT GAAGTATTAA TAAAAGTTCT CGTCTCTAAT CGTCCTTCAC GGGACAATTA
AF087485_C-FP-105126-Sp_USA_Vainio.....
EF174437_UASWS0297_Poland_Belbahri..... C.
DQ320131_NS234A1_Lithuania_Vasiliauskas..
AF087484_B-P160_Canada_Vainio.....
AF087487_A-P011_Canada/Finland_Vainio.... C.
EF174444_UASWS0305_Poland_Belbahri..... CC.
EF174436_UASWS0296_Poland_Belbahri..... C.
EF174446_UASWS0307_Poland_Belbahri..... CC. A.
EF174439_UASWS0299_Poland_Belbahri.....
AF087486_C-93318/1_Finland_Vainio.....

DQ320133_Rotstop_Finland_Vasiliauskas... ACCCTGACTT T
AF087485_C-FP-105126-Sp_USA_Vainio.....
EF174437_UASWS0297_Poland_Belbahri.....
DQ320131_NS234A1_Lithuania_Vasiliauskas..
AF087484_B-P160_Canada_Vainio.....
AF087487_A-P011_Canada/Finland_Vainio....
EF174444_UASWS0305_Poland_Belbahri.....
EF174436_UASWS0296_Poland_Belbahri.....
EF174446_UASWS0307_Poland_Belbahri.....
EF174439_UASWS0299_Poland_Belbahri.....
AF087486_C-93318/1_Finland_Vainio.....

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<sup>a</sup> NCBI Blast sequence similarities of 100% are found (i) between sequences DQ320133 and EF174449, EF174447, EF174441, EF174440, EF174438 by Belbahri et al. 2006 (unpublished), and AM084460, AM084822 by Kausrud et al. 2005 or (ii) between accessions AF087485 and EF174448, EF174445, EF174443, EF174442 by Belbahri et al. 2006 (unpublished), or (iii) between accessions DQ320131 and DQ068963 by Menkis et al. 2005.

## APPENDIX 2

List of recent fungal community fingerprinting studies conducted using SSU rDNA primers designed in study IV.

<b>Study</b>	<b>Fungal community</b>	<b>PCR</b>	<b>Primer specificity and sequence data</b>	<b>DGGE resolution and general comments</b>
Liiri et al. (2001)	Soil: microcosms with birch seedlings and ash treatment	FR1-gc/NS1. PCR as in study IV. <sup>a</sup>	Successful amplification, no sequence data	Band number N.R. <sup>b</sup> . Changes in the fungal DGGE profiles induced by ash application.
Pennanen et al. (2001)	Birch branches, laboratory mini-ecosystems, forest humus	FR1-gc/NS1 and FR1-gc/FF390. PCR as in study IV.	Successful amplification, no sequence data. No unspecific amplification from three tested soil eukaryotes ( <i>Tetrahymena</i> , <i>Cognettia</i> , <i>Acrobelloides</i> ).	5-14 dominant bands per sample from mini-ecosystems and field samples. High DGGE reproducibility from ten replicate soil samples.
Liiri et al. (2002)	Soil: microcosms with silver birch seedlings	FR1-gc/NS1. PCR as in study IV.	Successful amplification; no sequence data.	Band number N.R. Changes in microarthropod fauna did not affect the fungal DGGE profiles.
Björklöf et al. (2003)	Soil: birch rhizosphere on humus and peat, inoculated with <i>Pseudomonas</i>	FR1-gc/FF390. PCR for 35 cycles with 58.5°C annealing. Taq DNA polymerase.	Successful amplification, no sequence data.	Band number N.R. Humus and peat-specific DGGE profiles.
Gomes et al. (2003)	Soil: bulk and maize rhizosphere in the tropics	FR1-gc/NS1. PCR for 35 cycles with 48°C annealing. Taq DNA polymerase.	Successful amplification from all samples. Sequence data from 39 clones, related to Ascomycota ( <i>Alternaria</i> , <i>Aspergillus</i> , <i>Bionectria</i> , <i>Chaetomium</i> , <i>Cladosporium</i> , <i>Cucurbita</i> , <i>Gibberella</i> , <i>Paraphaeosphaeria</i> , <i>Phaeosphaeria</i> , <i>Setosperia</i> , <i>Raciborsklomyces</i> ) Basidiomycota ( <i>Bullera</i> , <i>Cryptococcus</i> , <i>Sporidiobolus</i> ), Zygomycota ( <i>Mortierella</i> ). Similarity to reference sequences 96.0-100%.	In total 12 dominant band types detected with SSU-DGGE. DGGE resolution not optimal: additional OTU's revealed by ARDRA. Fungal community profiles strongly affected by plant age. High reproducibility (low variability between replicates).



<b>Study</b>	<b>Fungal community</b>	<b>PCR</b>	<b>Primer specificity and sequence data</b>	<b>DGGE resolution and general comments</b>
Kowalchuk et al. (2003)	Soil: management practices against <i>Pythium</i> root rot in flower bulb cultivation	FR1-gc/FF390 or NS1-gc/NS2+10 PCR as in study IV. Expand High Fidelity polymerase.	Successful amplification. Six sequences: <i>Chaetomium</i> (99.4%), <i>Geomyces</i> (99%), <i>Kluyveromyces</i> (99.4%), <i>Acremonium</i> (99.7%), <i>Candida</i> (99.7%), <i>Ascotricha</i> (98.1%).	In total 7 distinct OTU's with FR1+FF390 and 3 OTU's with NS1/NS2+10. Fungal DGGE profiles similar between untreated and sterilized or flooded soils.
Perkiömäki & Fritze (2003)	Soil: microcosms treated with wood ash, cadmium and acid	FR1-gc/FF390. PCR as in study IV.	Successful amplification; no sequence data.	In total 34 separate DGGE bands from soil samples. Changes in seven indicative fungal DGGE OTUs induced by ash treatment.
Perkiömäki et al. (2003)	Soil: microcosms treated with ash, acid and metal	FR1-gc/FF390. PCR as in study IV.	Successful amplification, no sequence data.	Band number N.R. Treatments did not decrease DGGE band number; one OTU affected by metal addition.
Zuccaro et al. (2003)	Plant: fungi associated with <i>Fucus</i> algae	Semi-nested protocol: 1 <sup>st</sup> PCR with EF3/ NS1 , 2 <sup>nd</sup> PCR with FR1-gc/NS1 and 47°C annealing, TaqBead Hot start polymerase.	Successful amplification from 15 cultivated Diaporthales, Dothideales, Eurotiales, Helotiales, Xylariales and mitosporic fungi. Environmental sequences from Ascomycota ( <i>Neophaeosphaeria</i> 99%, <i>Corallospora</i> 98%, <i>Lulworthiales</i> 96%) and animal species ( <i>Hydrozoa</i> , <i>Arthropoda</i> , <i>Annelida</i> ).	7-11 major bands detected from algal samples. Cultivation and direct molecular analysis revealed a distinctly different fungal community composition.
Girvan et al. (2004)	Soil: winter wheat microbial communities with fertilizer and pesticide treatment	FR1-gc/FF390. PCR as in study IV. Superscript II reverse transcriptase.	Successful amplification, no sequence data.	Band number N.R. Variability between replicate samples high, microbial community was primarily determined by season.

<b>Study</b>	<b>Fungal community</b>	<b>PCR</b>	<b>Primer specificity and sequence data</b>	<b>DGGE resolution and general comments</b>
Green et al. (2004)	Soil: rhizosphere of cucumber and compost-amended potting mix	FR1-gc/NS1. PCR for 35 cycles with 57°C annealing. Nested PCR: 1 <sup>st</sup> PCR with NS1AG/NS8 or NS1AG/PyNS8, 2 <sup>nd</sup> PCR using FR1-gc/NS1 with 61°C annealing. Sigma RedTaq polymerase.	Successful amplification from all cultured pyrenomycete isolates ( <i>Colletotrichum</i> , <i>Trichoderma</i> , <i>Fusarium</i> , <i>Thielaviopsis</i> ), and <i>Botrytis</i> , <i>Saccharomyces</i> , <i>Tuber</i> , <i>Rhizoctonia</i> . Bands from cultivated oomycete ( <i>Phytophthora</i> ) and plant ( <i>Cucumis</i> ) DNA. Sequence data from 16 environmental band types from direct or nested PCR reactions related to Ascomycota: <i>Papulosa</i> , <i>Pseudallescheria</i> , <i>Nigrosabulum</i> , <i>Petriella</i> , <i>Trichoderma</i> , <i>Coccidioides</i> , <i>Saccobolus</i> (sequence similarity 95-99%). Direct PCR yielded products from flagellate DNA ( <i>Heteromita</i> , Cercozoa; 89-97%).	Band number from environmental samples N.R. Pyrenomycete-targeted PCR revealed species not detected by direct analysis with FR1-gc/NS1.
Pennanen et al. (2004)	Soil: microbe communities supplemented with glucose and L-asparagine	FR1-gc/FF390. PCR as in study IV. Expand High Fidelity polymerase and M-MLV reverse transcriptase.	Successful amplification and generation of cDNA from SSU rRNA, no sequence data.	Band number N.R. High substrate amendments affected one DGGE band type. Fungal rDNA and rRNA profiles similar.
Rantalainen et al. (2004)	Soil: Microcosms with pine seedlings and enchytraeid worms, inoculated with fungi and bacteria	PCR as in study IV.	Successful amplification, no sequence data.	Presence or absence of inoculated fungal taxa checked from soil samples by DGGE pattern comparisons to cultured strains. Two of eleven taxa indistinguishable by DGGE.
Abang et al. (2005)	<i>Colletotrichum</i> isolates from diseased yam and weed plants hosts	FR1-gc/NS1. PCR for 25 cycles with 48°C annealing, <i>Taq</i> Stoffel fragment polymerase.	Successful amplification, no sequence data.	Two DGGE band types corresponding two morphotypes of <i>C. gloeosporioides</i> . Morphotypes not separable by ITS-RFLP analysis.
Diouf et al. (2005)	Soil: biogenic soil sheetings of three different termite species and two control soils	FR1-gc/FF390. PCR for 35 cycles with 55°C annealing. Pharmacia Ready-To-Go <i>Taq</i> polymerase.	Successful amplification, no sequence data.	In total 20-28 DGGE bands in termite sheetings or control soils. DGGE revealed fungi not found by cultivation. LSU-DGGE produced a somewhat different view.

<b>Study</b>	<b>Fungal community</b>	<b>PCR</b>	<b>Primer specificity and sequence data</b>	<b>DGGE resolution and general comments</b>
Hiddink et al. (2005)	Soil: effects of mixed cropping on disease suppressiveness of soils	FR1-gc/FF390. Touch-down PCR for 37 cycles and 55-47°C annealing. AmpliTaq polymerase.	Successful amplification, no sequence data.	21±6 or 27±6 bands per lane detected from soils with different crops. No significant differences between DGGE profiles from mixed or mono-cropped soils.
Lilja et al. (2005)	Wood: fungi occurring in cankers of container seedlings of Norway spruce	FR1-gc/NS1 and FR1-gc/FF390 PCR as in study IV.	Successful amplification. More minor bands detected using FR1-gc/NS1 compared to FR1-gc/FF390. Sequence data from two cultivated isolates and four clones: <i>Sirococcus</i> (100%), <i>Phoma</i> (100%), <i>Cordyceps</i> (99%), <i>Raciborskiomyces</i> (99%).	0-5 or 1-9 bands detected from each canker using primer pair FR1-gc/FF390 or FR1-gc/NS1. Direct rDNA analysis revealed additional diversity compared to cultivation. RAMS fingerprinting from cultivated isolates revealed each dominant DGGE band type to represent a single species.
Pennanen et al. (2005)	Mycorrhiza: effects of mounding on spruce ectomycorrhizal diversity	FR1-gc/FF390 and FR1-gc/NS1 PCR as in study IV.	PCR success rate 83% and 48% for FR1-gc/FF390 and FR1-gc/NS1, respectively.	1-5 band types found from root tips of each spruce seedling. DGGE fingerprints indicated a few differences between ECM communities on mounds or untreated spots.
Rantalainen et al. (2005)	Soil: colonization of sterilized humus patches embedded in mineral soil	FR1-gc/FF390. PCR as in study IV.	Successful amplification, no sequence data.	Species richness per patch varied from 4 to 12. Use of habitat corridors increased the species richness of soil fungal community.
Bezemer et al. (2006)	Soil: greenhouse experiment with <i>Senecio jacobea</i>	FR1-gc/FF390. PCR as in study IV, Expand High Fidelity polymerase.	Successful amplification, no sequence data.	Band number N.R. Composition of fungal communities in unsown plots differed significantly from those in sown plots.
Chung, et al. (2006)	Soil: Effects of elevated CO <sub>2</sub> and O <sub>3</sub> on fungal community composition	FR1/FF390. PCR for 30 cycles with 50°C annealing. Expand High Fidelity polymerase.	Successful amplification, sequence data from 16 prominent OTU's: 10 Basidiomycota, 4 Ascomycota, 2 Zygomycota.	In total 39 OTU's identified. Identical DGGE banding pattern in five replicate samples. Elevated O <sub>3</sub> significantly altered the fungal community structure. Two indicative OTU's.
Costa et al. (2006)	Soil: rhizosphere of strawberry and oilseed rape	Nested protocol: 1 <sup>st</sup> PCR with NS0/EF3, 2 <sup>nd</sup> PCR with FR1-gc/NS1 for 20 cycles with 48°C annealing. Taq Stoffel fragment.	Successful amplification, no fungal sequences.	Band number N.R. Internal variability within bulk and rhizosphere soil replicates was high. Significant differences between strawberry and oilseed rape rhizosphere fungal profiles from the same sampling site.

<b>Study</b>	<b>Fungal community</b>	<b>PCR</b>	<b>Primer specificity and sequence data</b>	<b>DGGE resolution and general comments</b>
Davis et al. (2006)	Soil: weed seed mortality in soils with contrasting agricultural management	FR1-gc/FF390. Touch-down PCR for 30 cycles with 60-50°C annealing	Successful amplification, no sequence data.	Band number N.R. Distinct differences in the composition of fungal communities between the study soils.
Garbeva et al. (2006)	Soil: microbial diversity of soil under different agricultural regimes	FR1-gc/NS1. PCR as in study IV.	Successful amplification, no sequence data.	Significant differences in the fungal diversity of different grassland or arable land treatments as revealed by DGGE patterns.
Götz et al. (2006)	Soil: fungal endophytes in potato roots	FR1-gc/NS1. PCR for 35 cycles with 48°C annealing. Taq DNA polymerase.	Successful amplification, sequence data from 34 clones affiliated with <i>Volutella</i> (99.4%), <i>Colletotrichum</i> (99.3%), <i>Verticillium</i> (98.3-100%), <i>Septoria</i> (99.8%), <i>Paraphaeosphaeria</i> (99.7%) <i>Acremonium</i> (100%) and <i>Phialophora</i> (100%). One chimeric sequence.	5-6 dominant bands and several faint bands in all replicates. Low degree of variability between DGGE patterns of replicate samples. Some DGGE band types were not observed among cultured isolates from the same samples. Cloned amplicons were classified using DGGE and ARDRA.
Kang & Mills (2006)	Soil: sample size effects on community structure	FR1-gc/FF390. PCR for 30 cycles with 50°C annealing. AmpliTaq polymerase.	Successful amplification, no sequence data.	Band number N.R. Major differences in the fungal communities recovered by using different sample sizes.
Kauhanen et al. (2006)	Plant: cultured endophytic fungi from Siberian larch needles	FR1-gc/FF390 and FR1-gc/NS1 PCR as in study IV.	Successful amplification, sequence data from 59 samples and 10 OTUs related to <i>Phoma</i> (100% and 99%), <i>Hypoxyton</i> (99%), <i>Leucostoma</i> (99%), <i>Endoconidioma</i> (99%), <i>Aureobasidium/Discosphaerina</i> (99%), <i>Anguillospora</i> (99%), <i>Heyderia</i> (98%), <i>Monilinia</i> (95-98%).	Most morphological OTUs had distinct DGGE mobility, while the most common OTU showed intra-group variation (product length and DGGE mobility). Verification of cultivated morphotype groups (OTUs) by SSU-DGGE and ITS-RFLP.
Kowalchuk et al. (2006)	Soil: <i>Senecio jacobaea</i> rhizosphere, coastal sand dune soil	FR1-gc/FF390. PCR for 35 cycles with 47°C annealing. Expand High Fidelity polymerase.	Successful amplification, no sequence data.	In total 32 different band positions distinguished by DGGE. 5-17 bands per sample (average 11). Fungal diversity lower in the rhizosphere of plants with high alkaloid content.

<b>Study</b>	<b>Fungal community</b>	<b>PCR</b>	<b>Primer specificity and sequence data</b>	<b>DGGE resolution and general comments</b>
Kühbacher et al. (2006)	Human gut: microbial responses to probiotic therapy	FR1-gc/NS1. PCR as in study IV.	Successful amplification, no fungal sequences.	The mean number of bands varied from 8 to 13 between different treatment groups. Wide interindividual variation in banding patterns. A marked reduction in fungal diversity in patients treated with probiotic therapy.
Malosso et al. (2006)	Soil: maritime Antarctic soils	FR1-gc/FF390. PCR for 30 cycles with 50°C annealing. Taq DNA polymerase. UF1/S3 for ARDRA.	Successful amplification from all enriched soil samples. Sequence determination from 43 DGGE band types: 18 Ascomycota (88-100%), 16 Basidiomycota (85-100%), 4 Zygomycota (91-100%), 5 <i>Amoeba</i> .	24 and 19 band types from two different soils. DGGE, cultivation and ARDRA revealed a somewhat different fungal community composition.
Oros-Sichler et al. (2006)	Soil: 36 sites with agricultural soils (sugar beet, barley, rye, wheat, potato, rape, pea, maize, fallow system)	Semi-nested PCR: 1 <sup>st</sup> PCR with EF3/NS1, 2 <sup>nd</sup> PCR with FR1-gc/NS1 for 20 cycles with 48°C annealing. Direct PCR as in study IV, Taq polymerase.	Direct amplification with FR1-gc/NS1 failed from most soil samples. 24 of 101 cultured isolates failed to amplify using FR1-gc/NS1 (and 7 of 101 failed with EF3/NS1). GC-clamp seemed to interfere with direct PCR. Successful amplification using nested protocol, no sequence data.	Community fingerprints using nested PCR protocol showed high and consistent complexity with up to 11 dominant bands. Characteristic bands for each soil site and clear differentiation of fungal communities from different sites. Certain extent of variability in replicate DGGE fingerprints.
Van der Wal et al. (2006)	Soil: abandoned arable sandy soils and heathland soil supplemented with various carbon substrates	FR1-gc/FF390. Touch-down PCR for 35 cycles with 55-47°C annealing Expand High Fidelity polymerase.	Successful amplification, sequence data affiliated with <i>Cryptococcus</i> , <i>Fusarium</i> , <i>Mortierella</i> , <i>Humicola</i> , <i>Byssosascus</i> / <i>Oidiodendron</i> , <i>Hymenoscyphus</i> , <i>Chaetomium</i> , <i>Thelebolus</i> / <i>Hyphozyma</i> , <i>Aleuria</i> / <i>Wilcoxina</i> / <i>Cheilymenia</i> / <i>Otidea</i> . Reference sequence similarity ≥ 98%.	Band number N.R. Control and glucose treatments differed strongly from the wood and cellulose treatments in their DGGE profiles. Cultivated isolates predominately <i>Zygorhynchus</i> , <i>Mortierella</i> , <i>Mucor</i> , <i>Umbelopsis</i> , <i>Absidia</i> , <i>Acremonium</i> , <i>Humicola</i> , <i>Penicillium</i> , <i>Trichoderma</i> , <i>Sporothrix</i> and <i>Thielaviopsis</i> .
Blackwood et al. (2007)	Plant: decomposing leaf litter in deciduous forests (maple, basswood, oak)	FR1-gc/FF390. PCR for 35 cycles with 50°C annealing. Expand High Fidelity polymerase.	Successful amplification, sequence data from ten DGGE bands related to Basidiomycota ( <i>Auricularia</i> , <i>Multiclavula</i> , <i>Boletus</i> ), Ascomycota ( <i>Aspergillus</i> , <i>Ophioceras</i> , <i>Pachyphloeus</i> , <i>Neourmula</i> , <i>Rhizisma</i> , <i>Anguillospora</i> ) and Zygomycota ( <i>Piptocephalis</i> ).	Band number N.R. Overall forest floor-inhabiting fungal communities differed among forest ecosystems.

<b>Study</b>	<b>Fungal community</b>	<b>PCR</b>	<b>Primer specificity and sequence data</b>	<b>DGGE resolution and general comments</b>
Drigo et al. (2007)	Soil: rhizosphere of <i>Carex</i> and <i>Festuca</i>	FR1-gc/FF390. Touchdown PCR for 37 cycles with 55-47 °C annealing. Expand High Fidelity polymerase.	Successful amplification, no sequence data.	Band number N.R. The greatest degree of variation in DGGE profiles was explained by soil origin and plant species, while elevated CO <sub>2</sub> level explained only 10% of the total variation.
Hoshino & Matsumoto (2007)	Soil: bulk soil and spinach rhizosphere, effects of fumigation to fungal community structure.	Direct PCR with FR1-gc/FF390 for 40 cycles with 50°C annealing. Nested protocol: 1 <sup>st</sup> PCR with AU2/AU4, 2 <sup>nd</sup> PCR with FR1-gc/FF390 for 30 cycles with 45°C annealing. KODplus polymerase.	Direct PCR with FR1-gc/FF390 amplified oomycete ( <i>Phytophthora</i> ) and plant ( <i>Spinacia</i> ) templates from rhizosphere as well as <i>Mortierella</i> (99.7%), <i>Myrothecium</i> (100%) and Chytridiomycota (96.8%). Bulk soil yielded no unspecific products <i>via</i> direct PCR. Nested protocol excluded products from plant and oomycete DNA, whereas amplified Cercozoa (84.8-94.2%), Metazoa (82%) and Fungi (Chytridiomycota, Zygomycota, Basidiomycota, Ascomycota, similarity range 83.7-100%).	Direct PCR products revealed 2 and 5 major bands in bulk and rhizosphere soil, while 11 and 14 bands were detected using nested PCR. Drastic change of nested PCR-DGGE profiles using chloropicrin treatment.
Kardol et al. (2007)	Soil: microcosm with ex-arable soil and grass cultures	FR1-gc/FF390. Touchdown PCR with 40 cycles and 55C-47°C annealing. Expand High Fidelity polymerase.	Successful amplification, no sequence data.	DGGE profiles with 3-7 detectable bands from inoculum samples and 3-13 bands from rhizosphere. High within-treatment variation. Plant biomass production was connected to composition of the dominant fungal species.
Lagomarsino et al. (2007)	Soil: effects of elevated CO <sub>2</sub> and N addition on soil fungi in a poplar plantation	FR1-gc/FF390. PCR as in study IV. BioTherm DNA polymerase.	Successful amplification, no sequence data.	DGGE profiles with 3-15 fungal bands. Clear separation between unfertilized and fertilized soils, independent of elevated CO <sub>2</sub> treatment.

<b>Study</b>	<b>Fungal community</b>	<b>PCR</b>	<b>Primer specificity and sequence data</b>	<b>DGGE resolution and general comments</b>
Ott et al. (2007)	Human coronary atherosclerotic plaques	Nested protocol: 1 <sup>st</sup> PCR with NS0/EF3, 2 <sup>nd</sup> PCR with FR1-gc/NS1 for 25 cycles with 48°C annealing. A third PCR with BF2/TR1 was used for cloning.	Successful amplification, sequence data from 320 clones affiliated with <i>Yarrowia</i> , <i>Candida</i> , <i>Gibberella</i> , <i>Rhodospordium</i> , <i>Rhodotorula</i> , <i>Ceratobasidium</i> , <i>Cryptococcus</i> , <i>Penicillium</i> , <i>Glyphium</i> , <i>Microxyphium</i> , <i>Fusarium</i> with sequence similarity of $\geq 97\%$ . 5% of the sequences could not be assigned to known fungal representatives.	The median fungal richness (number of DGGE bands) of all individuals was 5 (range from 2 to 9) and the overall interindividual variability of fungal profiles was high.
Sekiguchi et al. (2007)	Soil: effects of cattle manure and green manure on soil fungal community	FR1-gc/NS1. PCR for 35 cycles with 47 °C annealing. Ex Taq polymerase.	Successful amplification, sequence data from five DGGE bands related to Basidiomycota ( <i>Psathyrella</i> ), Ascomycota ( <i>Fusarium</i> , <i>Chaetomium</i> ) and Zygomycota ( <i>Mortierella</i> ) with a sequence similarity of 98-99%.	In total 18 distinctive fungal bands (12-16 bands in each lane) detected. Fungal profiles obtained from cattle manure did not differ significantly from the control. Fluctuations in the relative intensity of several bands observed due to green manure application.
Yergeau et al. (2007)	Soil: vegetated and fell-field soils from Falkland Island to the Antarctic Peninsula	FR1-gc/FF390. Touchdown PCR for 37 cycles with 55-47°C annealing. Expand High Fidelity polymerase.	Successful amplification, no sequence data.	Band number N.R. Little or no intraplot variation from five replicate samples. Vegetation type and latitude influences on the fungal community structure.
Sekiguchi et al. (2008)	Soil: comparative analysis of soils between organic and conventional cherry tomato farms	FR1-gc/NS1. PCR for 35 cycles with 47 °C annealing. Ex Taq polymerase.	Successful amplifications, sequence data from 30 DGGE bands related to <i>Mucor</i> , <i>Mortierella</i> , <i>Fusarium</i> , <i>Neocosmospora</i> , <i>Eleutherascus</i> , <i>Macrolepiota</i> , <i>Trichocladium</i> , <i>Chaetomium</i> , <i>Gibberella</i> , <i>Cordyceps</i> , <i>Sordaria</i> , <i>Aleuria</i> with a sequence similarity of 98-99%. One Metazoa ( <i>Enchytraeus</i> ) sequence.	Band number N.R. No obvious differences in fungal populations between organic and conventional systems of farming. Principal component analysis revealed several characteristic bands in the DGGE profiles.

<sup>a</sup> PCR conditions using Dynazyme II DNA polymerase and FR1-gc/NS1: 8 min at 95°C and 35 cycles of (30 s at 95°C, 45s at 47°C and 3 min at 72°C) and 10 min at 72°C; FR1-gc/FF390: 8 min at 95°C and 30 cycles of (30s at 95°C, 45s at 50°C, 2 min at 72°C) and 10 min at 72°C.

<sup>b</sup> Not reported by the authors

## APPENDIX 3

Taxonomical affiliation and chimera check analysis for the SSU rDNA sequences determined during study V.

DGGE band (OTU)	Rotstop response <sup>a</sup>	GenBank Accession	Chimera check point <sup>b</sup> / full length	Taxonomical affiliation	Accession	Similarity (Blast identity/ overlap) <sup>c</sup>
FF390-29	-	AF541991	60/351	<i>Helicogloea lagerheimii</i> (Atractiellales)	AY124476	100% (351/351)
NS1-27b	+	AF541993	1100/1647	<i>Dothideomycete</i> from <i>Larix</i> needles <i>Helicoma isiola</i> (Tubeufiaceae, Dothideomycetes) <i>Lophium mytilinum</i> (Mytiliniaceae, Dothideomycetes)	AY190261 AY856935 DQ678030	99.44% (1594/1603) 99.44% (1592/1601) 99.48% (1526/1534)
NS1-11b	+	AF541989	720/1613	<i>Botryobasidium obtusisporum</i> (Cantharellales) <i>B. simile</i> <i>B. botryosum</i>	DQ898739 DQ898740 AY662667	99.26% (1603/1615) 99.01% (1601/1617) 99.01% (1601/1617)
NS1-24a	+	AF541987	710/1611	<i>Tyromyces chioneus</i> (Polyporales)	AF334938	99.13% (1601/1615)
FF390-14	-	AF541990	130/354	<i>Resupinatus alboniger</i> (Tricholomataceae, Agaricales) <i>Panaeolus foenicisii</i> (Bolbitiaceae, Agaricales) <i>P. sphinctrinus</i> <i>Agaricus bisporus</i> (Agaricaceae, Agaricales)	DQ851586 DQ851578 DQ459375 AY787216	98.29% (345/351) 98.30% (346/352) 98.30% (346/352) 98.29% (345/351)
FF390-44	+	AF541992	130/350	Same as for OTU FF390-14		
NS1-17	-	AF541994	330/1649	<i>Cryptococcus</i> sp. (mitosporic Tremellales) <i>Fellomyces lichenicola</i> (mitosporic Tremellales) <i>Kockovaella schimae</i> (mitosporic Tremellales)	EF363152 AB032661 AB005482	97.34% (1610/1654) 97.28% (1608/1653) 97.28% (1607/1652)
FF390-30 <sup>d</sup>	+	AF541988	1-247+717-829 Intron 248-716	<i>Scytalidium lignicola</i> (Meliniomyces) <i>Chalara strobilina</i> (Pezizales) <i>Lipomyces kockii</i> (Saccharomycetales, Ascomycota)	AY762623 AF222516 DQ519007	98.61% (245/248+111/113) 98.06%(245/248+109/113) 72-74% (128/176+105/149)

<sup>a</sup> Rotstop response: + OTU was shown to increase due to Rotstop treatment; - OTU diminished due to Rotstop treatment.

<sup>b</sup> Chimera check point: sequence division point suggested by the Chimera check program (version 2.7) of the Ribosomal Database Project (RDP-II, Cole et al. 2003) website (<http://rdp8.cme.msu.edu/cgis/chimera.cgi>). Total sequence length is shown after the slash. All sequences showed similar affiliations using both fragments and were thus considered non-chimeric (data not shown).

<sup>c</sup> Blast identity/overlap: number of identical nucleotides and total overlap of the compared sequences provided by the NCBI Blast program.

<sup>d</sup> For OTU FF390-30 the sequence consisted of two separate segments with high similarities with the given reference sequences (Blast similarities for these segments are shown separately in parenthesis) and an intronic sequence of 469 bp between them.