View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Jukuri

Mycol. Res. **109** (1): 103–114 (January 2005). © The British Mycological Society
DOI: 10.1017/S0953756204001406 *Printed in the United Kingdom.*

Direct analysis of ribosomal DNA in denaturing gradients: application on the effects of *Phlebiopsis gigantea* treatment on fungal communities of conifer stumps

Eeva J. VAINIO, Anna-Maija HALLAKSELA, Katriina LIPPONEN and Jarkko HANTULA*

Finnish Forest Research Institute, P.O. Box 18, FIN-01301, Vantaa, Finland.
E-mail: jarkko.hantula@metla.fi

Received 3 November 2003; accepted 2 September 2004.

The aim of this study was to test the usefulness of direct PCR-amplification in analysing fungal diversity in stumps. The analysis was conducted on stumps treated against *Heterobasidion* spp. using a commercial formulation of *Phlebiopsis gigantea* (Rotstop), and carried out using denaturing gradient gel electrophoresis (DGGE) of small subunit (SSU) ribosomal DNA (rDNA) fragments PCR-amplified directly from wood DNA samples using two separate fungus-specific primer pairs. On average, two (range 0–9) different amplification products were observed by DGGE in single wood samples of approximately 500 mm^3. The PCR products were classified into operational taxonomical unit (OTU) groups based on their DGGE mobility. Six OTUs could be affiliated with a known species based on a reference fungal collection of 37 species: *Heterobasidion annosum, H. parviporum. Hypholoma capnoides*, *P. gigantea*, *Resinicium bicolor* and *Stereum sanguinolentum*. Sequence analyses did not give further identifications. OTU profiles from old (6 yr-old) and fresh (1-year-old) Scots pine and Norway spruce stumps from treated and untreated forest plots were compared statistically, and some significant differences were observed in the species composition between the treated and untreated plots. However, the frequency of most of the OTUs did not seem to be affected, and the treatment did not seem to have reduced the overall level of fungal diversity. Based on these results, direct PCR-amplification seems to be useful in analyses of fungal communities in decaying conifer stumps.

INTRODUCTION

Pathogenic fungi belonging to the genus *Heterobasidion* cause severe root and butt rot in conifers of the northern temperate regions. During summertime loggings, basidiospores of these species readily infect fresh stump surfaces, from where the infection can spread vegetatively into the stump roots and further to surrounding healthy trees using root contacts. However, the stump surface infections can be efficiently prevented using *Phlebiopsis gigantea* biocontrol treatment (Risbeth 1952, 1963, Hodges 1964, Greig 1976, Kallio & Hallaksela 1979, Korhonen *et al*. 1994), and in Finland such treatment by private forest owners is financially supported by the Government.

P. gigantea is one of the most common fungal species found in coniferous wood remains in the Boreal region, especially in managed forests (Käärik & Rennerfelt 1958, Meredith 1959, Kallio 1965, Petäistö 1978, Eriksson, Hjortstam & Ryvarden 1981). The spore infection of this fungus is very effective, and it is a highly competitive primary colonizer of wood. The use of a commercial formulation of *P. gigantea* (Rotstop) allows practically complete prevention of *Heterobasidion annosum* infections in Scots pine stumps (Korhonen *et al*. 1994). In Norway spruce stumps, the efficacy of *P. gigantea* treatment is somewhat lower (Lipponen 1991, Korhonen *et al*. 1994, Nicolotti, Gonthier & Varese 1999). There are also indications that the use of *P. gigantea* might reduce the frequency of certain other fungal species cultured from conifer stumps (Kallio 1971, Kallio & Hallaksela 1979). These species include among others *Stereum sanguinolentum*, *Peniophora pithya* and *Sistotrema brinkmannii*, all of which occur very commonly in decomposing conifer stumps. This suggests that large-scale treatments with *P. gigantea* might present an ecological threat in the form of decreasing the diversity of natural communities of wood-inhabiting fungi.

The survival of introduced *P. gigantea* strains has been monitored in North America using RAPD markers (Roy *et al*. 1997), and the presence of inoculated strains was demonstrated in red pine stumps one year after treatment. In Europe, we used RAMS genetic fingerprinting markers to detect a biocontol genotype of *P. gigantea* (Rotstop) in Norway spruce

* Corresponding author.

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0953756204001406

Table 1. Stump treatment experiments. The numbers of stumps analysed in each plot are given. 'Old' and 'fresh' refer to stumps from trees felled six and one years before sampling, respectively. Four randomly collected samples were analysed from each stump.

Treatment/ tree species	No. of old stumps in treated plots (OT)	No. of fresh stumps in treated plots (FP)	No. of old stumps in untreated plots (OU)	No. of fresh stumps in untreated plots (FU)	No. of old stumps in separate control area (OC)	No. of fresh stumps in separate control area (FC)
Scots pine	8	4	8	4	–	5
Norway spruce	6	3	6	3	5	5

stumps six years after treatment (Vainio, Lipponen & Hantula 2001). However, the effect of *P. gigantea* inoculum on the overall diversity of natural communities of other stump decomposing fungi still remains unresolved.

Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified small subunit (SSU) ribosomal DNA (rDNA) fragments has proven to be a suitable method for the analysis of complex bacterial as well as eukaryal communities (Ferris, Muyzer & Ward 1996, Heuer *et al.* 1997, Vallaeys *et al.* 1997, Kowalchuk, Gerards & Woldendorp 1997a, Kowalchuk *et al.* 1997b, Marsh *et al.* 1998, Smalla *et al.* 1998, Smit *et al.* 1999, Vainio & Hantula 2000a,). The SSU rDNA gene shows very limited within-species variation, but different species can usually be separated from each other (Berbee & Taylor 1993, Olsen & Woese 1993, Mitchell, Roberts & Moss 1995). Using DGGE, SSU rDNA-fragments can be differentiated according to their sequences (in addition to length polymorphisms), and several different sequence types obtained from a single sample can be analysed simultaneously (Muyzer, de Waal & Uitterlinden 1993).

The direct PCR amplification of DNA extracted from environmental samples is not affected by the lack of suitable culturing media, or by slow-growing species being overgrown by faster ones. This method also gives quantitative information, as the amount of DNA obtained from each sample should directly reflect the amount of fungal mycelia it contains, although it is at least theoretically sensitive to differences in the amplification efficacies between different DNA fragments.

In this study the DGGE method was used to analyse fungal SSU rDNA fragments PCR-amplified directly from wood DNA samples. Species profiles obtained from treated and untreated Scots pine and Norway spruce stumps were compared to investigate whether the direct analysis could be applied in determining whether the use of Rotstop had reduced the diversity of wood-inhabiting microfungal communities.

MATERIAL AND METHODS

Stump treatment experiments

Two stands both located in Taipalsaari (southeastern Finland; 62° N, 28° E) were used. The Scots pine stand consisted of eight different plots, four of which had been treated with a commercial preparation of *Phlebiopsis gigantea* (Rotstop, Kemira Oy) during a thinning on 10 June 1992, while four plots had been left untreated. A stump treatment device fitted to a harvester made by a Finnish manufacturer, Ponsse, was used to apply the Rotstop preparation. A second felling was made on 6 August 1997, consisting of one tree aged about fifty years from each test plot, and the resulting stumps were left untreated. During the second felling, five trees were also cut from a separate Scots pine stand with a comparable tree structure and habitat located next to the test stand. The resulting stumps were used as outer controls in order to compare the degree of natural geographic variation to the effect of Rotstop treatment. In total, 29 stumps were sampled approximately one year after the second felling on 9 Sept. 1998 as shown in Table 1. The term pretreated is used below when referring to the fresh untreated stumps within the treated plots.

The Norway spruce test stand included six different plots: three had been treated with Rotstop during thinnings on 4 June and 2 July 1992, and three had been left untreated. In addition, five trees had been cut from a separate control Norway spruce stand located next to the test stand. During the second felling (August 6th, 1997), one tree aged about 70 years was cut from each test plot and five trees were felled from the control stand. All the resulting stumps were left untreated. In total, 28 stumps were sampled one year after the second felling (9 Sept. and 1 Dec. from the test stand and control stands, respectively) as shown in Table 1.

Sample preparation and DNA extraction

The outermost layer (*ca* 5 cm) of the stumps was discarded (in order to avoid fungi accidentally occurring on the stump, but not growing in the wood) and discs cut below the fresh surface were used for sampling.

The sample discs were stored in +4 °C for 1–5 d prior to sampling. Wood chips were carved with a sterile knife from inside the discs after the outer surface had been removed, and *ca* 500 mm^3 of wood material was taken for each sample. Four samples were collected randomly from different parts of each disc (in total 228 samples from 57 stumps) and stored in −80 ° prior to DNA extraction.

Downloaded from https:/www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https:/www.cambridge.org/core/terms.
https://doi.org/10.1017/S0953756204001406

Extraction of DNA directly from wood samples was carried out using a multistep procedure described in Vainio & Hantula (2000a). This procedure includes homogenization of the wood chips in eppendorf tubes using a sterilised glass rod, metal tweezers and quartz sand, and lysis of the fungal cells in extraction buffer. The lysate was purified with phenol and chloroform extractions followed by the High Pure PCR Template Preparation Kit (Boehringer Mannheim, IN), and selective precipitation of the DNA using polyethylene glycol.

PCR amplification and denaturing gradient gel electrophoresis (DGGE)

PCR amplifications of the partial SSU rDNA fragments were carried out as described in Vainio & Hantula (2000a) using primer pairs FR1+NS1 and FR1+FF390. Primer pair FR1+NS1 was used to produce long PCR-fragments covering most of the entire SSU rDNA molecule (*ca* 1650 bp) and primer pair FR1+FF390 for obtaining short fragments of *ca* 390 bp. A GC-clamp (Vainio & Hantula 2000a) was used in primer FR1 to allow optimal separation of the fragments in DGGE. The amplification products were analysed by the D GENE™ system (Bio-Rad) as described in Vainio & Hantula (2000a).

In order to be able to determine the relative migration rates of the amplification products and to ensure the reproducibility of the denaturing gradients, a collection of five (primer pair FR1+NS1) or six (FR1+FF390) PCR-amplification products from different fungal species was used as a mobility standard. The amplification products were initially named and classified into a mobility group using these standard species (the information about the initial groups may be useful in becoming studies, and is therefore given in this paper, although the initial grouping itself was not used in the statistical analyses). For primer pair FR1+NS1 the initial groups were named as follows: L, faster than *Peniophora pithya*; P, slower than *P. pithya*; G, as *Phlebiopsis gigantea* or slower; H, as *H. annosum* or slower; N, as *Nectria* sp. or slower; and T, as *Trichoderma* sp. or slower. For primer pair FR1+FF390 the initial groups were named as follows: L, faster than *Heterobasidion annosum*; H, as *H. annosum* or slower; G, as *P. gigantea* or slower; C, as *Chondrostereum purpureum* or slower; A, as *Amylostereum areolatum* or slower; B, as *Armillaria borealis* or slower; O, as *Armillaria ostoyae* or slower; or S, very slow. After this, the bands were compared on parallel lanes until they could be classified into a specific operational taxonomical unit (OTU). As all individual species cannot be differentiated from each other when only one primer pair is considered (Vainio & Hantula 2000a), further subgroups were also determined based on unique OTU combinations obtained by comparing the two separate banding patterns produced from each sample using the two different primer pairs.

For the identification of species, a reference fungal collection including the following wood-inhabiting fungi was used: *Amylocystis lapponica*, *Amylostereum areolatum*, *A. chailletii*, *Armillaria borealis*, *A. cepistipes*, *A. ostoyeae*, *Ascocoryne* sp., *Bjerkandera adusta*, *Chondrostereum purpureum*, *Coniophora arida*, *Cylindrobasidion evolvens*, *Exophiala* sp., *Fomitopsis pinicola*, *Gliocladium* sp., *Heterobasidion annosum*, *H. parviporum*, *Hypholoma capnoides*, *Ischnoderma benzoinum*, *Merulius lacrymans*, *Nectria* sp., *Neobulgaria premnophila*, *Panellus mitis*, *Penicillium* sp., *Peniophora pithya*, *Phaeolus schweinitzii*, *Phialophora* sp., *Phlebiopsis gigantea*, *Polyporus borealis*, *P. brumalis*, *Resinicium bicolor*, *Rhinocladiella* sp., *Sistotrema brinkmannii*, *Stereum sanguinolentum*, *Trametes zonata*, *Trichaptum* sp., *Trichoderma* sp., *Verticicladiella procera*. The collection numbers for the fungal isolates are listed in Vainio & Hantula (2000a).

For example OTU NS1-26b (with an initial code N3) migrated as fast or slower than *Nectria* sp. (it was the third fastest of those bands), and could be separated from OTU NS1-26a with primer pair FR1+FF390 (and therefore corresponds to OTU FF390-8 with an initial code of H7). As a band with similar mobility was also amplified from a culture of *Stereum sanguinolentum*, we concluded that OTU NS1-26b and FF390-8 correspond to this species.

Cloning and sequencing

Directly amplified PCR-fragments were cloned in pCR 2.1 plasmids with Original TOPO TA cloning kit (Invitrogen, Groningen) according to the manufacturers recommendations. The correct clone was selected by comparing the migration of cloned inserts to that of the original amplification product in DGGE (for details see Vainio & Hantula 2000b).

Statistical analyses

Only frequencies of each band was measured (and thus not differences in band intensities). Fisher's exact test was used for analysing whether the DGGE band distributions (frequencies of different OTUs) showed statistical differences according to the plot treatment. The frequency of each OTU among all plots representing a certain treatment type (treated, untreated or control) and stump type (fresh pines, old pines, fresh spruces, old spruces) was determined, and the obtained distribution profiles were compared using the Fisher's exact test of the StatXact program (Mehta & Patel 1991).

Shannon-Weaver and Gini heterogeneity indices (Peet 1974) were used for analysing whether the Rotstop treatment had influenced the general diversity of the OTU profiles. Statistical differences between the heterogeneity index profiles were tested using the Student's t-test.

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.
https://doi.org/10.1017/S0953756204001406

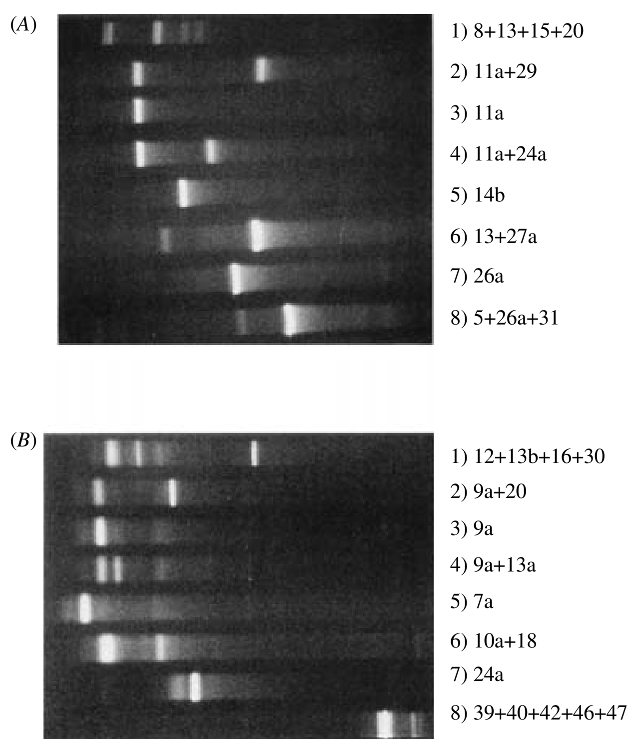

Fig. 1. Examples of amplification products from different wood samples obtained using primer pair FR1+NS1 (*A*) or FR1+FF390 (*B*). The bands were classified into OTU groups as listed after the lane number. Lanes (1) old treated pine, plot 4 (p4), stump 1 (st1), sample 1 (sa1); (2) fresh untreated pine, p4, sa4; (3) fresh control pine, st1, sa2; (4) old control spruce, st 3, sa2; (5) old treated spruce, p1, st2, sa3; (6) old treated spruce, p3, st2, sa3; (7) fresh pretreated spruce, p1, sa2; (8) fresh pretreated spruce, p3, sa1.

RESULTS

Occurrence of fungal amplification products within the wood samples

The highest number of different amplification products observed by DGGE in an individual wood chip sample was nine (found from one old untreated pine stump using primer pair FR1+NS1, and from one fresh pretreated spruce stump using primer pair FR1+FF390). Generally, the average number of bands amplified from each sample was somewhat higher using primer pair FR1+FF390 (average 2.25) compared to FR1+NS1 (1.89, Table 4). Examples of band profiles amplified from different samples are shown in Fig. 1.

The percentage of colonised samples containing at least one detectable band varied from 70–100% (average 86.2%) depending on the plot treatment and stump age, usually being higher within the treated plots compared to the untreated or control plots (Table 4). Accordingly, the average number of bands amplified from each sample was generally highest within the treated plots. Among the pine samples, fewer amplification products were obtained from fresh stumps (an average of 1.20 or 1.33 bands per sample using primer pair FR1+NS1 or FR1+FF390, respectively) than old stumps (2.78 or 2.67). In contrast, the average number of bands amplified from each spruce sample was higher within the fresh stumps (an average of 2.06 or 2.98 bands using primer pair FR1+NS1 or FR1+FF390, respectively) compared to old stumps (1.80 or 2.16).

Classification of PCR-products into different DGGE mobility groups (OTUs)

Using primer pair FR1+NS1, the amplification products could be divided into 38 different OTUs (OTUs NS1-1 to NS1-38, Table 2), while 48 different OTUs were identified for primer pair FR1+FF390 (OTUs FF390-1 to FF390-48, Table 3). In order to identify OTU combinations corresponding to an individual species, samples which produced only one amplification product with both primer sets were considered. If the same unique OTU combination (for example NS1-14 and FF390-5) occurred as a single PCR-product in at least two separate samples from different stumps, this combination was considered to represent a unique species (single observations were not taken into account as they could occur randomly due to possible differences in the amplification efficacy of the two different primer sets). Using these criteria, six different unique OTU combinations could be identified (Tables 2–3): NS1-11/FF390-9 (Fig. 1, lanes 3), NS1-14/FF390-5, NS1-24/FF390-13 (Fig. 1, lanes 4, upper bands), NS1-26/FF390-24 (Fig. 1, lanes 7), NS1-26/FF390-8 and NS1-27/FF390-10 (Fig. 1, lanes 6, upper band/lower band, respectively). Based on these combinations, the corresponding OTU groups could be further divided into subgroups (groups NS1-11a, 14c, 24a, 26a, 26b and 27a, and groups FF390-9a, 5a, 13a, 24a, 8 and 10a).

Identification of species

A reference collection containing 37 different species of wood-inhabiting fungi was used for comparing the OTU groups with known species. Based on these comparisons, four of the unique OTU combinations could be affiliated with a specific reference species: NS1-26/FF390-8 with *Stereum sanguinolentum*; NS1-27/FF390-10 with *Hypholoma capnoides*; NS1-26/FF390-24 with *Resinacium bicolor*; and NS1-11/FF390-9 with *Phlebiopsis gigantea*. In addition to this, the occurrence of *Heterobasidion annosum* and *H. parviporum* was checked, although the corresponding OTU combinations (NS1-14/FF390-6 and NS1-14/FF390-7, Tables 2–3) were not found as single PCR products from two separate samples as described above for the unique OTU combinations. The remaining reference species could not be identified as clear OTU combinations among the mixed samples containing more than one amplification product.

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0953756204001406

Frequency of the OTUs among different samples

Frequencies of the different OTUs varied considerably, ranging from single observations (11 or 9 OTUs using primer pair FR1+NS1 or FR1+FF390, respectively) to OTUs that accounted for 11% of all bands analysed (OTU NS1-11a). Six common OTUs, each of which accounted for over 5% of all bands, and occurred in more than 10% of the samples, were observed using primer pair FR1+NS1 (NS1-11a, 13, 26c, 11b, 33 and 8, listed from the largest group to the smallest one). Using the same criteria, four common OTUs (FF390-9a, 44, 41, 16) could be identified for primer pair FR1+FF390.

Identified species

Phlebiopsis gigantea formed the most common individual OTU group for both primer pairs (NS1-11a or FF390-9a), and occurred in 20.6% of all samples. It was found very frequently within fresh pine stumps (occurring in 43, 56, and 65% of all samples within the fresh pretreated, fresh untreated and fresh control stumps, respectively). However, it was not found among any of the old pine stumps (whether treated or untreated). Within fresh spruce stumps, *P. gigantea* occurred in 17, 33 and 20% of all samples within the fresh pretreated, fresh untreated and fresh control stumps, respectively, while the corresponding percentages for old spruce stumps were 0 (old treated), 17 (old untreated) and 20 (old control). Thus, the fungus could not be found from any of the old spruce stumps that had been treated six years before the sample collection, although it occurred within the untreated and control stumps.

The remaining OTU combinations that could be affiliated with a specific reference species were found relatively rarely, and no clear difference was observed between the treated and untreated plots. *Heterobasidion annosum* had colonised 3.5% of all samples, and it was found both from treated and untreated old pine and spruce stumps, and also from fresh pretreated spruce samples. *H. parviporum* had infected old spruce stumps within the untreated and control plots as well as fresh pretreated spruce samples, its overall occurrence being 2.2% of all samples. *Resinicium bicolor* (occurring in 6.1% of all samples) seemed to occur frequently in old treated pine stumps, but on the other hand it was relatively common in fresh control spruce stumps. *S. sanguinolentum* had colonised 2.2% of all samples, and was found only in untreated fresh and old spruce stumps, while *H. capnoides* was found only in old stumps (both pine and spruce), but its frequency (4.8% of all samples) did not seem to be correlated with the Rotstop treatment.

Unidentified species

The majority of the common OTU groups could not be identified as a specific species, and most of them seemed to occur more frequently in old stumps compared to fresh ones. Thus, OTUs NS1-8, NS1-11b, NSI-13, NS1-26c and FF390-44, were most frequent within old pine stumps, and the first three groups were also found commonly within old spruce stumps. In addition, OTU FF390-16 was common in old spruce stumps. Only two of the common unidentified OTUs (NS1-33, FF390-41) were most frequent within fresh pine stumps.

The frequency of most of the OTUs did not seem to be correlated with the Rotstop treatment (Tables 2–3). However, there were a few OTUs that appeared to get either more abundant or rarer due to the treatment. Thus, OTUs NS1-11b and FF390-30 seemed to be more common in treated old spruce stumps compared to the untreated ones, while OTU FF390-14 appeared to suffer from the treatment within old pine stumps. Other groups that seemed to a certain extent respond positively to the treatment include NS1-27b, FF390-39, FF390-41, FF390-44, and possibly declined groups include NS1-15, NS1-17, and FF390-29. When both primer pairs were considered, one of the unidentified OTU combinations (NS1-24/FF390-13) seemed to be more frequent in treated old spruce stumps compared to the untreated ones (occurring in 42, 12.5 and 5% of samples within the treated, untreated and control plots, respectively).

Sequences of OTUs affected by Rotstop treatment

Some of the DGGE-bands, the occurrence of which seemed to be most clearly affected by the Rotstop treatment, were cloned and sequenced. None of these sequences were 100% identical to the GenBank database of known sequences and therefore no further identifications were made. However, the taxonomic relatives of these OTUs could be identified. OTU FF390-30 (GenBank accession no, AF541988) had a similarity of 99% to *Chlara strobilina* (AF222516), which is a mitosporic ascomycete. OTU NS1-24a (AF541987) was related with 98% similarity to several species of the basidiomycetous order *Aphyllophorales*: *Tyromyces chioneus* (AF334938), *Amauroderma* sp. (AF255199), *Ganoderma boninense* (AF255198), and *Anthrodia xantha* (AF334902). The sequence of OTU NS1-11b (AF541989) had a similarity of 98% to *Botryobasidium isabellinum* (AF026610) and *B. subcoronatum* (AF026609), which belong to the basidiomycetous order *Stereales*. The sequences of OTU FF390-14 (AF541990) and FF390-44 (AF541992) were highly similar (99%) to each other, and were related with 98% similarity to basidiomycetous fungi in several orders: *Boletales* (*Coniophora olivacea* (AJ488905) and other *Coniophora* species), *Aphyllophorales* (*Ossicaulis lignatilis* (AF334923) and *Tretopileus sphaerophorus* AB006005)), *Nidulariales* (*Crucibulum laeve* (AF026624)), and *Agaricales* (*Agaricus bisporus* (L36658)). OTU FF390-29 (AF541991) had a similarity of 98% to *Helicogloea variabilis*, which belongs to basidiomycetous order *Atractiellales*. OTU NS1-27b

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.
https://doi.org/10.1017/S0953756204001406

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0953756204001406

Table 2. Classification of PCR amplification products obtained using primer pair FR1+NS1 into different OTU groups based on DGGE. The unsplit groups are printed in bold, and subgroups are separated by letters. The OTU groups affiliated with known reference species are indicated with letter combinations printed in italics: *Pg*=*P. gigantea*; *Ha*=*H. annosum*; *Hp*=*H. parviporum*; *Rb*=*R. bicolor*; *Ss*=*S. sanguinolentum*; *Hc*=*H. capnoides*.

			Plot treatment											
			Scots pine					Norway spruce						
			1 yr-old (fresh)			6 yr-old (old)		1 yr-old (fresh)			6 yr-old (old)			
			Pretreated (FP)	Untreated (FU)	Control (FC)	Treated (OT)	Untreated (OU)	Pre-treated (FP)	Un-treated (FU)	Control (FC)	Treated (OT)	Un-treated (OU)	Control (OC)	∑
No. of stumps			4	4	5	8	8	3	3	5	6	6	5	57
No. of samples			16	16	20	32	32	12	12	20	24	24	20	228
OTU	Initial group	Species												
NS1-1.	**A1**		0	0	0	0	0	1	2	0	0	0	0	3
NS1-2.	**A2**		0	0	0	0	0	0	1	0	0	0	0	1
NS1-3.	**P1**		0	0	0	0	0	2	3	0	0	1	0	6
NS1-4.	**P2**		0	0	0	0	1	0	0	0	0	0	0	1
NS1-5.	**P3a**		0	0	0	1	0	4	0	0	0	0	0	5
NS1-6.	**P3b**		0	0	0	2	0	0	0	0	1	0	0	3
NS1-7.	**P4**		**0**	0	0	2	0	0	0	0	0	1	1	4
NS1-8.	**P5**		**1**	0	0	5	9	0	0	1	8	1	1	26
NS1-9.	**P6**		**0**	0	0	0	1	0	0	0	1	0	0	2
NS1-10.	**P7**		0	1	0	0	0	0	0	0	0	0	0	1
NS1-11a.	G0/G0	*Pg*	7	9	13	0	0	2	4	4	0	4	4	47
NS1-11b.	G0/not-G0		0	0	0	9	13	0	0	0	10	1	2	35
NS1-12.	**G1**		**0**	0	0	0	1	0	0	0	0	0	0	1
NS1-13.	**G2**		**0**	0	0	13	7	2	3	1	5	2	9	42
NS1-14a.	H0/H0	*Ha*	0	0	0	2	2	1	0	0	1	2	0	8
NS1-14b.	H0/H5	*Hp*	0	0	0	0	0	2	0	0	0	2	1	5
NS1-14c.	H0/L5		0	0	0	0	0	0	0	0	1	5	0	6
NS1-14d.	H0/not-H0,H5,L5		0	0	0	2	2	0	1	4	1	0	8	18
NS1-15.	**H1**		**0**	0	0	2	7	0	2	0	1	0	0	12
NS1-16.	**H2a**		**0**	0	0	0	0	0	0	0	0	1	0	1
NS1-17.	**H2b**		**0**	0	0	1	6	0	0	0	3	0	0	10
NS1-18.	**H3**		**0**	0	0	0	1	0	0	0	0	0	0	1
NS1-19.	**H4**		**0**	0	0	0	0	0	0	1	0	0	0	1
NS1-20.	**H5**		**0**	0	0	1	0	0	0	0	0	0	0	1
NS1-21.	**H6**		**0**	0	0	2	0	0	0	0	0	0	0	2
NS1-22.	**H7**		**0**	0	0	0	1	0	0	0	0	0	0	1
NS1-23.	**H8**		**0**	0	0	4	0	0	0	0	0	0	0	4
NS1-24a.	N0/C0		0	0	0	3	1	0	0	0	10	3	1	18
NS1-24b.	N0/not-C0		0	0	0	2	4	1	0	0	0	0	0	7
NS1-25.	**N1**		**0**	0	0	0	2	0	0	0	0	0	0	2

NS1-26a.	N3/B0	*Rb*	0	0	0	5	0	1	0	6	1	1	0	14
NS1-26b.	N3/H7	*Ss*	0	0	0	0	0	0	1	0	0	3	1	5
NS1-26c.	N3/not-B0,H7		0	1	0	7	15	5	3	0	2	1	2	36
NS1-27a.	N6a/G1	*Hc*	0	0	0	4	3	0	0	0	0	3	1	11
NS1-27b.	N6a/not-G1		6	1	1	1	3	0	1	0	0	0	3	16
NS1-28.	**N6b**		**0**	0	0	0	0	0	1	0	0	0	0	1
NS1-29.	**N7a**		**2**	2	0	0	1	4	3	0	0	0	0	12
NS1-30.	**N7b**		**0**	0	0	4	0	0	0	0	0	0	0	4
NS1-31.	**T0**		**1**	0	0	1	2	8	5	0	3	0	1	21
NS1-32.	**T1**		**0**	0	0	4	0	0	0	0	0	0	0	4
NS1-33.	**T2a**		**9**	4	2	1	6	0	2	0	3	0	0	27
NS1-34.	**T2b**		**0**	0	1	0	0	0	0	0	0	0	0	1
NS1-35.	**T3a**		0	0	0	2	0	0	0	0	0	0	0	2
NS1-36.	**T3b**		0	0	0	0	0	0	0	0	2	0	0	2
NS1-37.	**T4**		0	0	0	0	0	0	0	0	1	0	1	2
NS1-38.	**T5**		0	0	0	4	5	0	0	0	0	0	0	9

(AF541993) was related with 98% similarity to ascomycetous fungi (*Dothideomycetes* et *Chaetothyriomycetes* incertae sedis) *Phyllosticta pyrolae* (AB041250), *Guignardia endophyllicola* (AB041249), other *Guinardia* species and *Bulgaria inquinans* (AJ224362; order *Leotiomycetes*). The sequence of OTU NS1-17 (AF541994) was most similar (97%) to that of *Kockovaella scimae* (AB005482) and *Fellomyces lichenicola* (AB032661), which are basidiomycetous fungi belonging to order *Tramellales*.

Statistical comparison of OTU profiles between the treated and untreated plots

Due to the two separate primer pairs used, two different OTU groupings were obtained, both consisting of unsplit groups and subgroups (Tables 2–3). The statistical analyses were conducted using two different methods: the Fisher's exact test was used to reveal differences in the species composition (i.e. OTU profiles), while the heterogeneity indices (Shannon-Weaver and Gini) were used to measure general species diversity (both species richness and evenness).

Among the fresh pine stumps, the pretreated and control plots revealed to be statistically different in their overall diversity using primer pair FR1+FF390 (Table 6). However, no statistical differentiation was observed between the pretreated and untreated plots, and an even more similar species composition (as indicated by the Fisher's exact test, Table 5) was found between the untreated and control plots, although the control plots showed a lower overall diversity (Table 6).

Among the old pine stumps, the treated and untreated plots differed significantly from each other based on the Fisher's exact test (Table 5). However, the heterogeneity indices showed no statistical differentiation, although a slightly lower level of diversity was observed within the treated plots.

Among the fresh spruce stumps, statistically significant differentiation was observed between the control plots compared to both untreated and pretreated plots (see Tables 5 and 6). This was based on the control plots showing a low level of diversity, which was reflected to both the heterogeneity indices (especially Shannon-Weaver) and the Fisher's exact test. In contrast, the pretreated and untreated plots did not differ significantly. As with the fresh pine stumps, differences in the species composition were highest between the pretreated and control plots (Table 5).

Among the old spruce stumps, the heterogeneity indices were quite similar between the different treatments, but in this case the untreated plots showed the lowest diversity. According to the Fisher's exact test, a high differentiation was observed in the species composition between the treated and control as well as the treated and untreated plots (Table 5). Significant differences were also found between the species compositions of untreated and control plots.

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0953756204001406

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0953756204001406

Table 3. Classification of PCR amplification products obtained using primer pair FR1+FF390 into different OTU groups based on DGGE. The unsplit groups are printed in bold, and subgroups are separated by letters. The OTU groups affiliated with known reference species are indicated with letter combinations printed in italics: *Pg*, *Phlebiopsis gigantea*; *Ha*, *Heterobasidion annosum*; *Hp*, *H. parviporum*; *Rb*, *Resinacium bicolor*; *Ss*, *Stereum sanguinolentum*; *Hc*, *Hypholoma capnoides*.

			Plot treatment											
			Scots pine					Norway spruce						
			1 yr-old (fresh)			6 yr-old (old)		1 yr-old (fresh)			6 yr-old (old)			
			Pretreated (FP)	Untreated (FU)	Control (FC)	Treated (OT)	Untreated (OU)	Pre-treated (FP)	Un-treated (FU)	Control (FC)	Treated (OT)	Un-treated (OU)	Control (OC)	∑
No. of stumps			4	4	5	8	8	3	3	5	6	6	5	57
No. of samples			16	16	20	32	32	12	12	20	24	24	20	228
OTU	Initial group	Species												
FF390-1.	**L1**		0	0	0	0	0	0	0	0	1	0	0	1
FF390-2.	**L2**		0	0	0	0	0	0	0	0	2	0	0	2
FF390-3.	**L3**		0	0	0	2	0	0	0	0	0	1	3	6
FF390-4.	**L4**		0	0	0	0	0	0	0	0	0	0	1	1
FF390-5a.	L5/H0		0	0	0	0	0	0	0	0	1	5	0	6
FF390-5b.	L5/not-H0		0	0	0	0	4	0	0	0	0	0	0	4
FF390-6a.	H0/H0	*Ha*	0	0	0	2	2	1	0	0	1	2	0	8
FF390-6b.	H0/not-H0		0	0	0	2	7	1	1	0	6	0	0	17
FF390-7a.	H5/H0	*Hp*	0	0	0	0	0	2	0	0	0	2	1	5
FF390-7b.	H5/not-H0		1	0	0	0	0	1	2	0	0	1	2	7
FF390-8.	H7/N3	*Ss*	0	0	0	0	0	0	1	0	0	3	1	5
FF390-9a.	G0/G0	*Pg*	7	9	13	0	0	2	4	4	0	4	4	47
FF390-9b.	G0/not-G0		0	0	0	2	0	0	0	0	0	0	0	2
FF390-10a.	G1/N6a	*Hc*	0	0	0	4	3	0	0	0	0	3	1	11
FF390-10b.	G1/not-N6a		0	0	0	1	0	0	0	1	0	0	0	2
FF390-11.	**G2**		0	0	0	1	0	2	1	3	0	0	3	10
FF390-12.	**G3**		0	0	0	1	0	0	0	0	0	0	0	1
FF390-13a.	C0/N0		0	0	0	3	1	0	0	0	10	3	1	18
FF390-13b.	C0/not-N0		0	0	0	1	0	0	2	0	0	0	0	3
FF390-14.	**C2**		0	0	0	1	10	0	1	0	2	0	3	17
FF390-15.	**A0**		0	0	0	0	4	0	1	0	3	0	0	8
FF390-16.	**A1**		0	1	0	3	9	0	0	0	9	1	7	30
FF390-17.	**A2**		0	0	0	2	0	0	0	1	0	0	1	4
FF390-18.	**A3**		0	0	0	3	3	1	1	0	0	1	3	12
FF390-19.	**A4**		0	0	0	3	6	0	1	2	2	0	4	18
FF390-20.	**A5a**		2	1	0	5	7	0	1	0	4	0	0	20
FF390-21.	**A5b**		0	0	0	0	2	0	0	0	3	0	0	5
FF390-22.	**A6a**		0	0	0	2	5	0	0	0	0	1	0	8
FF390-23.	**A6b**		0	0	0	0	0	0	0	2	0	0	2	4
FF390-24a.	B0/N3	*Rb*	0	0	0	5	0	1	0	6	1	1	0	14
FF390-24b.	B0/not-N3		0	0	0	1	0	0	1	0	5	1	0	8
FF390-25.	**B5**		0	0	0	0	0	0	0	0	1	0	0	1

FF390-26.	B6	0	0	0	0	1	0	0	0	1	0	0	2
FF390-27.	01	0	0	0	0	1	0	0	0	1	0	0	2
FF390-28.	02	0	0	0	1	0	0	0	0	0	0	1	2
FF390-29.	03	0	0	0	0	6	0	0	0	0	0	0	6
FF390-30.	04	0	0	0	3	2	0	0	0	9	0	0	14
FF390-31.	06	0	0	0	1	0	0	0	0	0	1	2	4
FF390-32.	07a	0	0	0	0	0	0	0	0	0	1	0	1
FF390-33.	07b	0	1	0	5	3	0	0	0	1	0	1	11
FF390-34.	07c	0	0	0	0	0	0	0	0	1	1	0	2
FF390-35.	08a	0	0	0	0	0	1	0	0	0	1	0	2
FF390-36.	08b	0	0	0	1	0	0	0	0	0	0	0	1
FF390-37.	09	1	1	0	1	5	0	1	0	1	0	0	10
FF390-38.	011	0	0	0	0	1	0	0	0	0	0	0	1
FF390-39.	S1	0	0	0	0	0	8	3	0	0	0	0	11
FF390-40.	S2	0	0	0	0	0	7	3	0	0	0	0	10
FF390-41.	S3	10	4	2	5	3	2	4	0	2	0	1	33
FF390-42.	S4a	0	0	0	0	0	6	3	0	0	0	0	9
FF390-43.	S4b	0	0	0	0	0	0	0	0	0	1	0	1
FF390-44.	S5	6	1	0	10	13	2	4	0	0	1	0	37
FF390-45.	S7	4	0	0	0	2	3	3	0	0	0	0	12
FF390-46.	S8	2	1	0	0	0	6	2	0	1	0	0	12
FF390-47.	S9	0	0	0	0	0	7	3	0	1	0	0	11
FF390-48.	S10	0	0	0	0	0	0	0	0	1	0	0	1

DISCUSSION

Mature decay fungus communities usually develop a mosaic structure (patch-like distribution of separate individuals) due to antagonistic mycelial interactions (Rayner & Boddy 1988). In this study, however, up to nine different PCR amplification products were observed by DGGE in a single 500 mm^3 wood sample (the average number being approximately two). This suggests that the samples were often occupied by more than one fungal mycelium, as the successful amplification of DNA from wood samples probably requires more DNA than what can be practically extracted from single spores or single hyphal branches. Thus, it seemed that many of the fungi had colonised relatively small patches of wood or possibly even intermingled within the same wood region. This kind of distribution is characteristic to many microfungal non-decay fungi, that may occupy small interaction zones between the mycelia of decay fungi (Rayner & Boddy 1988). In addition, some of the bands might represent species that are difficult to cultivate or are easily overgrown by faster-growing species on agar plates.

The average number of bands amplified from each sample was somewhat higher using primer pair FR1+FF390 compared to FR1+NS1, which might indicate a higher amplification efficacy (possibly due to termination of the amplification product of FR1+NS1 during the PCR). However, the larger number of OTU groups obtained using primer pair FR1+FF390 indicated also a higher DGGE separation capacity for this primer pair, which is consistent with previous results (Vainio & Hantula 2000a). Thus, the smaller number of bands obtained using primer pair FR1+NS1 may be partly due to some of the amplification products remaining undetected by DGGE due to having identical migration rates despite minor sequence variations. The higher DGGE separation capacity probably also explains the higher level of overall diversity revealed using primer pair FR1+FF390. This was indicated by the heterogeneity indices and also by the Fisher's exact test that usually showed higher statistical differentiation between the treatments using primer pair FR1+FF390 compared to FR1+NS1.

All the OTU combinations that could be affiliated with a specific reference species (*Stereum sanguinolentum*, *Hypholoma capnoides*, *Resinicium bicolor*, *Phlebiopsis gigantea*, *Heterobasidion annosum* and *H. parviporum*) represented very common wood-decay fungi. The frequent occurrence of *P. gigantea* within the fresh pine stumps and the somewhat lower colonisation of fresh spruce stumps is consistent with previous studies (Käärik & Rennerfelt 1958, Kallio 1965, Petäistö 1978). However, the Rotstop treatment had not increased the overall occurrence of *P. gigantea* within the next stump generation (fresh untreated stumps within the previously treated plots). Furthermore, *P. gigantea* could not be found from any of the old spruce stumps that had been treated six years

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.
https://doi.org/10.1017/S0953756204001406

Table 4. Occurrence of amplification products in the wood samples. Colonised samples contained at least one amplification product, but blank samples did not produce any bands in PCR amplification.

Tree species	Stump age	Stump treatment type	No. of Samples	No. of Blank samples	Percentage of colonised samples	Average no. of bands per sample NS1	Average no. of bands per sample FF390
Spruce	1	pretreated	12	0	100.00	2.67	4.42
Spruce	1	untreated	12	3	75.00	2.67	3.58
Spruce	1	control	20	6	70.00	0.85	0.95
Spruce	6	treated	24	0	100.00	2.25	2.92
Spruce	6	untreated	24	2	91.67	1.29	1.46
Spruce	6	control	20	1	95.00	1.85	2.10
Pine	1	pretreated	16	2	87.50	1.63	2.06
Pine	1	untreated	16	4	75.00	1.13	1.19
Pine	1	control	20	6	70.00	0.85	0.75
Pine	6	treated	32	3	90.63	2.63	2.22
Pine	6	untreated	32	2	93.75	2.94	3.13
Sum			228	29			
Average					86.23	1.89	2.25

Table 5. Statistical comparison of species compositions between different treatments using the Fisher's exact test (exact *P*-value or Monte Carlo estimate[b] of *P*-value). Statistically significant probabilities are printed in bold.

Host tree	Stump age	Stump treatment types compared	*P*-value FR1+NS1	*P*-value FR1+FF390
Pine	1	pretreated (4) – untreated (4) – control (4)	0.1916	0.1509
Pine	1	pretreated (4) – untreated(4)	0.2259	0.2194
Pine	1	pretreated (4) – control (4)	0.1133	0.0602
Pine	1	untreated (4) – control (4)	0.7653	0.9840
Pine	6	treated (8) – untreated (8)	**0.0013**[b]	**0.0012**[b]
Spruce	1	pretreated (3) – untreated (3) – control (3)	**0.0033**	**0.0042**[b]
Spruce	1	pretreated (3) – untreated (3)	0.2944	0.4490
Spruce	1	pretreated (3) – control (3)	**0.0002**	**0.0000**
Spruce	1	untreated (3) – control (3)	**0.0025**	**0.0019**
Spruce	6	treated (5) – untreated (5) – control (5)	**0.0000**[b]	**0.0000**[b]
Spruce	6	treated (6) – untreated (6)	**0.0000**	**0.0000**
Spruce	6	treated (5) – control (5)	**0.0000**	**0.0000**
Spruce	6	untreated (5) – control (5)	**0.0015**	**0.0049**

Table 6. Average values of Shannon-Weaver and Gini indices for the treated/pretreated, untreated and control plots and statistical comparison between the treatments. Symbols +, – and c are used for the treated/pretreated, untreated and control plots, respectively. The heterogeneity index value is followed by superscript symbols ($^{+-c}$), which indicate treatments that did not differ statistically significantly ($P>0.05$) from the current value (for example, value 1.056^{-c} indicates that this index obtained from the pretreated plots did not differ significantly from the untreated or control plots). The statistically significant values are also printed in bold. The stump numbers used for the index calculations correspond to those given in Table 5.

Host tree	Stump age	Primer used with FR1	Shannon-Weaver Treated/ pretreated (+)	Shannon-Weaver Untreated (–)	Shannon-Weaver Control (c)	Gini Treated/ pretreated (+)	Gini Untreated (–)	Gini Control (c)
Pine	1	NS1	1.056^{-c}	0.918^{+c}	0.470^{+-}	0.618^{-c}	0.514^{+c}	0.278^{+-}
Pine	1	FF390	**1.201**$^{-}$	0.976^{+c}	**0.300**$^{-}$	**0.669**$^{-}$	0.531^{+c}	**0.205**$^{-}$
Pine	6	NS1	2.190^{-}	2.247^{+}	–	0.859^{-}	0.879^{+}	–
Pine	6	FF390	2.244^{-}	2.305^{+}	–	0.884^{-}	0.888^{+}	–
Spruce	1	NS1	1.737^{-}	1.987^{+}	**1.059**	0.792^{-c}	0.849^{+c}	0.639^{+-}
Spruce	1	FF390	2.126^{-}	2.083^{+}	**1.290**	0.869^{-}	0.863^{+}	**0.722**
Spruce	6	NS1	1.686^{-c}	1.268^{+c}	1.295^{+-}	0.791^{-c}	0.685^{+c}	0.737^{+-}
Spruce	6	FF390	1.993^{-c}	1.408^{+c}	1.767^{+-}	0.851^{-c}	0.709^{+c}	0.814^{+-}

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.
https://doi.org/10.1017/S0953756204001406

before the sample collection, although it occurred within the untreated and control stumps. It must be noted, that based on culturing experiments, *P. gigantea* (more specifically, the Rotstop genotype) could be isolated from these stumps when typical orange-brown decay columns were selected for the analysis (Vainio *et al*. 2001). Thus, the fungus occurred only sporadically within the stumps when six years had passed from the treatment, and was therefore not detected among the random wood samples. Within the old pine stumps, *P. gigantea* had probably disappeared altogether during the decay succession, as it was not found in any of the samples whether treated or untreated. In contrast, *H. annosum* was found both in treated and untreated old pine and spruce stumps. Thus, the treatment had not completely prevented the occurrence of this fungus within the treated stumps, while *H. parviporum* was found only in the untreated old spruce stumps.

Most of the largest OTU groups could not be identified as a specific species. This might be partly due to some of the reference species remaining undetected among the mixed samples containing more than one amplification product. In addition, the reference collection used in this study was somewhat biased towards primary decomposer species, while most of the unidentified common OTU groups seemed to occur more commonly in old stumps. Thus, some of the unidentified groups probably represented species that are characteristic to the later stages of stump decomposition (Käärik & Rennerfelt 1958, Meredith 1960).

Based on statistical tests, the Rotstop treatment did not appear to have caused long-lasting effects that would have influenced diversity of new fungal infections to fresh pine and spruce stumps when six years had passed from the treatment. On old spruce and pine stumps the treatment appeared to have influenced mainly the species composition of the stumps, but not reduced their overall fungal diversity. Thus, while some OTU groups seemed to suffer from the treatment (four OTUs), other groups appeared to favour treated plots over the untreated ones (seven OTUs). It therefore seems that although Rotstop acts as a strong competitor during the primary succession of the stumps, the wood material decomposed by *P. gigantea* offers a suitable substrate for certain species.

In conclusion, the analysis of fungal communities in conifer stumps by DGGE analysis of directly amplified SSU rDNA turned out to be a fruitful method. It may be considered as another view (in addition to mycelial isolations) to fungal diversity, and therefore can also be used to complement the more traditional approach. The only drawback we observed was the inability to identify fungi based on BLAST search in GenBank. This should, however, be a temporary problem as DNA databases are developing rapidly. However, it must be noted that SSU rDNA may not allow the separation of all closely related species due to its conservative nature. As a biological conclusion, the stump treatment did not seem to have reduced the overall fungal diversity within the treated plots, and the usage of Rotstop is therefore not likely to produce a highly monomorphic stump microbiota. However, the data analysed here was relatively small, and therefore cannot be considered as comprehensive. Therefore possible changes in the species composition of fungal communities should be continually monitored in treated forests, especially if large-scale stump treatments covering several tree generations are carried out.

ACKNOWLEDGMENTS

We acknowledge Marja-Leena for skilful technical assistance, and Kari Korhonen for valuable comments during the study. The study was financially supported by the Maj and Tor Nessling foundation and the University of Helsinki.

REFERENCES

Berbee, M. L. & Taylor, J. W. (1993) Dating the evolutionary radiations of the true fungi. *Canadian Journal of Botany* **71**: 1114–1127.

Eriksson, J., Hjortstam, K. & Ryvarden, L. (1981) *The Corticiaceae of North Europe*. Vol. 6. *Phlebia-Sarcodontia*. Fungiflora, Oslo.

Ferris, M. J., Muyzer, G. & Ward, D. M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology* **62**: 340–346.

Greig, B. J. W. (1976) Biological control of *Fomes annosus* by *Peniophora gigantea*. *European Journal of Forest Pathology* **6**: 65–71.

Heuer, H., Krsek, M., Baker, B., Smalla, K. & Wellington, E. (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* **63**: 3233–3241.

Hodges, C. S. (1964) The effect of competition by *Peniophora gigantea* on the growth of *Fomes annosus* in stumps and roots. *Phytopathology* **54**: 623.

Käärik, A. & Rennerfelt, E. (1958) Investigations on the fungal flora of spruce and pine stumps. *Meddelander från Statens Skogsforskningsinstitut* **47**(7): 1–88.

Kallio, T. (1965) Studies on the biology of distribution and possibilities to control *Fomes annosus* in southern Finland. *Acta Forestalia Fennica* **78**: 1–18.

Kallio, T. (1971) Protection of spruce stumps against Fomes annosus (Fr.) Cooke by some wood-inhabiting fungi. *Acta Forestalia Fennica* **117**: 1–20.

Kallio, T. & Hallaksela, A.-M. (1979) Biological control of *Heterobasidion annosum* (Fr.) Bref. (*Fomes annosus*) in Finland. *European Journal of Forest Pathology* **9**: 298–308.

Korhonen, K., Lipponen, K., Bendz, M., Johansson, M., Ryen, I., Venn, K., Seiskari, P. & Niemi, M. (1994) Control of *Heterobasidion annosum* by stump treatment with 'Rotstop', a new commercial formulation of *Phlebiopsis gigantea*. *Proceedings of the 8th International Conference on Root and Butt Rots*: 675–685.

Kowalchuk, G. A., Gerards, S. & Woldendorp, J. W. (1997a) Detection and characterization of fungal infections of *Ammophila arenaria* (Marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. *Applied and Environmental Microbiology* **63**: 3858–3865.

Kowalchuk, G. A., Stephen, J. R., De Boer, W., Prosser, J. I., Embley, T. M. & Woldendorp, J. W. (1997b) Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0953756204001406

DNA fragments. *Applied and Environmental Microbiology* **63**: 1489–1497.

Lipponen, K. (1991) Stump infection by *Heterobasidion annosum* and its control in stands at the first thinning stage. *Folia Forestalia* **770**: 1–12.

Marsh, T. L., Liu, W.-T., Forney, L. J. & Cheng, H. (1998) Beginning a molecular analysis of the eukaryal community in activated sludge. *Water Science and Technology* **37**: 455–460.

Mehta, C. & Patel, N. (1991) *StatXact – Statistical Software for Exact Nonparametric Inference – User Manual*. Version 2. Cytel Software Cambridge, MA.

Meredith, D. S. (1959) The infection of pine stumps by *Fomes annosus* and other fungi. *Annals of Botany* **23**: 455–476.

Meredith, D. S. (1960) Further observations on fungi inhabiting pine stumps. *Annals of Botany* **24**: 63–78.

Mitchell, J. I., Roberts, P. J. & Moss, S. T. (1995) Sequence of structure? A short review on the application of nucleic acid sequence information to fungal taxonomy. *Mycologist* **9**: 67–75.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695–700.

Nicolotti, G., Gonthier, P. & Varese, G. C. (1999) Effectiveness of some biocontrol and chemical treatments against *Heterobasidion annosum* on Norway spruce stumps. *European Journal of Forest Pathology* **29**: 339–346.

Olsen, G. J. & Woese, C. R. (1993) Ribosomal RNA: a key to phylogeny. *FASEB Journal* **7**: 113–123.

Peet, R. K. (1974) The measurement of species diversity. *Annual Review of Ecology and Systematics* **5**: 285–307.

Petäistö, R.-L. (1978) *Phlebia gigantea* and *Heterobasidion annosum* in pine stumps on cutting areas in Suomenniemi and Savitaipale. *Folia Forestalia* **373**: 1–9.

Rayner, A. D. M. & Boddy, L. (1988) *Fungal Decomposition of Wood: its biology and ecology*. John Wiley & Sons, Bath.

Risbeth, J. (1952) Control of *Fomes annosus* Fr. *Forestry* **25**: 41–50.

Risbeth, J. (1963) Stump protection against *Fomes annosus*. III. Inoculation with *Peniophora gigantea*. *Annals of Applied Biology* **52**: 63–77.

Roy, G., Cormier, M., Hamelin, R. C. & Dessureault, M. (1997) Comparison of RAPD technique and somatic incompatibility tests for the identification of *Phlebiopsis gigantea* strains. *Canadian Journal of Botany* **75**: 2097–2104.

Smalla, K., Wachtendorf, U., Heuer, H., Liu, W.-T. & Forney, L. (1998) Analysis of BIOLOG GN substrate utilization patterns by microbial communities. *Applied and Environmental Microbiology* **64**: 1220–1225.

Smit, E., Leeflang, P., Glandorf, B., van Elsas, J. D. & Wernars, K. (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology* **65**: 2614–2621.

Vainio, E. J. & Hantula, J. (2000a) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycological Research* **104**: 927–936.

Vainio, E. J. & Hantula, J. (2000b) Genetic differentiation between European and North American population of *Phlebiopsis gigantea*. *Mycologia* **92**: 436–446.

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.

Vallaeys, T., Topp, E., Muyzer, G., Macheret, V., Laguerre, G., Rigaud, A. & Soulas, G. (1997) Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiology Ecology* **24**: 279–285.

Corresponding Editor: N. Hallenberg

Downloaded from https://www.cambridge.org/core. Open University Library, on 21 Jan 2017 at 20:07:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.
https://doi.org/10.1017/S0953756204001406