

Relationship between wood-inhabiting fungi determined by molecular analysis (denaturing gradient gel electrophoresis) and quality of decaying logs

Tiina Rajala, Mikko Peltoniemi, Taina Pennanen, and Raisa Mäkipää

Abstract: We investigated the fungal communities inhabiting decaying logs in a seminatural boreal forest stand in relation to host tree species, stage of decay, density, diameter, moisture, C to N ratio, Klason lignin content, and water- and ethanol-soluble extractives. Communities were profiled using denaturing gradient gel electrophoresis fingerprinting of the rDNA ITS1 region coupled with sequencing of fungal DNA extracted directly from the wood. In addition, polypore fruit bodies were inventoried. Logs from different tree species had different fungal communities and different physicochemical properties (e.g., C to N ratio, density, ethanol extractives, and diameter). Ascomycetes comprised a larger portion of communities inhabiting deciduous birch (*Betula* spp.) and European aspen (*Populus tremula* L.) logs compared with those living on coniferous Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* L.). A relationship between mycelial community structure and density of decaying spruce logs suggested a succession of fungi with mass loss of wood. The fruit body inventory underestimated fungal diversity in comparison with the culture-free denaturing gradient gel electrophoresis analysis that also detected inconspicuous but important species inhabiting decaying wood.

Résumé : Nous avons étudié les communautés de champignons lignicoles dans les billes en décomposition dans un peuplement forestier boréal semi-naturel en relation avec l'espèce d'arbre hôte, le stade de décomposition, la densité, le diamètre, l'humidité, le rapport C à N, le contenu en lignine Klaxon ainsi que les produits d'extraction solubles dans l'eau et solubles dans l'éthanol. Le profilage des communautés a été effectué à l'aide d'une technique d'empreinte génomique de la région ITS1 de l'ADNr par électrophorèse en gel de gradient dénaturant couplée au séquençage de l'ADN fongique extrait directement du bois. De plus, les fructifications des polypores ont été inventoriées. Les billes de différentes espèces d'arbres contenaient différentes communautés fongiques et avaient différentes propriétés physico-chimiques (p. ex. le rapport C à N, la densité, les produits d'extraction à l'éthanol et le diamètre). Les ascomycètes constituaient une plus grande proportion des communautés lignicoles dans les billes des essences feuillues comme le bouleau (*Betula* spp.) et le peuplier européen (*Populus tremula* L.) comparativement aux communautés lignicoles des essences résineuses comme l'épicéa commun (*Picea abies* (L.) Karst.) et le pin sylvestre (*Pinus sylvestris* L.). Une relation entre la structure des communautés mycéliennes et la densité des billes d'épicéa en décomposition indiquait qu'il y avait une succession de champignons en lien avec la perte de masse du bois. L'inventaire des fructifications sous-estimait la diversité fongique comparativement à l'analyse par électrophorèse en gel de gradient dénaturant qui ne nécessitait pas de mise en culture et qui détectait aussi les espèces lignicoles dans le bois en décomposition qui, quoiqu'importantes, passent inaperçues.

[Traduit par la Rédaction]

Introduction

Fungal decomposers and dead wood play an important role in forest ecosystems in the context of biodiversity, CO₂ dynamics, nutrient cycling, and forest regeneration (Rayner and Boddy 1988; Stenlid et al. 2008). Decaying wood sustains biodiversity by providing habitats and energy for fungi, insects, and many other organisms. The total number of saprophytic species in Finland is estimated at 4000–5000, of which roughly a third are fungi (Siitonen 2001). As an increasing amount of Finnish forestland becomes intensively managed, the amount of coarse wood debris, and conse-

quently the number of wood decomposers, is expected to decline. In Finland, 37% of assessed polypore species are already classified as threatened or near threatened and the reduction of suitable habitat is suspected to be the main cause (Rassi et al. 2001; Siitonen 2001). The effects that this loss of diversity will have on ecosystem processes, such as decomposition and nutrient recycling, are poorly understood.

Evaluating the potential impacts of forest management and climate change to fungal diversity and decomposition requires an understanding of their habitat preferences and succession dynamics. Previous laboratory experiments have

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indicated that fungal species affect wood decomposition rate (Worrall et al. 1997; Hakala et al. 2004; Chi et al. 2007) and that while species richness can increase the rate (Tiunov and Scheu 2005; Toljander et al. 2006), interactions between fungi may have a negative impact on the process (Fukami et al. 2010). Studies of fungal habitats and succession have shown that climatic conditions and the amount and quality of dead-wood substrate (e.g., tree species, log size, stage of decay, amount of bark, moisture content, history of fungal infections, type of stem breakage) affect the fungal community (e.g., Renvall 1995; Lumley et al. 2001; Hottola and Siitonen 2008; Jönsson et al. 2008; Fukasawa et al. 2009a, 2009b; Fukami et al. 2010). However, our understanding of wood-decomposing fungi is mainly limited to those that form visible fruit bodies and those that can be cultured in the laboratory. Consequently, many important species may escape detection.

Molecular methods provide a window into the fungal community living inside logs (Jasalavich et al. 2000; Vainio and Hantula 2000a; Adair et al. 2002) that can be hidden from traditional approaches. Fungal DNA can be extracted directly from wood and selectively amplified with specific polymerase chain reaction (PCR) primers. Culture is not required, and therefore, those species that are difficult to grow in vitro can be detected. In this way, direct DNA analyses provide a more comprehensive and instantaneous description of the fungal community compared with fruit body inventories and culture isolation studies. Furthermore, the importance of fungi that form fruit bodies in relation to those that remain as mycelia in decaying logs is unclear. Culture-free molecular methods have been used to study wood-inhabiting fungal communities in boreal forest ecosystems (Johannesson and Stenlid 1999; Allmér et al. 2006; Kulháňková et al. 2006) but our knowledge of communities of wood-inhabiting fungi and their relation to substrate quality remains in its infancy.

Our objective was to investigate the entire fungal community inhabiting decaying wood in a boreal forest stand. Explicitly, we asked (i) is substrate quality related to the community structure of wood-inhabiting fungi, (ii) is the state of decomposition connected to the community structure of wood-inhabiting fungi, and (iii) are communities of wood-inhabiting fungi dominated by polypore species? We studied variation among fungal communities inhabiting decaying logs in a seminatural boreal forest dominated by Norway spruce (*Picea abies* (L.) Karst). A gradient of decomposition was established using logs that had fallen at different times and were consequently at different stages of decay. Fungal community was investigated by amplifying total DNA extracted directly from decomposing wood with selective primers and analyzing the products via denaturing gradient gel electrophoresis (DGGE) fingerprinting and sequencing. The occurrence of polypore fruit bodies was also recorded.

Material and methods

Study site

The study site is an unmanaged seminatural forest in Evo, southern Finland (67°90'N, 34°01'E; altitude 155 m). Mean annual precipitation is 489 mm and the effective tempera-

ture sum is 1189 °C (with threshold of +5 °C). Mean temperatures in January and July are -7 and 16.5 °C, respectively. The study site represents a mesic heath forest according to Cajander's site type classification (Cajander 1926; Hotanen et al. 2008) and the dominant tree species is Norway spruce. The size of the sampled plot was 1600 m². Tree stand characteristics are presented in Table 1. Classification of decay stage was based on hardness of the logs (see Table 2 footnote) (Mäkinen et al. 2006).

Sampling and fruit body inventory

In October 2007, we took wood samples from all dead fallen logs within the study plot with a diameter >5 cm at breast height (1.3 m). Logs represented different decay stages and thus their soil contact varied. A total of 37 logs were sampled of which 18 were Norway spruce, four were Scots pine (*Pinus sylvestris* L.), 10 were birch (*Betula* spp.), and five were European aspen (*Populus tremula* L.) (Table 2). Discs of approximately 5 cm thickness were removed from each log using a handsaw at breast height or 1 m if breast height could not be determined (Fig. 1). The diameter of each sample disc was measured. Discs were packed into plastic bags and stored at -20 °C until processing. In the laboratory, sawdust samples of frozen discs were taken using an electric drill (bit diameter 8–10 mm) (Fig. 1). The outermost layers of the discs were removed before drilling and the drill bit was sterilized between samples by flaming with ethanol. The resulting sawdust and shavings were collected and stored at -20 °C until DNA extraction (see DNA extraction and PCR section) and chemical analyses (see Wood physicochemical analyses section).

Fruit bodies of all polypores and three corticoid and one hydroid basidiomycete growing in the study plot on dead and live wood were recorded (Appendix A, Table A1) (note that all target species in the fruit body inventory are considered polypores in this paper). Fruit bodies were identified in the field or transported to the laboratory for microscopic examination. In addition, fruit bodies of 23 species found at the study site were collected as DNA reference samples.

Molecular analyses

DNA extraction and PCR

Total DNA was extracted from sawdust samples (50–100 mg) using an E.Z.N.A.TM SP Plant DNA Mini kit (Omega Bio-tek, Inc., Norcross, Georgia). Homogenization and cell lysis were carried out using quartz sand and a Fast-Prep[®] cell disrupter (Qbiogene, CEDEX, France) for 3 × 20 s at 4 m·s⁻¹ and incubating at 65 °C for 60 min. Otherwise, the manufacturer's instructions were followed. If deemed necessary, DNA was further purified by precipitating with 0.6 volume of 20% (w/v) polyethylene glycol 8000 – 5 mol·L⁻¹ NaCl followed by incubation on ice for 20 min and centrifugation (16 000g) for 20 min. Pelleted nucleic acids were then washed with 70% (v/v) ethanol, dried, and resuspended in TE-buffer (6 mmol·L⁻¹ Tris-HCl (pH 8.0) and 1 mmol·L⁻¹ EDTA). DNA was extracted from fruit body reference samples according to Vainio et al. (1998). A total of 50 DNA samples of 23 species were prepared. The protocol included cell disruption, three phenol – chloroform – isoamyl alcohol (25:24:1) extractions, chloro-

Table 1. Tree stand characteristics at the 1600 m² study site in Evo, Finland.

	No. of trees	Volume, m ³	Diameter at breast height, cm
Dead logs			
Norway spruce (<i>Picea abies</i>)	21	2.71	11.8 (1.3)
Scots pine (<i>Pinus sylvestris</i>)	5	0.64	13.0 (1.3)
Birch (<i>Betula</i> spp.)	10	1.72	16.0 (0.7)
European aspen (<i>Populus tremula</i>)	5	3.2	23.2 (6.5)
Dead snags			
Norway spruce	20	2.24	12.2 (0.9)
Scots pine	2	0.25	12.9 (2.2)
Birch	2	0.94	21.7 (0.3)
European aspen	1	0.61	24.7 (0)
Living trees			
Norway spruce	105	46.1	20.6 (0.7)
Scots pine	9	7.2	28.2 (2.1)
Birch	16	10.3	25.2 (1.4)
European aspen	10	13.2	28.1 (3.7)

Note: Values are means with standard error in parentheses.

Table 2. Number of sampled logs of each species, their stage of decay, diameter, and fungal diversity.

Tree species	Decay stage	No. of logs	Diameter at breast height, cm	Richness ₁	Richness ₂
Norway spruce (<i>Picea abies</i>)	1	10	11.8 (1.6)	0.6 (0.2)	5 (0.7)
	2	4	7.7 (1.6)	1.7 (0.3)	8.0 (3.1)
	3	4	15.4 (5.0)	2 (0.6)	7.3 (1.3)
	All (1–3)	18	12.1 (1.4)	1.1 (0.2)	6.2 (0.8)
Scots pine (<i>Pinus sylvestris</i>)	1	1	16.9 (0)	2 (0)	9 (0)
	2	1	14.6 (0)	2 (0)	15 (0)
	3	2	10.5 (6.5)	1.5 (0.5)	7.5 (1.5)
	All (1–3)	4	13.0 (1.3)	2 (0.3)	9.8 (1.9)
Birch (<i>Betula</i> spp.)	1	1	25.0 (0)	3 (0)	1 (0)
	2	1	20.8 (0)	0 (0)	2 (0)
	3	3	16.2 (1.6)	1.3 (0.9)	4.3 (0.3)
	4	3	14.1 (1.7)	0.3 (0.3)	7.3 (0.7)
	5	2	11.8 (0.4)	0 (0)	9.5 (1.5)
	All (1–5)	10	16.0 (1.4)	0.8 (0.4)	5.7 (1.0)
European aspen (<i>Populus tremula</i>)	1	1	47.5 (0)	1 (0)	6 (0)
	2	2	15.9 (2.9)	1 (1.0)	8 (2.0)
	3	2	18.4 (6.7)	1.5 (1.5)	3.5 (0.5)
	All (1–3)	5	23.2 (6.5)	1.2 (0.6)	5.8 (1.2)

Note: Values are means with standard errors in parentheses. Log decay stage was estimated using the following classification: (1) wood hard, bark intact, a knife penetrates only a few millimetres into the wood, (2) wood relatively hard, bark often still intact, a knife penetrates 1–2 cm into the wood, (3) wood relatively soft, bark partly lost, a knife penetrates 3–5 cm into the wood, (4) wood soft, bark mainly lost, a knife penetrates totally into the wood, and (5) wood very soft, can be moulded by hand, often only a slight rise on the ground remains (e.g., Mäkinen et al. 2006). Richness₁ is the average number of fungal species on a log according to polypore fruit bodies; richness₂ is the average number of DGGE bands in a sawdust sample of a single log.

form – isoamyl alcohol (24:1) extraction, precipitation with polyethylene glycol, and drying.

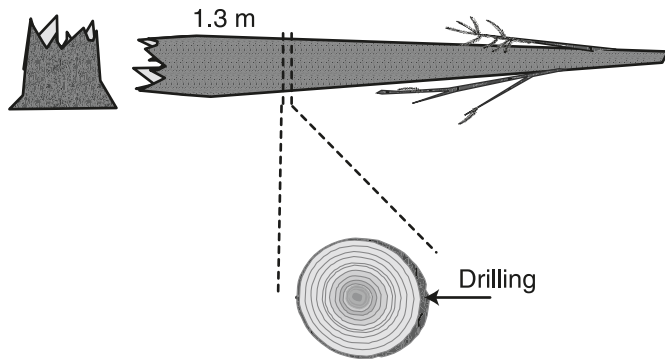
The internal transcribed spacer 1 (ITS1) regions of the rDNA were amplified via PCR using ITS1F (Gardes and Bruns 1993) and ITS2 (White et al. 1990) primers. A GC clamp (Vainio and Hantula 2000b; Korkama et al. 2006) was added to the 5' end of the ITS1F primer to generate PCR products suitable for DGGE analysis. PCR was carried out in a 50 µL reaction containing 5 µL of 10× reaction buffer (75 mmol·L⁻¹ Tris–HCl (pH 9.0), 2 mmol·L⁻¹ MgCl₂, 50 mmol·L⁻¹ KCl, 20 mmol·L⁻¹ (NH₄)₂SO₄), 200 µmol·L⁻¹ each dNTP, 0.5 µmol·L⁻¹ each primer, and 0.2 U of Biotools DNA polymerase (B&M Laboratories, Madrid, Spain). Cycling parameters were an 8 min hot-start at 95 °C followed

by 35 cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 10 min. All PCR products were electrophoresed on 1% (w/v) agarose gels in 1× TAE buffer (40 mmol·L⁻¹ Tris–HCl (pH 8) and 1 mmol·L⁻¹ EDTA), stained with ethidium bromide, and visualized with UV light.

Denaturing gradient gel electrophoresis analysis

DGGE analyses of DNA fragments of wood-inhabiting fungal mycelia and fruit bodies were carried out using the DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, California). Polyacrylamide gels (7.5% (w/v) acrylamide–bisacrylamide (37.5:1)) were prepared with an 18%–58% denaturing gradient using a gradient for-

Fig. 1. Wood samples of decaying logs were taken by removing a disc from each log and drilling them from the outer surface through the sapwood and heartwood.



mer (model 485) (Bio-Rad Laboratories) and a peristaltic pump (Minipuls 3) (Gilson SAS, Villiers-le-Bel, France) with a flow rate of $4.8 \text{ mL}\cdot\text{min}^{-1}$. An equal amount of each PCR product was loaded onto the gel and electrophoresis was performed in $1\times$ TAE buffer at 75 V and 60°C for 16 h. Gels were stained with SYBR[®] Gold (Molecular Probes, Eugene, Oregon) and visualized with blue light on a SafeImager[™] transilluminator (Invitrogen, Carlsbad, California). Bands at the same position in the gel are hereafter referred to as an operational taxonomic unit (OTU). Several bands of each OTU were excised from DGGE gels of sawdust samples. Excised bands were placed separately into $100 \mu\text{L}$ of sterile water and left at 4°C overnight. Eluted bands were then reamplified with 23 cycles and rerun in DGGE against the initial samples to check that only the target bands were amplified. If not, the band of interest was re-excised, reamplified, and rerun in DGGE. Bands that could not be isolated after a maximum of five excisions remained unidentified.

DNA sequencing

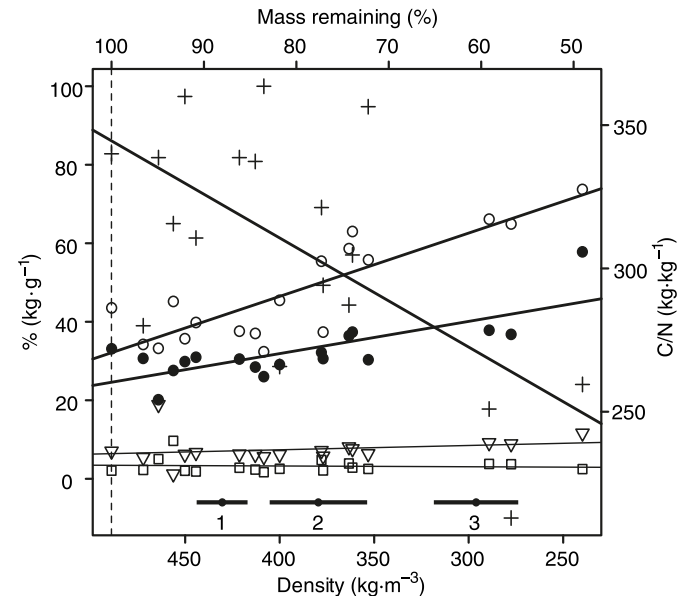
A total of 117 excised DGGE bands were amplified with primer pair ITS1F-ITS2 as described above. PCR products were purified (HighPure PCR Product Purification Kit) (Roche, Mannheim, Germany) and sequenced with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, California) using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit and PCR primers. Sequence editing and assembly was performed with Geneious Pro 4.7 software (Biomatter Ltd, Auckland, New Zealand) (Drummond et al. 2009). Fungal sequences were identified via comparison with the GenBank (NCBI) database using the BlastN algorithm (Altschul et al. 1997). Ninety-two original sequences were deposited with GenBank (accession Nos. HMO15671–HMO15762).

Wood physicochemical analyses

To study the relationship between wood quality and fungal community structure, the sawdust samples and the wood discs were subjected to physicochemical analyses described below. Relationships between the measured attributes are shown in Fig. 2.

Organic chemical fractions of dead wood were determined gravimetrically by separating ethanol and hot water extractives (polar compounds, e.g., carbohydrates and phe-

Fig. 2. Quality of Norway spruce (*Picea abies*) logs (left y-axis: solid circles, Klason lignin; open circles, moisture; squares, water extractives; triangles, ethanol extractives; right y-axis: crosses, C/N) as a function of density; note the reversed x-axis. Mean densities of logs in decay stages 1, 2, and 3 are marked above the lower x-axis and the low horizontal lines indicate standard errors of these means. The upper x-axis shows the remaining mass of logs in the course of decomposition assuming that all logs had initially the same density as the densest log of the sample set.



nolics) and a H_2SO_4 -insoluble fraction (Klason lignin). We followed a modified procedure of Ryan et al. (1990) and Wieder and Starr (1998), which is described more fully in Karsisto et al. (2003) and Vávřová et al. (2009). Briefly, air-dried and milled sawdust samples were sonicated with ethanol, vacuum filtered, dried, and weighed. The residue was then sonicated with water followed by filtration, drying, and weighing. Extractive-free samples were hydrolysed with 72% H_2SO_4 for a 60 min sonication and with 4% H_2SO_4 for 60 min at 125°C and 1.2 bar (120 kPa). After filtration and drying, the remaining mass was assumed to contain lignin plus ash.

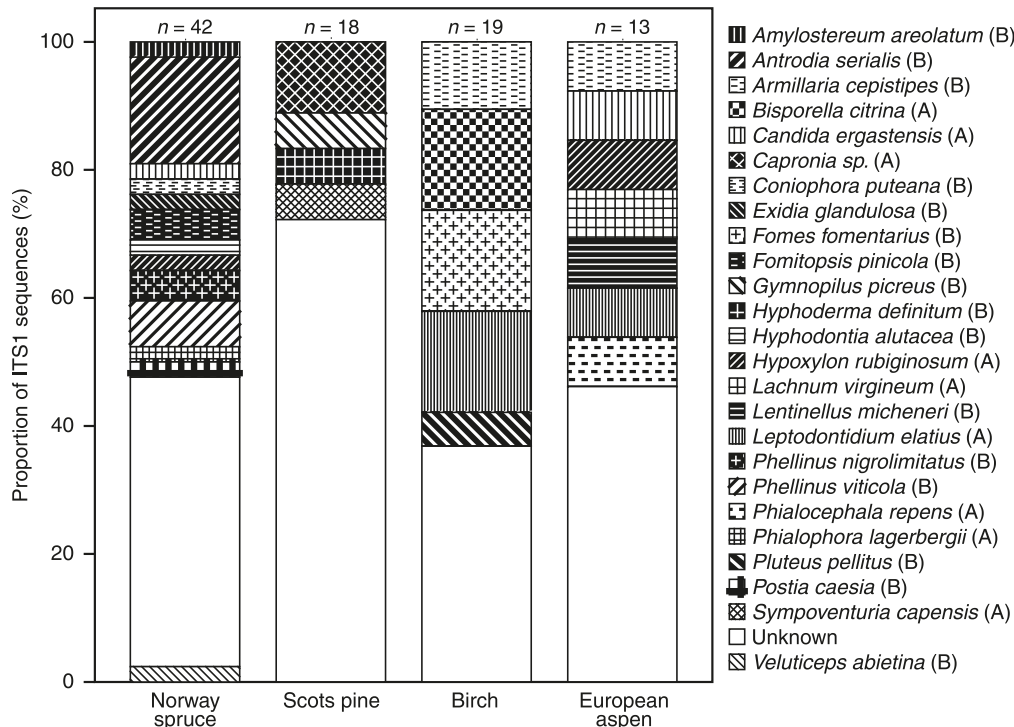
Total C and N contents of dead wood were determined from air-dried and milled samples by a dry combustion method using the LECO CHN-1000 elemental analyzer (ISO 10694, ISO 13878) (Finnish Forest Research Institute, Central Laboratory, Vantaa, Finland).

Wood density was measured by the water-displacement method (Olesen 1971) as described by Mäkinen et al. (2006). Briefly, discs were soaked in water and the volume was recorded as displaced water. Dry mass of the sample discs was determined and the density was calculated as the dry mass of the sample divided by its volume. Prior to soaking, samples were fresh weighed to determine their moisture content at the time of sampling.

Data analyses

DGGE gel images were analyzed using GelCompar II software version 5.1 (Applied Maths BVBA, Belgium) and a binary matrix (presence-absence data) was produced with a band-matching optimization of 0% and band position tol-

Fig. 3. Species of wood-inhabiting fungi detected inside fallen logs of Norway spruce (*Picea abies*) ($n = 18$), Scots pine (*Pinus sylvestris*) ($n = 4$), birch (*Betula* spp.) ($n = 10$), and European aspen (*Populus tremula*) ($n = 5$). DNA sequences of the ITS1 region <97% similar to species in GenBank were marked as unknown. A and B in parentheses after species names refer to ascomycete and basidiomycete, respectively. Values above the bars correspond to the number of fungal sequences obtained from each tree species.



erance of 1%. A binary matrix of polypore species found as fruit bodies was constructed by counting the occurrence of a species on a log or snag as a single record regardless of the number observed.

Variation in fungal communities among decaying logs was analyzed by nonmetric multidimensional scaling (NMDS) using the Jaccard distance measure. We chose to use three- and four-dimensional solutions after considering the decrease in stress value with increasing dimensions. GAM surfaces of wood quality factors were fitted to ordination plots. A relationship between fungal community structure and wood quality was explored further with canonical correspondence analysis in which the marginal significance of parameters was estimated with a permutation test (i.e., significance of a parameter when all other parameters are already in the model). For fungal DGGE data and polypore fruit body data, a multivariate ANOVA permutation test was conducted with function *adonis*. Statistical analyses were performed in the *vegan* package (Oksanen et al. 2009) of R (R Development Core Team 2009).

Results

Species richness of fungal communities determined by DGGE and polypore fruit bodies

Analysis of wood-inhabiting fungi with molecular methods (culture-free DGGE) indicated a greater diversity than a fruit body inventory of polypore species at the time of sampling (Table 2). On average, 6.4 DGGE bands were clearly observed in the fingerprint of a single sawdust sample drilled from a log (50–100 mg, ~0.5 cm³). The highest num-

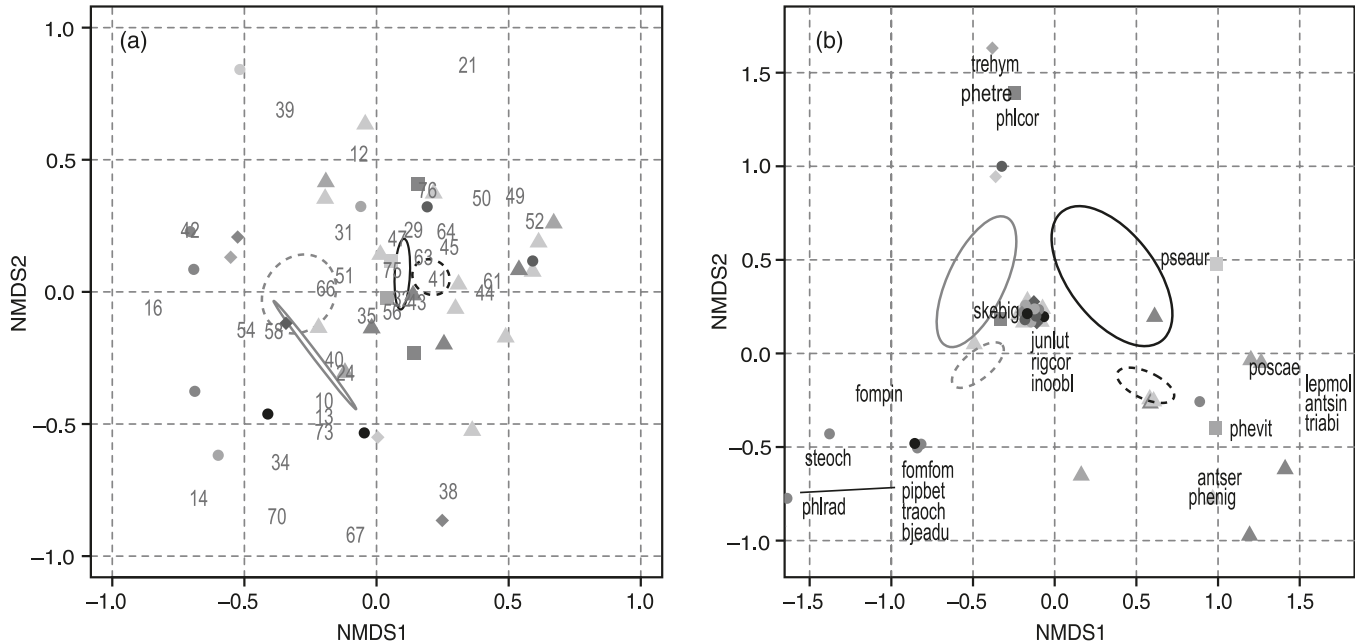
ber of bands was 17, observed in the fingerprint of a spruce log in decay stage 2 with a diameter of 6 cm; fruit bodies of only a single polypore species were observed on the surface of this log. According to the fruit body inventory, a maximum of five species were recorded on a single log, although this number was typically two or less. We found no correspondence between the number of species found as fruit bodies and as DGGE bands (Table 2).

The average number of DGGE bands of a sawdust sample was lowest in decay stage 1 logs (average wood density 435 kg·m⁻³) and highest in decay stage 5 (average wood density 117 kg·m⁻³). The diversity of polypore fruit bodies on a log was highest at decay stage 3 and lowest at decay stage 5. Data limited to Norway spruce logs and decay stages 1–3 indicated that fungal richness (according to DGGE and polypore fruit bodies) increased with loss of wood density. The number of DGGE bands in spruce sawdust samples increased but not significantly with decreasing wood density (Spearman $r_s = -0.442$, $P = 0.066$, $n = 18$), and the diversity of polypore fruit bodies on a spruce was negatively correlated with wood density ($r_s = -0.724$, $P = 0.002$, $n = 16$). Species richness according to DGGE and polypore fruit bodies did not differ among tree species (Table 2).

Identification of fungal communities

All fungi found inside fallen logs belonged to either Basidiomycota or Ascomycota. Basidiomycetes comprised 60% of sequenced DGGE bands in conifers but only 30% in deciduous logs and represented 57%, 44%, and 80% of sequenced DGGE bands in Norway spruce logs of decay

Fig. 4. NMDS plots of mycelial communities determined by (a) culture-free DGGE and (b) polypore fruit bodies in decaying logs of Norway spruce (*Picea abies*) (triangles), Scots pine (*Pinus sylvestris*) (squares), birch (*Betula* spp.) (circles), and European aspen (*Pinus tremula*) (diamonds). The level of shading of the log symbol indicates the decay stage of a log (increasing with decay), numbers in Fig. 4a refer to OTU identities (see Appendix A, Table A2), and dispersion ellipses illustrate standard errors of the weighted average of plot scores in spruce (black broken line), pine (black line), birch (grey broken line), and aspen (grey line). In Fig. 4b, only live fruit bodies were included. In Fig. 4a, spruce differed from aspen ($P = 0.01$) and birch ($P = 0.003$) and in Fig. 4b, spruce differed from pine ($P = 0.03$), aspen ($P = 0.002$), and birch ($P = 0.004$). Abbreviations for fruit bodies: antser, *Antrodia serialis*; antsin, *Antrodia sinuosa*; bjeadu, *Bjerkandera adusta*; fomfom, *Fomes fomentarius*; fompin, *Fomitopsis pinicola*; inoobl, *Inonotus obliquus*; junlut, *Junghuhnia luteoalba*; lepmol, *Leptoporus mollis*; phenig, *Phellinus nigrolimitatus*; phetre, *Phellinus tremulae*; phevit, *Phellinus viticola*; phlcor, *Phlebia cornea*; phlrad, *Phlebia radiata*; pipbet, *Piptoporus betulinus*; poscae, *Postia caesia*; procar, *Protomerulius caryae*; pseaur, *Pseudomerulius aureus*; rigcor, *Rigidoporus corticola*; skepig, *Skeletocutis biguttulata*; steoch, *Steccherinum ochraceum*; traoch, *Trametes ochracea*; trehym, *Trechispora hymenocystis*; triabi, *Trichaptum abietinum*.



stages 1, 2, and 3, respectively. Similarly, within birch logs, the proportion of basidiomycetes was highest in decay stages 3 and 4 (43% and 40%).

The dominant OTU (according to DGGE analysis), observed in 72% of Norway spruce logs, was OTU50. According to DGGE analysis of reference fruit body specimens and sequences of excised bands, OTU50 represents *Phellinus viticola* (Appendix A, Table A2). The probability of occurrence of OTU50 was 60% in spruce logs of decay stage 1 and 100% in stages 2 and 3. The second most frequently observed OTU was OTU43 (most likely *Antrodia serialis*) (Appendix A, Table A2), which occurred in 61% of spruce logs. The fruit body reference sample of *Phlebia radiata* mimicked OTU43 in DGGE gels but this species mostly associates with deciduous trees (Kotiranta et al. 2009) and is thus an unlikely inhabitant of spruce logs. OTU43 occurred in spruce logs of all decay stages and its probability of occurrence did not show an association with any particular one.

In several cases, identical bands from different tree species were not the same fungal species, but within a tree species, variation in OTU identity was lower (Appendix A, Table A2). Similarly, our analysis of fruit body reference samples revealed that different fungal species were not always resolved in DGGE. For example, the rDNA ITS1 fragment of *Phellinus nigrolimitatus* and *Fomitopsis pinicola*

had the same mobility in DGGE (Appendix A, Table A2), even though their ITS1 sequence similarity was only 59%.

In total, 23 fungal species were recorded as fruit bodies on 79 logs and snags of Norway spruce, Scots pine, birch, and European aspen. Only one polypore species was growing on live trees and that was the heart rot fungus *Phellinus tremulae* on European aspen. The remaining species were all found on dead logs. On spruce logs, 10 species were observed as fruit bodies of which the most frequently observed were *P. viticola*, *Postia caesia*, and *A. serialis* (24%, 13%, and 8% of spruce logs, respectively). None of the fruit bodies of *P. viticola*, *P. caesia*, and *A. serialis* were detected on spruce logs of decay stage 1 and were most abundant on logs in decay stages 2–4.

Patterns of fungal communities with quality of wood substrate

Figure 3 illustrates the difference in the fungal communities among tree species based on the number of sequenced DGGE bands. *Antrodia serialis*, *P. viticola*, and *P. nigrolimitatus* were frequently found in sawdust of spruce logs. *Bisporella citrina*, *Fomes fomentarius*, and *Leptodontidium elatius* were typical of birch logs. Applying a similarity criterion >97% in BlastN searches, many sequences remained unknown at the species level. Particularly, species in most heavily decayed logs (birches) remained unknown.

Also, NMDS illustrated that the fungal community inhabiting deciduous birch and aspen was distinct from that found on coniferous spruce and pine along axis 1 ($P = 0.001$) (Fig. 4a), and this effect was insensitive to the number of NMDS dimensions chosen. Canonical correspondence analysis verified that the effect of tree species significantly explained fungal community structure ($P = 0.01$). Even though coniferous logs were denser ($P = 0.002$), the mean C to N ratio was higher ($P < 0.001$), the fraction of ethanol extractives was smaller ($P = 0.031$), and the diameter was smaller ($P = 0.012$) compared with deciduous logs, none of these variables were significant in a canonical correspondence analysis model in which they were included as covariates but tree species was not. OTUs typical of deciduous dead trees were 16, 42, 14, 54, 58, 34, 70, 67, 39, and 38, including fungi most similar to *Dwayaangam colodena*, *Arxula adenivorans*, *L. elatius*, *B. citrina*, *Lecythophora* sp., *Pluteus pellitus*, *F. fomentarius*, and *Hypoxyton rubiginosum* (Fig. 4a). Contrary to deciduous dead wood, OTUs typical of conifers were mainly basidiomycetes (Fig. 4a).

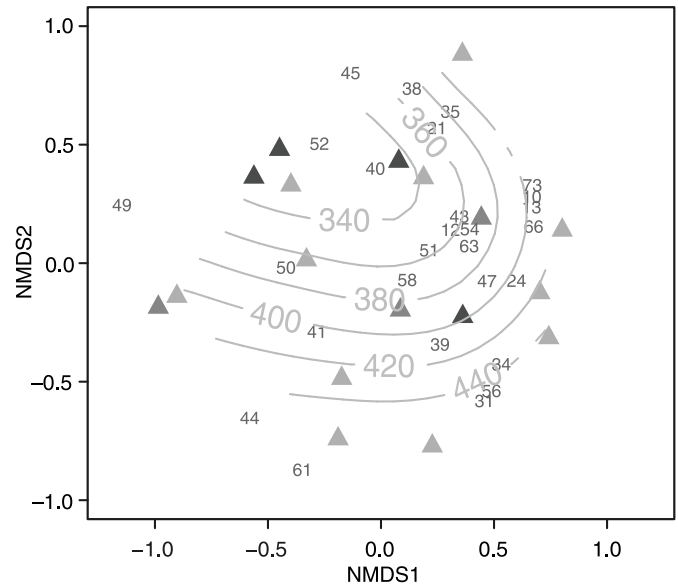
Although species inferred from fruit bodies and sawdusts were dissimilar, tree species were also separated in three-dimensional NMDS according to the composition of polypore fruit bodies ($P < 0.001$) (Fig. 4b). *Fomes fomentarius* was the only species recognized both as mycelial DNA and fruit body on deciduous logs (Fig. 4). Typical polypores of Norway spruce logs, such as *P. viticola*, *P. nigrolimitatus*, *P. caesia*, and *A. serialis*, were also common inside spruce logs (Fig. 4). In contrast, fruit bodies of *Trichaptum abietinum* were associated with spruce (Fig. 4b) but this species was not detected with DGGE (Appendix A, Table A2).

Because nearly half of all sampled logs were Norway spruce (Table 2), a binary matrix of DGGE data for fungi inhabiting this species was chosen for further analysis with NMDS and GAM surface fit of wood qualities. A four-dimensional NMDS ordination of fungal community structure showed a significant and nonlinear relationship with wood density (Fig. 5). Other qualities of spruce logs were not significantly connected with fungal community structure. However, as spruce wood becomes less dense, several other properties are affected (Fig. 2).

Discussion

The data obtained by analyzing DNA from decaying logs sampled as sawdust implied that communities of fungi inhabiting decaying wood are more diverse than one can infer from the number of fruit bodies observed growing on logs (Boddy et al. 2008). Compared with fruit body inventory, isolation on agar media may reveal a more comprehensive picture of fungal community. However, the main advantage of the culture-free molecular identification approach is that, in principle, all species can be detected. Although the molecular approach used in this study (i.e., culture-free DGGE coupled with Sanger sequencing) provides a quantitative and reasonably broad view of wood-inhabiting fungi, it may still underestimate diversity with respect to more advanced molecular methods such as high-throughput 454-pyrosequencing (Ovaskainen et al. 2010). Polyporous and corticoid basidiomycetes, which are traditionally detected as fruit bodies (e.g., Renvall 1995; Lindblad 1998; Penttilä et al.

Fig. 5. NMDS plot of mycelial communities in decaying logs of Norway spruce (*Picea abies*) (triangles) and statistically significant ($P = 0.03$) GAM surface fit of wood density ($\text{kg}\cdot\text{m}^{-3}$). The level of shading of the log symbol indicates the decay stage of a log (increasing with decay). Numbers refer to OTU identities (see Appendix A, Table A2).



2004; Hottola and Siitonen 2008; Olsson 2008) and are presumed to be the primary wood decomposers (Rayner and Boddy 1988), represented a relatively small part of the fungal species observed in this study. Roughly equal proportions of ascomycetes and basidiomycetes were sequenced from fungal DNAs extracted directly from the wood. Fungal DNA sequences typically belonged to orders Leotiales, Fomitopsidales, and Agaricales. Yet, a large proportion of fungi determined by DGGE and sequencing were of unknown identity because their DNA sequences could not be matched to any of the identified fungal species deposited in the public nucleotide database. Members of Ascomycota, in particular, often could not be identified to specific or even generic level. Similarly, Ovaskainen et al. (2010) were able to identify only half of the fungi inhabiting dead wood when they compared 454 sequence data with a high-quality reference library containing well-annotated sequences of 1145 species of wood-decaying and mycorrhizal fungi. The findings thereby indicate that wood-inhabiting fungi are still poorly known.

Fungal communities differed among tree species

In spite of the limited resolution provided by DGGE analysis (for a review, see Muyzer and Smalla 1998; Anderson and Cairney 2004; but for contrasting viewpoints, see Anderson et al. 2003; Kulhánková et al. 2006; Novinscak et al. 2009), the community of wood-inhabiting fungi determined by DGGE was clearly affected by tree species (Fig. 4a). This finding was also supported by a polypore fruit body inventory of the study site (Fig. 4b) and is congruent with previous findings concerning the effect of tree species on fungal community structure (Lumley et al. 2001; Kulhánková et al. 2006; Kotiranta et al. 2009). While the distinction of fungal communities was most apparent between

deciduous and coniferous trees, species composition also differed among tree species, implying that tree diversity enhances fungal diversity. Even though this study confirmed differences in the physicochemical attributes of wood among tree species (e.g., Palviainen et al. 2008), none of the measured variables significantly explained the separation of fungal communities among tree species, possibly due to a high correlation among the physicochemical properties themselves and (or) the low number of logs in each category.

According to the DGGE analysis of sawdust samples, deciduous logs harbored relatively more ascomycetes than logs from conifers. Interestingly, polypore basidiomycetes observed as fruit bodies were typically detected in DGGE of the internal tissue of Norway spruce whereas the DGGE analysis of deciduous logs often failed to recover them. This could be related to an inadequate sample (see also Allmér et al. 2006). Another reason why the DGGE approach failed to detect sequences of polypore basidiomycetes in deciduous logs may be the high proportion of ascomycetes and other inconspicuous species in comparison with the polypores.

Change in fungal community along with decomposition

An analysis of fungal diversity inhabiting Norway spruce through a gradient of decay indicated a succession of species as decomposition progressed. We observed a weak relationship between community structure of wood-inhabiting fungi and mass loss of spruce (Fig. 5). A similar fungal succession during the process of wood decay has been found through fruit body (Renvall 1995; Fukasawa et al. 2009a) and isolation (Lumley et al. 2001; Fukasawa et al. 2009b) approaches, but our results reveal the response of the entire fungal community, not only part of it.

According to DGGE, the proportion of basidiomycetes in the fungal community inhabiting spruce logs peaked during intermediate stages of decay. A similar trend was observed for birch-inhabiting fungi even though ascomycetes dominated those communities. Ascomycetes utilizing forest litter are generally considered to be less substrate specific than basidiomycetes (Allmér et al. 2009) and thus the greater amount of extractable compounds in deciduous wood might explain their abundance. However, the identities and functional roles of the wood-inhabiting ascomycetes are poorly known.

Species richness determined by DGGE increased with wood decay and peaked in the most decayed logs, which is consistent with the findings of earlier isolation studies (Lumley et al. 2001; Fukasawa et al. 2009b). Species richness of polypore fruit bodies per log, in contrast, reached a maximum earlier during intermediate stages of decay. A similar successional trend of fruit body diversity on fallen trees has been detected in boreal conifer forests (Bader et al. 1995; Renvall 1995; Lindblad 1998) and in temperate deciduous forests (Fukasawa et al. 2009a). Congruent with Fukasawa et al. (2009b), our ordination analyses indicated that the communities detected from the logs in more advanced stages of decay were more similar to each other than early decay stage communities. This finding may reflect that, as decay proceeds, the influence of airborne colonization decreases and more stabilized fungal communities eventually form. These late-stage fungi are the least known (see also

Renvall 1995) and their diversity seems to be greater than expected.

In this study, *P. viticola* and *A. serialis* were frequently observed both as fruit bodies and as DNA in Norway spruce logs. Previous fruit body inventories have shown that *P. viticola* and *A. serialis* associate with spruce logs in intermediate stages of decay (Renvall 1995; Kruys et al. 1999; Jönsson et al. 2008). Here, we observed fruit bodies of *P. viticola* and *A. serialis* growing on moderately decayed logs (stages 2–4). Mycelial DNA of *P. viticola* and *A. serialis* was also found in spruce logs of decay stage 1, which indicates that fungi are present at an early stage of decay but it takes time before they reach a critical mycelial biomass for fruit body formation. Since mycelial DNA analysis was restricted to spruce logs of decay stages 1–3, a question remains concerning the persistence of *P. viticola* and *A. serialis* after fruiting in logs at an advanced stage of decay.

Conclusions

Our investigation of the wood-inhabiting fungi from all of the decaying logs of the study site showed that (i) tree species harbor different fungal communities and ascomycetes tend to associate with deciduous and slightly decayed logs, (ii) wood density is related to the fungal community structure in Norway spruce logs, and (iii) well-known polypores comprise only a part of the wood-inhabiting fungal community.

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Appendix A

Tables A1 and A2 appear on the following pages.

Table A1. Total numbers of trees inhabited by live fruit bodies (numbers of trees inhabited either by live or dead fruit bodies are given in parentheses).

Fungal species	Total no. of trees	Norway spruce (<i>Picea abies</i>)	Scots pine (<i>Pinus sylvestris</i>)	Birch (<i>Betula</i> spp.)	European aspen (<i>Populus tremula</i>)
<i>Phellinus viticola</i>	11 (11)	9 (9)	1 (1)	1 (1)	
<i>Fomes fomentarius</i>	10 (21)			9 (20)	1 (1)
<i>Phellinus tremulae</i>	8 (9)				8 (9)
<i>Postia caesia</i>	7 (7)	5 (5)	2 (2)		
<i>Antrodia serialis</i>	5 (5)	3 (3)	2 (2)		
<i>Fomitopsis pinicola</i>	4 (7)	1 (2)		3 (5)	
<i>Trechispora hymenocystis</i>	4 (4)	1 (1)	1 (1)	1 (1)	1 (1)
<i>Phellinus nigrolimitatus</i>	2 (2)	2 (2)			
<i>Piptoporus betulinus</i>	2 (2)			2 (2)	
<i>Trichaptum abietinum</i>	2 (3)	2 (3)			
<i>Antrodia sinuosa</i>	1 (1)	1 (1)			
<i>Bjerkandera adusta</i>	1 (1)				1 (1)
<i>Junghuhnia luteoalba</i>	1 (1)	1 (1)			
<i>Leptoporus mollis</i>	1 (1)	1 (1)			
<i>Phlebia cornea</i>	1 (1)		1 (1)		
<i>Phlebia radiata</i>	1 (1)			1 (1)	
<i>Protomerulius caryae</i>	1 (1)			1 (1)	
<i>Pseudomerulius aureus</i>	1 (1)		1 (1)		
<i>Rigidoporus corticola</i>	1 (1)				1 (1)
<i>Skeletocutis biguttulata</i>	1 (1)		1 (1)		
<i>Steccherinum ochraceum</i>	1 (1)			1 (1)	
<i>Trametes ochracea</i>	1 (2)			0 (1)	1 (1)
<i>Inonotus obliquus</i>	0 (1)			0 (1)	

Table A2. Identification of OTUs (ITS1 DGGE bands in the same position in gels) by BlastN comparison of sequenced bands with fungal sequences obtained from GenBank and by comparison with the DGGE mobility of fruit body reference specimens.

OTU	No. of samples containing the OTU	GenBank accession No. of sequenced band ^a	Host tree of sequenced band (decay stage)	Closest species match in GenBank	Similarity % (base pairs)	Fruit body reference specimen(s) in OTU ^b
43	20	HM015746	Norway spruce (<i>Picea abies</i>) (1)	<i>Antrodia serialis</i>	98.1 (268)	<i>Antrodia serialis</i> (c)
		HM015733	Norway spruce (1)	<i>Antrodia serialis</i>	98.5 (267)	<i>Phlebia radiata</i> (d)
		HM015699	Norway spruce (1)	<i>Antrodia serialis</i>	98.9 (266)	
		HM015676	Norway spruce (1)	<i>Antrodia serialis</i>	99.6 (266)	
		HM015685	Norway spruce (1)	<i>Phialophora lagerbergii</i>	99.6 (266)	
		HM015707	Norway spruce (2)	<i>Antrodia serialis</i>	96.4 (274)	
		HM015762	Norway spruce (3)	<i>Antrodia serialis</i>	97.5 (285)	
		HM015690	Norway spruce (3)	<i>Hyphodontia alutacea</i>	98.5 (203)	
		HM015758	Scots pine (<i>Pinus sylvestris</i>) (3)	<i>Rhizoscyphus ericae</i>	91.2 (239)	
		HM015687	Birch (<i>Betula</i> spp.) (4)	<i>Spadicoides bina</i>	80.2 (243)	
		HM015684	Birch (5)	<i>Phialocephala repens</i>	100 (213)	
50	18	HM015677	European aspen (<i>Populus tremula</i>) (1)	<i>Phialocephala repens</i>	100 (232)	
		HM015711	Norway spruce (1)	<i>Postia caesia</i>	100 (259)	<i>Phellinus viticola</i> (c)
		HM015701	Norway spruce (1)	<i>Phellinus viticola</i>	98.2 (227)	
		HM015672	Norway spruce (1)	<i>Phialocephala</i> sp.	90.9 (231)	
		HM015702	Norway spruce (2)	<i>Phellinus viticola</i>	97.1 (104)	
		HM015751	Norway spruce (3)	Uncultured Agaricomycetes	77.9 (262)	
		HM015752	Scots pine (1)	<i>Capronia</i> sp.	98.5 (271)	
		HM015727	Scots pine (2)	<i>Tubulicrinis globisporus</i>	80.8 (307)	
		HM015714	Scots pine (2)	<i>Tubulicrinis globisporus</i>	81.0 (306)	
		HM015695	Scots pine (2)	<i>Tubulicrinis globisporus</i>	83.2 (131)	
		HM015756	Scots pine (3)	<i>Capronia</i> sp.	98.5 (270)	
39	14	HM015757	Birch (4)	<i>Tubulicrinis globisporus</i>	72.3 (260)	
		HM015744	Norway spruce (1)	<i>Phialocephala sphaeroides</i>	94.1 (547)	<i>Fomes fomentarius</i> (d)
		HM015713	Birch (3)	<i>Fomes fomentarius</i>	100 (260)	
		HM015693	Birch (3)	<i>Fomes fomentarius</i>	99.6 (261)	
		HM015722	European aspen (2)	<i>Hypomyces chrysospermus</i>	79.3 (300)	
58	13	HM015674	European aspen (2)	<i>Sarea difformis</i>	87.2 (117)	
		HM015673	Norway spruce (1)	<i>Amylostereum areolatum</i>	100 (251)	
		HM015683	Norway spruce (2)	<i>Hericium erinaceum</i>	77.6 (263)	
		HM015720	Birch (2)	<i>Bisporella citrina</i>	97.4 (227)	
		HM015671	Birch (3)	<i>Bisporella citrina</i>	97.8 (228)	
47	12	HM015731	Birch (3)	<i>Bisporella citrina</i>	95.6 (206)	
		HM015725	European aspen (2)	<i>Lecytophora</i> sp.	100 (237)	
		HM015710	Norway spruce (1)	<i>Fomitopsis pinicola</i>	98.0 (297)	<i>Phellinus nigrolimitatus</i> (c)
		HM015743	Norway spruce (1)	<i>Phellinus nigrolimitatus</i>	94.2 (258)	<i>Fomitopsis pinicola</i>
		HM015750	Norway spruce (1)	<i>Phellinus nigrolimitatus</i>	99.2 (120)	
		HM015734	Norway spruce (1)	<i>Phialocephala</i> sp.	91.3 (231)	
		HM015747	Norway spruce (1)	<i>Phialocephala</i> sp.	93.7 (189)	
		HM015681	Norway spruce (2)	<i>Penicillium coralligerum</i>	88.8 (80)	

Table A2 (continued).

OTU	No. of samples containing the OTU	GenBank accession No. of sequenced band ^a	Host tree of sequenced band (decay stage)	Closest species match in GenBank	Similarity % (base pairs)	Fruit body reference specimen(s) in OTU ^b
54	12	HM015745	Norway spruce (2)	<i>Phialocephala</i> sp.	91.3 (231)	
		HM015703	Norway spruce (2)	<i>Phialocephala</i> sp.	93.7 (189)	
		HM015680	Norway spruce (3)	<i>Antrodia serialis</i>	98.9 (267)	
		HM015691	Norway spruce (3)	<i>Fomitopsis pinicola</i>	99.0 (294)	
		HM015697	Scots pine (3)	<i>Calocera cornea</i>	83.7 (190)	
		HM015694	Birch (3)	<i>Leptodontidium elatius</i>	98.8 (244)	
		HM015723	Birch (3)	<i>Leptodontidium elatius</i>	98.8 (244)	
		HM015709	Birch (5)	<i>Leptodontidium elatius</i>	98.8 (244)	
		HM015724	European aspen (2)	<i>Leptodontidium elatius</i>	98.8 (244)	
		31	10	HM015732	Norway spruce (1)	
HM015717	Birch (4)			<i>Armillaria cepistipes</i>	99.3 (300)	
35	10	HM015721	European aspen (2)	<i>Armillaria cepistipes</i>	99.7 (299)	<i>Gloeoporus pannacinctus</i> (d)
		HM015689	Norway spruce (3)	<i>Xeromphalina campanella</i>	94.9 (274)	
49	10	HM015719	Birch (5)	<i>Apodus decidius</i>	81.8 (242)	
		HM015686	Birch (4)	<i>Armillaria cepistipes</i>	99.7 (299)	
51	10	HM015739	Norway spruce (3)	cf. <i>Hymenoscyphus</i>	96.1 (205)	
		HM015730	Birch (4)	<i>Lachnum virgineum</i>	98.2 (224)	
51	10	HM015675	European aspen (2)	<i>Lachnum virgineum</i>	97.8 (225)	
		HM015749	Norway spruce (1)	<i>Phialocephala</i> sp.	90.9 (231)	
41	9	HM015748	Norway spruce (3)	<i>Columnocystis abietina</i>	100 (261)	<i>Trametes ochracea</i> (d)
		HM015753	Scots pine (1)	<i>Cladophialophora</i> sp.	86.1 (274)	
44	9	HM015708	Birch (5)	<i>Leptodontidium elatius</i>	93.1 (245)	<i>Junghuhnia luteoalba</i> (c)
		HM015705	European aspen (1)	<i>Graphium penicillioides</i>	94.5 (200)	
44	9	HM015736	Norway spruce (1)	<i>Exidia glandulosa</i>	98.3 (237)	<i>Trichaptum abietinum</i> (c)
		HM015737	Norway spruce (1)	<i>Phialocephala sphaeroides</i>	89.2 (519)	
44	9	HM015716	Norway spruce (1)	<i>Phialocephala</i> sp.	88.4 (164)	<i>Piptoporus betulinus</i> (d)
		HM015742	European aspen (2)	<i>Lentinellus micheneri</i>	100 (232)	
45	9	HM015740	Norway spruce (1)	<i>Phellinus viticola</i>	98.7 (226)	
		HM015755	Scots pine (3)	<i>Rhizoscyphus ericae</i>	90.8 (240)	
34	8	HM015759	Scots pine (3)	<i>Calocera cornea</i>	83.0 (176)	<i>Rigidoporus corticola</i> (d)
		HM015741	Scots pine (2)	<i>Amorphotheca resiniae</i>	86.6 (205)	
38	8	HM015754	Norway spruce (3)	<i>Coniophora puteana</i>	99.3 (293)	<i>Protomerulius caryae</i> (d)
		HM015726	Scots pine (2)	<i>Gymnopilus picreus</i>	99.7 (287)	
56	7	HM015761	Birch (3)	<i>Pluteus pellitus</i>	98.8 (329)	
		HM015760	Norway spruce (1)	<i>Hypoxylon rubiginosum</i>	98.6 (213)	
52	—	HM015700	Birch (1)	<i>Fomes fomentarius</i>	99.6 (261)	<i>Sceletocutis biguttulata</i> (c)
		HM015704	European aspen (3)	<i>Hypoxylon rubiginosum</i>	100 (210)	
52	—	HM015682	Norway spruce (2)	<i>Hericium erinaceum</i>	77.3 (264)	
		HM015698	Scots pine (3)	<i>Sympoventuria capensis</i>	97.1 (69)	
52	—	HM015706	European aspen (1)	<i>Lecythophora</i> sp.	99.0 (209)	

Table A2 (concluded).

OTU	No. of samples containing the OTU	GenBank accession No. of sequenced band ^a	Host tree of sequenced band (decay stage)	Closest species match in GenBank	Similarity % (base pairs)	Fruit body reference specimen(s) in OTU ^b
						<i>Phellinus tremulae</i> (d)
66	6	HM015712	Norway spruce (1)	<i>Lecythophora</i> sp.	92.4 (238)	
		HM015688	Norway spruce (3)	<i>Lecythophora</i> sp.	92.8 (237)	
29	5	HM015692	Scots pine (3)	<i>Hyphoderma definitum</i>	99.5 (209)	<i>Pseudomerulius aureus</i> (c)
12	4	HM015735	European aspen (2)	<i>Candida ergastensis</i>	100 (256)	
40	4	—				
42	4	HM015738	European aspen (2)	<i>Dwayaangam colodena</i>	92.9 (210)	
61	4	—				
16	3	—				
32	3	—				
63	3	—				
24	2	—				
64	2	HM015696	Scots pine (2)	Uncultured Corticiales	99.6 (262)	
70	2	—				<i>Phlebia cornea</i>
76	2	HM015729	Scots pine (2)	Uncultured Corticiales	100 (261)	
10	1	HM015678	Norway spruce (2)	<i>Waltomyces lipofer</i>	80.8 (172)	
13	1	HM015679	Norway spruce (2)	<i>Candida ergastensis</i>	100 (274)	
14	1	HM015718	Birch (5)	<i>Arxula adeninivorans</i>	74.6 (244)	
21	1	—				<i>Leptoporus mollis</i> (c)
67	1	—				
73	1	—				
75	1	HM015715	Scots pine (2)	<i>Cryptococcus huempii</i>	89.1 (92)	
		HM015728	Scots pine (2)	<i>Cryptococcus huempii</i>	87.0 (92)	

^a—, no sequence obtained.

^bc, conifer specialist; d, deciduous specialist.