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RNA reveals a succession of active fungi during the decay of Norway spruce logs

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ABSTRACT

Current knowledge of the succession of fungi in decaying wood is mostly based on fruit bodies and *in vitro* culture. Here, we investigated the changing community of metabolically active fungi during the decomposition of fallen *Picea abies* logs by directly extracting and barcode sequencing precursor rRNA. We also compared rRNA-derived amplicons of the 18S and ITS regions in 21 isolates and discuss the use of RNA as a marker of metabolically active fungi. The richness of active fungi, revealed as separated bands in DGGE, peaked in logs at an advanced stage of decay. Soft-rot fungi were common in the early stages but white- and brown-rot fungi became dominant as decay progressed. Ectomycorrhizal fungi were detected at an early stage, and they became the most abundant group in the late stages of succession. A comparison of rRNA-derived amplicons revealed that although ITS was detected in the form of precursor rRNA, introns within 18S rDNA were already spliced. As such, rRNA- and rDNA-derived amplicons would yield different profiles of active and total communities if profiling method is affected by amplicon length.

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Introduction

Decaying plant litter is crucial to nutrient cycling, carbon dynamics, and the maintenance of biodiversity in forest ecosystems. Dead logs, twigs, branches, needles, leaves, cones and other decaying plant material provide habitat for many species. The number of species dependent on dead wood in Finland is at least 4000–5000 and roughly one third of them are fungi (Siitonen 2001). Fungi play a major role in litter decomposition in boreal forests due to their release of enzymes involved in the decomposition of recalcitrant components, such as lignin (Baldrian 2008). Soft-rot fungi form cavities by decomposing cellulose and hemicellulose and are involved in the modification of lignin (Daniel & Nilsson, 1998 and references therein). Brown-rot fungi efficiently decompose cellulose and hemicellulose but only

slightly alter lignin (Blanchette 1995), whereas white-rot fungi are able to decompose all wood polymers.

Modelling forest carbon balances requires an estimate of the decay rate of dead organic material. The effect of fungal species richness and community composition on the rate of decay has been demonstrated *in vitro* (Toljander *et al.* 2006; Fukami *et al.* 2010), but *in situ* experiments are needed to obtain a greater understanding about the relationship between community structure of fungal decomposers, resource quality and rate of decay. Traditionally, fungal communities in forest litter have been studied through fruit body inventories or isolation on culture media. Unfortunately, both of these methods tend to underestimate species richness and misrepresent fungal activity, because fungi may be inconspicuous, highly selective and slow growing. Furthermore, the current understanding of fungal succession during

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wood decomposition and the habitat preferences of fungal decomposers is mainly restricted to species that form visible fruit bodies (particularly polypores and corticioids in boreal forests) and those which can be cultured.

Advanced molecular techniques are now applied in environmental microbial studies. By extracting total DNA directly from environmental samples, principally all fungal species can be detected. Since culture-independent molecular methods have been used in fungal ecology, our understanding of fungal richness in soil and litter has changed dramatically (Horton & Bruns 2001; Anderson & Cairney 2004; Fierer et al. 2007; Lindahl et al. 2007; van der Heijden et al. 2008; Hirsch et al. 2010). High-throughput sequencing in particular has the potential to yield a comprehensive profile of fungal richness in forests (Urich et al. 2008; Buée et al. 2009; Jumpponen & Jones 2009; Öpik et al. 2009; Ovaskainen et al. 2010, 2011; Wallander et al. 2010). Despite these advances, true fungal richness and species identity remain poorly understood and the activity levels of community members are even less known.

By extracting total DNA from an environmental sample mainly living organisms can be detected. However, DNA can also originate from dormant and dead organisms (England et al. 1997; Demanèche et al. 2001). In contrast, RNA is synthesized only by metabolically active organisms. Based on the relationship between RNA transcription and growth, activity and survival (Weider et al. 2005), ribosomal RNA (rRNA) is treated as a suitable marker for the detection of active and functionally important species in an environmental sample, although rRNA in ribosome particles is not always very short-lived (Flårdh et al. 1992; Fukui et al. 1996). Messenger RNA (mRNA) degrades faster than rRNA, and its use, e.g., in the precise analysis of functional genes, requires more careful sampling and processing.

Nuclear small-subunit ribosomal (SSU) RNAs, i.e. 16S rRNA in bacteria and 18S rRNA in fungi, have been directly extracted from metabolically active bacteria (Ward et al. 1990; Felske et al. 1996; Nicol et al. 2004; Sharma et al. 2006; Bernard et al. 2007; Tourna et al. 2008) and fungi (Aneja et al. 2004, 2006, 2007; Girvan et al. 2004; Pennanen et al. 2004; Izumi et al. 2006) in environmental samples. Compared to 18S rRNA, the non-coding internal transcribed spacer (ITS) region is more variable among fungal species, and its use as an identification tag has led to it becoming the most abundant sequence in public nucleotide databases. Anderson & Parkin (2007) demonstrated that precursor ITS can be detected in an RNA pool extracted from soil fungi. They proposed that the ITS of rRNA reflects a more active part of the fungal community than 18S rRNA as it is constantly transcribed but quickly removed in the processing of mature rRNA (i.e., 18S, 5.8S and 28S rRNA in fungi). Thereafter, ITS of precursor RNA has been used to detect the fungal activity in soil (Bastias et al. 2007; Anderson et al. 2008) and needle litter (Korkama-Rajala et al. 2008). Although the processing of precursor rRNA to mature rRNA varies among fungi (Allmang et al. 2000), little is known of the possible differences in splicing time. Such differences, if demonstrated, might question the use of rRNA for pointing out active species in fungal communities based on the study of rDNA.

Here, we evaluate whether rRNA is a suitable marker for metabolically active fungi in the environment. We compared *in vitro* RT-PCR amplicons of 18S and ITS regions of rRNA

against corresponding rDNA PCR amplicons. In addition, we analyzed fungal community structure in decaying Norway spruce logs on the basis of ITS in directly extracted total RNA and DNA.

We tested the following hypotheses: (1) 18S rRNA and the ITS region are both suitable markers for revealing active species in a fungal community, (2) only part of the fungal community inhabiting dead wood is metabolically active at a particular time or under certain conditions, (3) the richness of active fungi is greatest at an intermediate level of decay when the decomposition rate is maximal, (4) metabolic activity and functionality of species is related to woody resource substrate quality, which changes during decomposition.

Material and methods

Study site and wood sampling

The study site was located in an unmanaged Norway spruce (*Picea abies*) dominated stand in Lapinjärvi, Southern Finland (60°39.413'N, 26°7.352'E, altitude 50 m, temperature sum 1300 °C d). The study site was relatively fertile, representing herb-rich heath forests (*Oxalis-Myrtillus* type according to Cajander's site type classification; Hotanen et al. 2008). The size of the sample plot was 5625 m² (75 m × 75 m) within which we measured all living (411 m³ per ha) and dead trees (112 m³ per ha of *P. abies*, 2.3 m³ per ha of *Pinus sylvestris*, 10 m³ per ha of *Betula* sp. and 0.3 m³ per ha of other tree species) and noted their locations and attributes.

In Oct. 2008, we sampled 50 fallen Norway spruce logs (diameter >5 cm at breast height) that represented different decay stages ranging from hard, recently fallen logs to soft, strongly decayed wood (decay stages 1–5 according to Mäkinen et al. 2006). We sawed discs (5 cm) at the midpoint of the largest fraction of each log. Distance from the sampling point to the first ground contact was measured. Discs were packed into plastic bags and kept frozen prior to processing. In the laboratory, frozen discs were drilled (bit diameter 10 mm) from the surface through sapwood and heartwood. The bark or outermost layer of each disc was removed before drilling, the drill bit was sterilized by flaming between samples and the resulting sawdust and shavings were collected and stored at –80 °C until extraction of nucleic acids.

Nucleic acid extraction from fungal isolates and decaying wood

Twenty one fungal isolates (Table 1) were grown on ½MMN media (Marx 1969) modified to contain CaCl₂·2H₂O 0.05 g, NaCl 0.025 g, MgSO₄·7H₂O 0.15 g, Fe-EDTA 0.0168 g, Thiamine-HCl 0.01 g, Malt extract 5 g, (NH₄)₂HPO₄ 0.25 g, KH₂PO₄ 0.5 g, glucose 1.25 g and agar 12 g per litre. Nucleic acids were extracted from cultured mycelia using E.Z.N.A.[™] Fungal RNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA). Samples were ground with quartz sand for 3 × 20 s at 4 m s⁻¹ prior to processing according to the manufacturer's protocol I. The eluted nucleic acids were a mixture of RNA and DNA, and the latter

Table 1 – Fungal isolates and observed length variation (+ observed, – not observed) between their rDNA- and rRNA-derived amplicons obtained by FF390/FR1 and ITS1F/ITS2 primer pairs

Isolate	Phylum	FF390/FR1	ITS1F/ITS2
<i>Antrodia serialis</i> 97K2 2b	Basidiomycota	–	–
<i>Cadophora finlandia</i> ARON 3669.S	Ascomycota	+	+
<i>Cadophora finlandia</i> ARON 3704.S	Ascomycota	+	+
<i>Cadophora finlandia</i> R-RS02	Ascomycota	+	+
<i>Cenococcum geophilum</i> R-C401	Ascomycota	–	+
<i>Cenococcum geophilum</i> R-FC01	Ascomycota	–	+
<i>Cenococcum geophilum</i> R-MF04	Ascomycota	–	+
<i>Cenococcum geophilum</i> R-NC06	Ascomycota	–	+
<i>Chondrostereum purpureum</i> AL1	Basidiomycota	–	–
<i>Fomitopsis pinicola</i> 981108 3R	Basidiomycota	–	–
<i>Heterobasidium parviporum</i> 5/a	Basidiomycota	–	–
<i>Lactarius rufus</i> F-SS06	Basidiomycota	–	–
<i>Lophodermium piceae</i> 94.107	Ascomycota	–	+
<i>Meliniomyces bicolor</i> R-FC06	Ascomycota	–	+
<i>Meliniomyces bicolor</i> R-MF01	Ascomycota	–	+
<i>Meliniomyces variabilis</i> R-MF11	Ascomycota	–	+
<i>Phialocephala fortinii</i> R-RS07	Ascomycota	–	–
<i>Phlebiopsis gigantea</i>	Basidiomycota	–	–
<i>Piloderma</i> sp. R-SP02	Basidiomycota	–	–
<i>Russula</i> sp. F-HP03	Basidiomycota	–	–
<i>Tylospora fibrillosa</i> R-NC07	Basidiomycota	–	–

was removed via treatment with DNase I (Fermentas, UAB, Vilnius, Lithuania).

DNA and RNA were extracted from sawdust samples of decaying wood (50–100 mg) using the E.Z.N.A.™ SP Plant DNA Mini kit and E.Z.N.A.™ SP Plant RNA Mini kit (Omega Bio-tek, Inc., Norcross, GA, USA). Homogenization and cell lysis was carried out as described above. DNA samples were purified further with PEG as in Vainio & Hantula (2000). RNA extraction followed the manufacturer's protocol II with DNase I digestion. Finally, RNA samples were subjected to an additional DNase I digestion step (Fermentas, UAB, Vilnius, Lithuania) to eliminate any remaining DNA.

cDNA synthesis and PCR amplification

cDNA was synthesized from 2 µl (fungal isolates; 0.13–1.8 µg) or 4 µl (sawdust samples; 12–280 ng) of pure RNA and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). 18S and ITS regions of the rRNA gene of each fungal isolate were reverse transcribed using FR1 (Vainio & Hantula 2000) and ITS2 primers (White et al. 1990), respectively (Fig 1). Primer ITS2 was used in the reverse transcription of all sawdust samples.

Fungal 18S and ITS regions of rRNA were amplified using FF390/FR1 (Vainio & Hantula 2000) and ITS1F/ITS2 (White et al. 1990; Gardes & Bruns 1993) primer pairs, respectively, using DNA and cDNA as template. Primers FF390/FR1 amplified a region near the 3' end of 18S rRNA gene whereas ITS1F/ITS2 primers amplified the 3' end of 18S, ITS1 and the 5' end of the 5.8S region of rRNA (Fig 1). DNase-treated RNA, which was not reverse transcribed, was also amplified to check the success of DNase digestion. A GC-clamp was added to the 5' end of the ITS1F primer (Korkama et al. 2007) and FR1 primer (Vainio & Hantula 2000) to generate PCR products suitable for DGGE analysis. PCR reactions contained 1 µl of template, 200 µM of

each dNTP, 0.5 µM of each primer, 5 µl of 10× reaction buffer (75 mM Tris–HCl, pH 9.0; 2 mM MgCl₂; 50 mM KCl; 20 mM (NH₄)₂SO₄, and 0.2 U of Biotools DNA polymerase (B&M Laboratories, Madrid, Spain)). Cycling parameters for 18S rRNA gene amplification were 5 min at 95 °C followed by 35 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 2 min, and a final extension of 72 °C for 10 min. ITS amplification followed the programme: 5 min at 95 °C, 35 cycles of 95 °C for 30 s, 57 °C for 45 s, 72 °C for 1 min, and a final extension of 72 °C for 7 min. All PCR products were electrophoresed on 1 % (w/v) agarose gels in 1× TAE buffer (40 mM Tris–HCl, pH 8; 1 mM EDTA), stained with ethidium bromide and visualized on a UV trans-illuminator. Negative control containing no template was always included in PCRs.

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analyses of 18S and ITS regions of rDNA- and rRNA-derived fragments were carried out using the DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA) as described for the FF390/FR1 primer pair by Vainio & Hantula (2000) and for ITS1F/ITS2 by Korkama et al. (2007). Polyacrylamide gels (7.5 % w/v acrylamide/

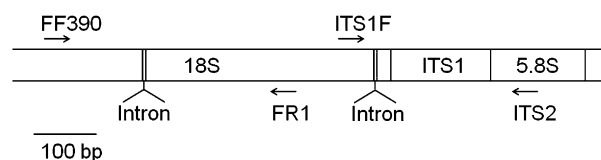


Fig 1 – A diagram showing the position of the primers used in PCR and RT-PCR and approximate position of introns detected in the 18S rRNA gene of some ascomycete isolates. FR1 and ITS2 primers were used in the RT reaction.

bisacrylamide 37.5:1) were prepared with a denaturing gradient of 45–60 % for FF390/FR1 amplicons and 18–58 % for ITS1F/ITS2 amplicons using a gradient former (Model 485, Bio-Rad Laboratories) and a peristaltic pump (Minipuls 3, Gilson SAS, Villiers-le-Bel, France). PCR products were loaded and electrophoresis was performed in 1× TAE buffer. Running parameters for the FF390/FR1 amplicons were 50 V, 58 °C and 18 hr, and for ITS1F/ITS2 amplicons 75 V, 60 °C and 16 hr. Gels were stained with SYBR[®] Gold (Molecular Probes, Eugene, OR, USA) and visualized with blue light on a SafeImager™ trans-illuminator (Invitrogen, Carlsbad, CA, USA). DGGE bands were excised and placed in sterile water over night. We aimed to select excised DGGE bands so that they represented rRNA-derived fragments from all mobility classes (OTUs, operational taxonomic units). The number of excised bands in each OTU was proportional to its frequency. Eluted bands were re-amplified and run again in DGGE. Single PCR products resulting from the successive refinement were sequenced directly.

DNA sequencing

PCR products were purified with the HighPure PCR Product Purification kit (Roche, Mannheim, Germany) and sequenced from both ends by the Macrogen Sequencing Service (Korea) using PCR primers and an ABI 3730XL DNA sequencer. Sequence editing and contig assembly was performed with Geneious Pro 4.7 software (Biomatter Ltd, Auckland, New Zealand; Drummond et al. 2009). Sequences were identified via comparison with the GenBank (NCBI) database using the BlastN algorithm (Altschul et al. 1997). Over 95 % sequence similarity was used as a limit to general level identification. Sequences were deposited with GenBank under accession numbers HQ441868–HQ441923.

Wood physio-chemical analyses

Wood density was measured by the water-displacement method as described by Mäkinen et al. (2006). Total C and N content of dead wood was determined from air-dried and milled samples by a dry combustion method using a LECO CHN-1000 elemental analyzer (ISO 10694, ISO 13878; Finnish Forest Research Institute, Central laboratory, Vantaa, Finland). Organic chemical fractions of dead wood were determined gravimetrically by separating ethanol and hot-water extracts (polar compounds, e.g., carbohydrates and phenolics) and a sulphuric acid-insoluble fraction (Klason lignin) as described in Rajala et al. (2010) and references therein.

Data analyses

DGGE gel images were analysed using the GelCompar II software, version 5.1 (Applied Maths BVBA, Belgium). The presence (1) or absence (0) of DGGE bands (OTUs) in each lane was determined with a band matching optimization of 0 % and band position tolerance of 1 %.

The binary data were further analyzed by non-metric multidimensional scaling (NMDS). Fungal community dissimilarities among logs were represented as Bray–Curtis distance measure. NMDS ordination of the dissimilarity

matrix was made in three dimensions to obtain an ordination graph in the reduced space.

Relationships between fungal community structures and wood quality variables were evaluated in the reduced space by fitting generalized additive models (GAM). A smooth term for each variable of interest was represented with a smooth thinplate regression spline. Ordinations were made using metaMDS and surface fittings were made using the *ordisurf* function of the *vegan* library (Oksanen et al. 2009) in R (R Development Core Team 2009).

Results

rRNA- and rDNA-derived profiles of fungal isolates

Eleven ascomycetes out of 21 tested basidiomycete and ascomycete isolates had introns that had been removed from precursor rRNA (Table 1). These were observed as longer rDNA-derived fragments compared to rRNA-derived fragments in agarose gel electrophoresis (Fig 2) and migrated differently in DGGE (Fig 2). In three of the isolates, the size difference among rDNA- and rRNA-derived fragments was obtained both by ITS1F/ITS2 and FF390/FR1 primer pairs. In eight isolates the length reduction from DNA to its transcriptase concerned only the amplicon obtained by the ITS1F/ITS2 primer pair. Sequence alignment revealed that introns, which were excised from precursor RNA, were exclusively in the 18S rDNA region (Fig 1). The DNA insertion detected by the ITS1F/ITS2 primers contained four conserved regions characteristic of group I introns (Cech 1988). ITS1 regions were identical among rRNA- and rDNA-derived fragments, indicating that the ITS region can be detected in precursor rRNA although other introns are already spliced.

Change of fungal community structure during wood decomposition

Despite the differences noted between rDNA- and rRNA-derived fragments, there were a few DGGE bands clearly visible in the precursor rRNA profile but not in the corresponding rDNA profile (Fig 3). Until decay stage 4, only a fraction of the rDNA-derived DGGE bands were observed in the precursor rRNA profiles of the same sample, whereas consistency between rDNA- and rRNA-derived profiles increased as decay continued (Fig 3). In a similar way, rRNA-based fungal richness increased with decreasing wood density whereas this trend was not observed in the rDNA-based DGGE analysis of fungal community (Fig 4).

NMDS ordination of the precursor rRNA- and rDNA-derived data separately suggested that fungal community structure changed with wood density (Fig 5). The relationship between rDNA-derived community structure and wood density was linear, whereas this trend was more complex and communities seemed to be more divergent at high densities for the rRNA-derived data (Fig 5). In addition to log density, diameter, length, approximate surface area, approximate volume, moisture, C content and C/N ratio were all related to fungal community structure (Table 2). Diameter and moisture seemed the most important factors as they were significantly

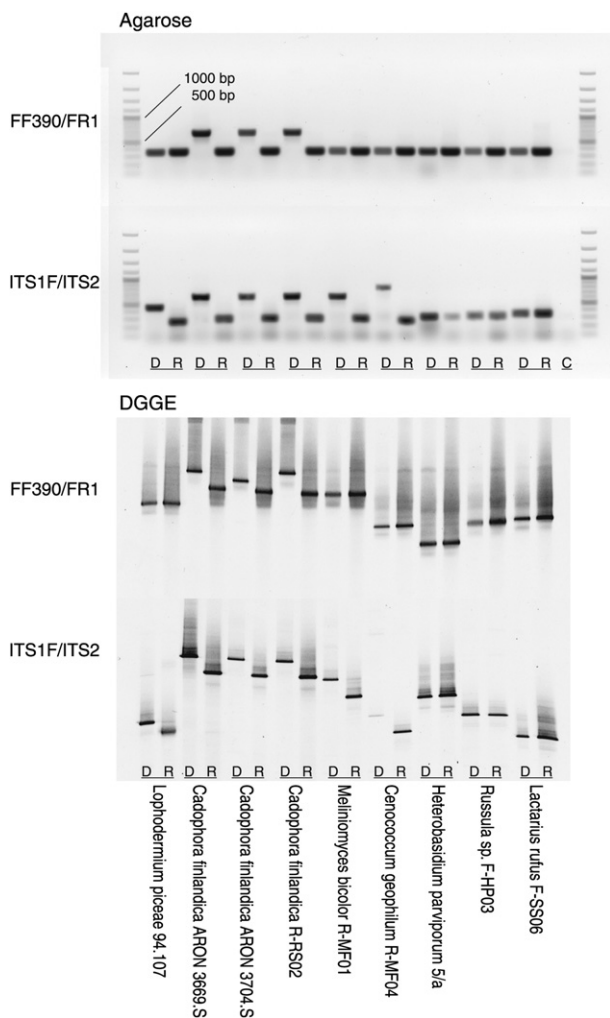


Fig 2 – Agarose gel electrophoresis and denaturing gradient gel electrophoresis (DGGE) of rDNA(D)- and rRNA(R)-derived fragments PCR and RT-PCR amplified by FF390/FR1 and ITS1F/ITS2 primer pairs. C refers to negative control. The longer rDNA-derived fragment compared to its corresponding rRNA-derived fragment was caused by introns that varied from ca. 280 to 310 bp when amplified with FF390/FR1 primers and 195 to 530 bp with ITS1F/ITS2 primers. Introns caused dissimilar migration of rDNA-derived and the corresponding rRNA-derived fragments in electrophoresis.

related both to the rDNA-derived and rRNA-derived richness estimates. Relationships between the fungal community and wood attributes were detected more often in the rDNA-derived data than in precursor rRNA-derived data (Table 2).

Succession of metabolically active fungal species during wood decomposition

The dominant life strategy of metabolically active fungi inhabiting dead wood changed during the decay process (Fig 6). Soft- and white-rot fungi dominated the community in the earliest stages and after that soft-rot fungi yielded habitat to

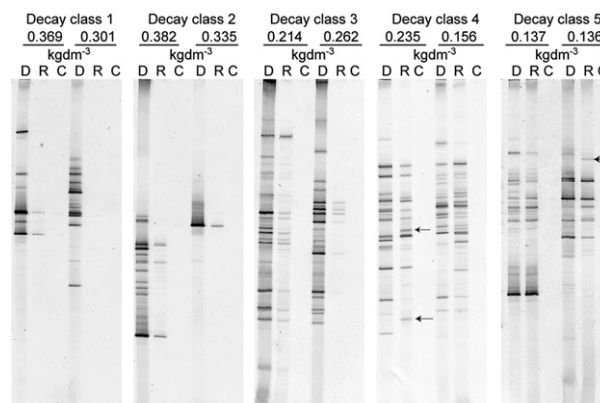


Fig 3 – Denaturing gradient gel electrophoresis of rDNA-(D) and rRNA-based (R) ITS1 region profiles of fungal communities inhabiting decayed Norway spruce logs, classified from decay stage 1–5. Two wood samples from each decay stage are presented and their wood density (kg dm^{-3}) is noted. C indicates PCR control of DNase-treated RNA. Arrows indicate rRNA-derived bands that were not detected in rDNA profiles.

brown-rot and mycorrhizal fungi. White-rot fungi constituted about 20 % of taxa until decay stage 5 and mycorrhizal fungi were the most abundant life strategy group in heavily decayed logs. Interestingly, the ectomycorrhizal genera *Lactarius* and *Russula* also colonized slightly decayed (stage 2) logs (Fig 7).

Overall, basidiomycetes dominated the community of metabolically active fungi inhabiting decaying Norway spruce logs (71 % of all sequenced DGGE bands). Ascomycetes were the main fungi in communities inhabiting slightly decayed (stage 1) logs; *Phellinus* and *Chalara* were the most abundant in decay stage 1 yielding to *Coniophora* and *Fomitopsis* in decay stage 2 (Fig 7). The most common fungi in heavily decayed logs were ectomycorrhizal species of *Tylospora* and *Russula* (Fig 7).

Most of the ascomycetes were unknown according to BlastN sequence similarity searches. Members of *Chalara* and *Phialocephala* were metabolically active in logs of decay stage 1. In more decayed logs, *Candida* was the only identified ascomycete genus.

Discussion

RNA as a marker for active fungal community

Analysis of rRNA molecules aims to detect active members of microbial communities due to the facts that rRNA is constantly synthesized in metabolically active cells, and that the majority of rRNA is folded in ribosomes that are the sites of protein synthesis. In bacterial cells positive relationship between rRNA content and activity has been noted (Wagner 1994). However, a similar correlation has not been proven for fungi and thus rRNA content cannot be taken as a quantitatively reliable marker for fungal activity. While secondary structure and structural proteins may delay the degradation of rRNA, the life span of precursor rRNA containing ITS is a few

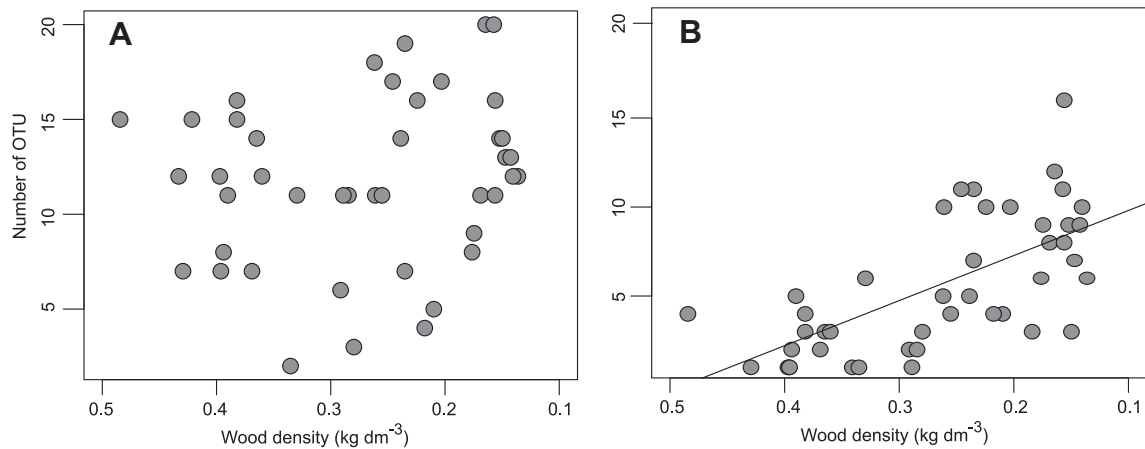


Fig 4 – Relationship between wood density (kg m^{-3}) and fungal richness (number of OTU) based on rDNA- (A) and rRNA-derived (B) ITS-DGGE.

minutes (Stryer 1988). Therefore, the presence of ITS in premature rRNA transcript indicates more recent fungal activity than 18S rRNA.

Two issues that are rarely taken into account in quantifying fungi are that rRNA gene copy number and genome size vary considerably among fungi. Concern over the potential bias created by rRNA copy number should be high, especially given that the number of fungal rRNA genes can vary from one to several thousand (Rooney & Ward 2005). Also plasmids containing rRNA genes have been found from certain fungi, implying that the total copy number of rDNA is affected by the copies of extrachromosomal plasmids (Huber & Rustchenko 2001). Fungal 18S rDNA genes have been used to estimate the quantity of endomycorrhizal fungi inhabiting root tissues (Simon et al. 1992), the relative amount of fungal DNA in leaf litter (Manerkar et al. 2008), and ectomycorrhizal activity (Parrent & Vilgalys 2009). Copy number variation among fungal species may be extensive compared to that of biochemical compounds such as ergosterol (Antibus & Sinsabaugh 1993) or phospholipid fatty acids (Haack et al. 1994), which are often criticized as unreliable fungal

biomass markers. Bailly et al. (2007) demonstrated discrepancies of rDNA and rRNA in community studies; rDNA and rRNA produced nearly equal proportions of fungal and protist 18S rDNA sequences, mRNA revealed almost 20-times as many fungal transcripts, while traditional methods based on cell volumes reported protist/fungal ratios as low as 10^{-3} in soil. The authors pointed out that these discrepancies between ribosomal and biomass methods may be largely due to unpredictable variation in copy number, as well as PCR and amplification bias. Direct extraction of mRNA from soil (e.g., McGrath et al. 2008) is not fully protected from this problem even though this method is used mainly to study functional genes that usually have fewer copies than ribosomal genes. However, these and other potential biases can be excluded incrementally. The first attempts to simultaneously determine the transcriptome of soil microbial communities and their functions without the problematic PCR step have been completed (Urich et al. 2008). Ribosomal genes, due to their variation and historical popularity, will continue to be an important means of molecular identification of fungal communities in their environments. Within the ribosomal

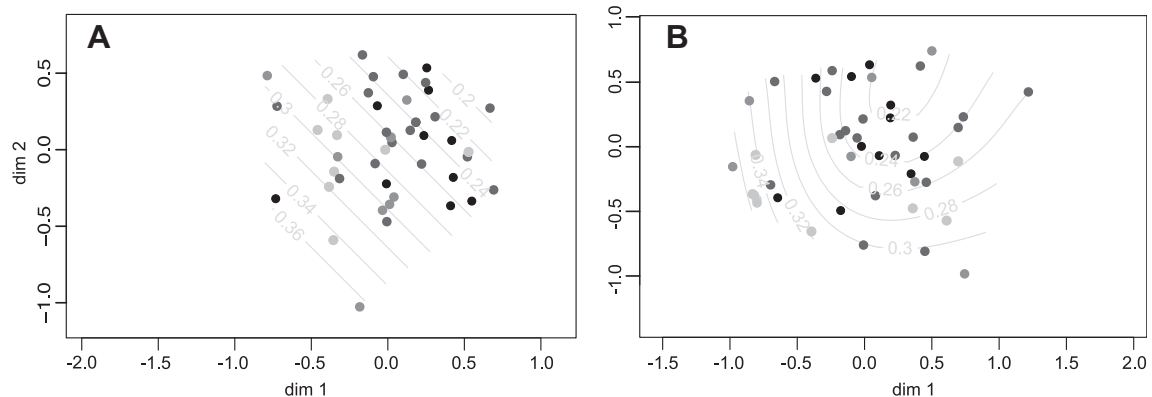


Fig 5 – NMDS plots of fungal community determined by rDNA- (A) and rRNA-derived (B) ITS-DGGE together with GAM-surface fits of wood density. Relationship between wood density and fungal community structure was significant ($p = 0.030$ in panel A, $p = 0.045$ in panel B). Symbols darken as the state of decay increases.

Table 2 – Significance levels of the relationships between fungal community structure and wood quality factors estimated by GAM-surface fittings on NMDS ordinations of rDNA- and rRNA-derived ITS-DGGE data

Properties of logs	rDNA	rRNA
Density	$p = 0.030$	$p = 0.045$
Diameter	$p = 0.005$	$p = 0.042$
Length	$p = 0.02$	$p = 0.144$
Surface area	$p = 0.026$	$p = 0.078$
Volume	$p = 0.049$	$p = 0.11$
C	$p = 0.03$	$p = 0.235$
N	$p = 0.155$	$p = 0.518$
C/N ratio	$p = 0.034$	$p = 0.078$
Lignin	$p = 0.077$	$p = 0.358$
Ethanol extracted	$p = 0.314$	$p = 0.131$
Water extracted	$p = 0.447$	$p = 0.635$
Moisture	$p = 0.015$	$p = 0.013$
Distance to soil	$p = 0.098$	$p = 0.321$

gene, interspecific variation of the quickly degraded ITS spacer is usually optimal, although cases of high intraspecific variation (Müller *et al.* 2007) have been observed. With these caveats in mind, we encourage the exploration of ITS as a marker for active fungi.

The use of precursor RNA for characterizing active fungi

We validated the quality of reverse transcribed cDNA originating from the ITS1 spacer situated between 18S and 5.8S regions and compared it to the coding 18S rRNA region and

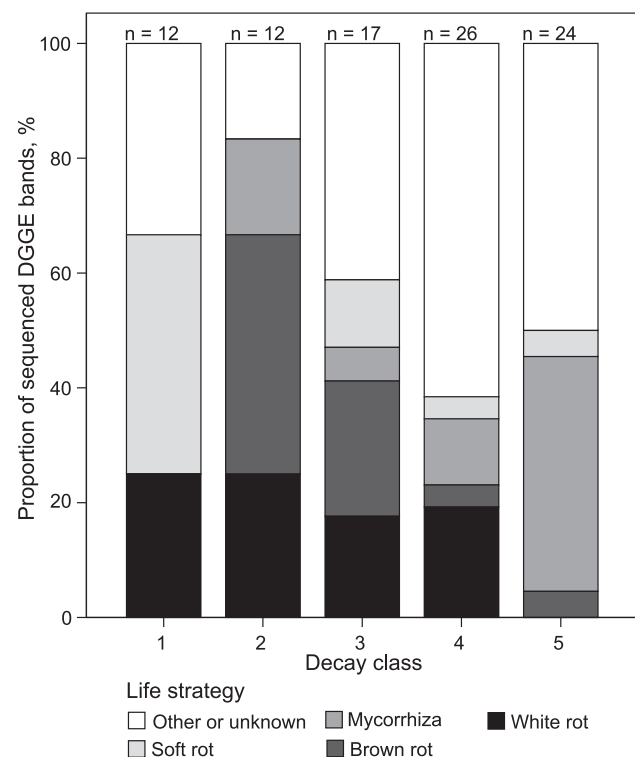


Fig 6 – Succession of life strategies of metabolically active fungi during wood decomposition, classified from decay stage 1–5.

respective rDNAs. Mycelium used as template was taken from pure fungal cultures actively growing on rich nutrient media and thus it was not surprising that both regions resulted in generous amounts of RT-PCR products. We noted that many of the fungi tested showed length variation between the DNA and the respective cDNA products. The 18S rDNA region is known to contain a diverse selection of introns (Hibbett 1996) and, accordingly, DNA inserts were always located within the 18S rDNA region. All of the tested ascomycete isolates, with the exception of the root endophytic dark septate endophytic (DSE) fungus *Phialocephala fortinii*, possessed 18S introns identified as Group I (Cech 1988; Perotto *et al.* 2000). The ascomycete isolates in our study belong to the subphylum Pezizomycotina. This subphylum contains species with the most Group I introns of all eukaryotes (Bhattacharya *et al.* 2005). Group I introns are widely found in the genomes of fungi and also in algae and unicellular eukaryotes (reviewed by Haugen *et al.* 2005). These introns can self-splice in the 35–40S precursor rRNA (Hibbett 1996), which is an earlier phase in rRNA processing than the splicing of the ITS1 region (Hibbett 1992; Allmang *et al.* 2000). This explains why all the fungal cDNA templates still contained the ITS region but introns in the coding region of 18S were already excised. The common appearance of self-splicing introns implies that in studies that do not make use of the phylogenetic information these introns provide, cDNA template might be more convenient than DNA template due to the absence of various inserts possibly causing technical problems in PCR amplification (Vrålstad *et al.* 2002; Damon *et al.* 2010) and community fingerprinting.

Group I introns were amplified with the ITS1F forward primer, which is fungal specific (Gardes & Bruns 1993) and often used to amplify fungal communities in environmental samples. The divergent mobilities of rDNA- and rRNA-derived amplicons of certain fungi implies that community profiling by DGGE, T-RFLP or other separation methods using this amplicon may lead to misinterpretation; bands/peaks missing from an RNA-based profile or appearance in the RNA profile may be due to the inserts being excised from rDNA, not due to the divergent community structures of active and total communities. In our data from wood-inhabiting fungal communities, this effect was detected in the form of a few rRNA-derived bands without a corresponding band in the rDNA-based fingerprint. Some of the fragments may also have originated from species with low abundance but high activity, or species that were present due to the uneven mixing of sawdust samples.

Taken together, our findings suggest that DNA- and RNA-based fingerprints should not be directly compared. However, it may well be that length variation between DNA and its cDNA remain a minor problem when targeting highly diverse fungal communities, since only a few studies exploring richness of soil fungi have detected differences between rDNA- and rRNA-derived fingerprints (Anderson & Parkin 2007; Bastias *et al.* 2007). Dissimilar mobilities of rDNA and the respective cDNA fragments may easily remain undetected in fingerprinting methods (e.g., DGGE and T-RFLP) that are unable to separate all the species efficiently in highly diverse communities (Pennanen *et al.* 2001). On the basis of test results and the advantages of precursor rRNA mentioned

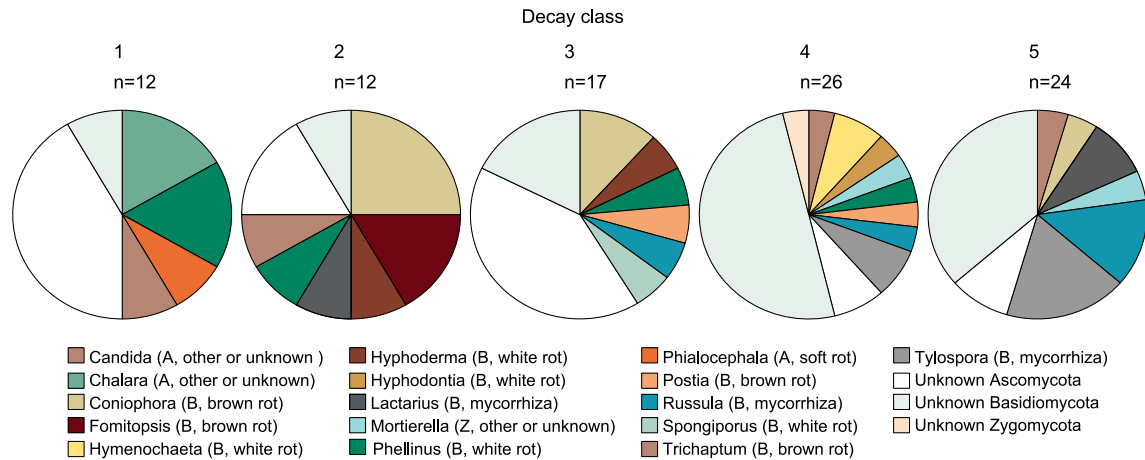


Fig 7 – Succession of metabolically active fungal genera during wood decomposition, classified from decay stage 1–5. A and B in parenthesis after the genera names refers to Ascomycota and Basidiomycota, respectively. Assumed life strategy of each fungal genus is also marked in parentheses.

above, we used the ITS region to measure metabolic activity in fungal communities of decaying Norway spruce logs.

Richness of active wood-inhabiting fungi increases with decay

As expected, fungal community structure in decaying logs changed with decomposition. The number of fungal taxa increased with decay according to the precursor rRNA-based analysis, but the rDNA-based estimate did not suggest an increase from slightly to highly decayed logs. Recently dead trees may have been colonized by endophytes or other latent fungi (see Parfitt et al. 2010) that were not metabolically active at the time of sampling and were undetected by the precursor rRNA analysis. On the other hand, divergence among rDNA- and rRNA-based fungal richness succession may result from limited resolution of DGGE fingerprinting. In heavily decayed logs, fungal richness may have been so high that different species were not reliably or adequately separated in DGGE. Hence, we propose that the rRNA-based analysis is preferable as it offers the means to eliminate an inactive and thereby less interesting part of the microbial community from environmental samples. This might be even more appropriate if the resolution of the method is poorly matched to the expected richness. Methods of higher resolution are needed in the case of more decayed wood and when using DNA template since it is evident that DGGE is biased towards the dominant component(s) of the fungal community (Ovaskainen et al. 2010).

Estimates of fungal richness based on conspicuous fruit bodies of polypores and corticoids (Aphyllporaceae) usually peak on moderately decayed coniferous logs (Bader et al. 1995; Renvall 1995; Lindblad 1998) when the decay rate is assumed to be highest (Mäkinen et al. 2006). In contrast, this study shows that the “total” number of metabolically active fungal species reaches a maximum in heavily decayed logs. Also, previous studies using directly extracted DNA (Ovaskainen et al. 2010; Rajala et al. 2010) or isolation on media (Lumley et al. 2001; Fukasawa et al. 2009b) have implied a maximal fungal richness in almost completely decayed logs. This

makes sense if we consider the remarkable fungal richness in forest soil (Buée et al. 2009). Many polypore and corticoid species are effective wood decomposers possessing the enzymes needed for decomposition of recalcitrant compounds such as lignin and cellulose (Hakala et al. 2004), which constitute as much as 70 % of the conifer’s dry mass (Sjöström & Westermarck 1998).

The fungal community responds to changes in the quality of decaying wood – and vice versa

Besides the increase in fungal richness, the species of fungi that were active changed during the course of wood decay. Ordination of the fungal community structure showed the relationship to wood density, which decreases during decomposition. In line with Fukasawa et al. (2009b) and our earlier results (Rajala et al. 2010), active fungal communities in decaying logs seemed to become increasingly similar during the succession. This may be partly due to the increase of species richness, which also leads to species in common among the logs. Then again, in the early stage of decay species composition may be highly variable as a result of random colonization via spores, specificity of initial colonizers, or the local community of endophytic fungal species that can vary among and within tree species (Todd 1988; Korkama-Rajala et al. 2008). Fukami et al. (2010) recently showed in a microcosm experiment that the history of initial colonization has a considerable effect on the structure and functionality of the community of wood decomposers. However, as the decomposition succession of a fallen tree proceeds, branches disappear, stem contacts the ground and consequently, soil becomes a prominent source of fungal colonization. We suggest that colonization via soil mycelia may partly stabilize the community composition of wood-inhabiting fungi. Still, soil microbes are patchily distributed (Pennanen et al. 1999; Lilleskov et al. 2004) and fungal colonization via soil is not uniform.

As wood decays and its density decreases, the moisture and lignin content increases and C/N ratio decreases (Lambert

et al. 1980; Palviainen et al. 2008; Krankina et al. 1999; Laiho & Prescott 2004). Similar to previous studies of fruiting (Fukasawa et al. 2009a) and isolated wood-inhabiting fungi (Lumley et al. 2001; Fukasawa et al. 2009b), all the above mentioned changes in wood chemical and physical properties were associated with fungal community structure as determined by DNA, whereas only moisture at the sampling time was significantly related to the active species determined by precursor rRNA. The result indicates that as a molecular marker precursor, rRNA is less stable and more difficult to handle compared to rDNA or other traditional methods. For example, fungal activity may respond rapidly to weather changes. In our opinion, this should be taken into consideration when using precursor rRNA as a marker in environmental studies, although the approach might be optimal for the *in situ* detection of rapid responses of fungal communities to fluctuating environmental conditions or resource quality.

Functional succession of metabolically active fungi during the decomposition process

Functionality of saprotrophic fungi is often considered to change during wood decay, following succession from soft-rot fungi to white- and brown-rot fungi (Rayner & Boddy 1988). Here, we found that metabolically active soft-rot fungi mainly colonized recently fallen spruce logs. Some of the ascomycetes classified as soft-rot fungi might have been latent in live spruces. For example, *Phialocephala* species have been found both from living and decaying trees, which might indicate the change in their functionality from endophytes to decomposers (Menkis et al. 2004). Also, certain needle endophytes are known to be active and efficient decomposers (Korkama-Rajala et al. 2008). Mycelia of white-rot fungi were present and active from the earliest stage of decay but they were undetectable from heavily decayed logs and presumably had been succeeded by ectomycorrhizal fungi. In boreal forest soil, ectomycorrhizal fungi constitute up to half of the fungal biomass (Finlay & Söderström 1989; Högberg & Högberg 2002) and they are frequently found in coarse woody debris (Tedesoo et al. 2003; Hashimoto & Yoneda 2006). Dead logs in contact with the soil can be colonized by ectomycorrhizal fungi via mycelial growth. What was more surprising is the finding that metabolically active ectomycorrhizal fungi were detected in slightly decayed (stage 2) spruce logs, which still had intact branches preventing the trunk from contacting the soil. Contamination is an unlikely explanation, as sampling tools were carefully sterilized between samples and negative PCR control reactions never amplified. In addition, the finding is in line with our latest results from five study sites and nearly 600 logs (T. Rajala, M. Peltoniemi, T. Pennanen, R. Mäkipää, unpublished). The first ectomycorrhizal species to be detected here were members of *Lactarius* and *Russula*, which likely colonized via spores. *Tylospora*, one of the most common ectomycorrhizal genera in Norway spruce (Taylor et al. 2000; Korkama et al. 2006; Toljander et al. 2006) from very early ages (T. Pennanen, M. Müller, T. Rajala, S. Timonen, R. Rikala, J. Hantula, J. Garbaye, unpublished), was frequently found in highly decayed spruce logs. Dead wood colonization via airborne spores might be less significant for *Tylospora* species that form resupinate fruit bodies under logs compared to

pileated *Lactarius* and *Russula*. The role of ectomycorrhizal *Lactarius*, *Russula* and *Tylospora* species as facultative decomposers has been suggested (Chambers et al. 1999; Bødeker et al. 2009) as they are found to possess peroxidase-encoding genes and enzyme activity *in vitro*. In natural forests, decaying logs are known to be a good regeneration base for coniferous seedlings, possibly due to lower competition with forest floor vegetation. Thus we propose that the activities of certain ectomycorrhizal fungi in decaying logs might also be related to the continuum of tree regeneration in old natural forests.

Conclusions

Coding regions of the rRNA gene have been used for molecular identification of active organisms. Although ITS is a non-coding region of the rRNA gene and is spliced during the synthesis of mature rRNA, it can be detected in the fungal RNA pool extracted directly from an environmental sample. Therefore, ITS of precursor rRNA is a more attractive marker of metabolically active fungi *in situ*, not only because it is variable among species and is well represented in public databases, but also due to its fast turnover time. The use of precursor rRNA as a fungal identification marker allows the most active part of the community to be studied, which may be particularly attractive if the proportion of inactive species is high and resolution of the profiling method is limited. The instability and sensitivity of precursor rRNA to environmental fluctuations should also be acknowledged in sampling and data evaluation. We demonstrated that comparison of inactive and active fungi may be biased by possible length variation between rDNA and corresponding cDNA fragments. Length variation between SSU 18S rDNA and cDNA is common among ascomycetes and is due to introns within the 18S rRNA gene, which become degraded in the early stages of RNA transcript processing.

Here, we analysed fungal succession in decaying spruce logs using precursor rRNA as a marker of the metabolically active species. Based on the data, richness of active fungi at the time of sampling increased with decay and peaked in highly decayed logs. Divergence between rDNA- and precursor rRNA-derived fungal richness in the early stage of succession suggests that only a part of the community was metabolically active in recently dead trees whereas richness of active species increased during decay. Functionality of wood-inhabiting fungi changed from soft- and white-rot fungi in the beginning of decay to dominance of white- and brown-rotting fungi in moderately decayed logs. Somewhat surprising was that ectomycorrhizal fungi were found early in the decay process (stage 2 logs) although they were widespread in the late stage of succession too.

The test data of precursor rRNA of fungal isolates demonstrate the requirement of validating new approaches, bearing in mind that in exploring the richness of an environment, *in vitro* validations will always cover just the foreseeable part of the microbial richness. Challenges for future research on fungal litter decomposers are to determine the relationship between fungal community structure and decay rate, as well as determining the functional role of endophytic, ectomycorrhizal and yet unknown fungal species in the

decomposition succession. Before taxonomic and functional annotations of genes related to decomposition are sufficiently recognized, precursor rRNA could be used to reveal specific roles of fungi in decaying forest litter.

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