

# Detection of active soil fungi by RT-PCR amplification of precursor rRNA molecules

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## Abstract

Microbial ecologists have used direct RT-PCR amplification of 16S rRNA molecules for the detection of active species of bacteria and archaea, and 18S rRNA molecules for the detection of active fungi. The drawback to this approach for fungi is that 18S rRNA sequences often do not provide sufficient taxonomic resolution to allow identification of taxa in mixed communities to genus or species level. Internal transcribed spacer (ITS) sequences are known to be more taxonomically informative than 18S rRNA sequences and are the common target in DNA based studies but are thought to be absent from RNA pools as they are cleaved after transcription of the large rRNA precursor molecule to leave the mature rRNA's for ribosome synthesis. Here we show, however, that fungal ITS regions can be detected in RNA pools by RT-PCR amplification of fungal precursor rRNA molecules. This suggests that precursor rRNA molecules reside in the cells of active fungi for sufficient time to allow RT-PCR amplification of ITS regions prior to their removal by post-transcriptional cleavage. The RT-PCR conditions for this approach were initially optimised using a range of fungi grown in pure culture prior to applying the approach to complex fungal communities in two contrasting soil types.

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## 1. Introduction

Fungi are an important component of the world's microbiota and are responsible for many of the key steps in a wide range of ecosystem processes. The application of molecular techniques that exploit sequence variation within ribosomal RNA (rRNA) genes and associated spacer regions has enabled the field of fungal ecology to progress rapidly over the last decade (Horton and Bruns, 2001; Anderson and Cairney, 2004). Fungal rRNA genes exist as a multi-copy gene family arranged as tandem repeats, with each major repeat containing the coding regions for the primary rRNAs and non-coding spacer regions. Within the rRNA gene cluster, the target regions most commonly used in ecological studies of fungal communities are the genes encoding 18S rRNA and 25/28S rRNA, and the internal transcribed spacer (ITS) region that incorporates the 5.8S rRNA gene. The highly variable nature of rapidly evolving rDNA spacer regions has made the ITS the most popular choice for species level iden-

tification of fungal taxa in environmental DNA pools (Anderson and Cairney, 2004). As a result, it is the region for which the largest amount of reference database sequence information is currently available for the molecular identification of fungi.

While rRNA genes and spacer regions are useful for the detection and identification of soil fungi, they can persist in environmental DNA pools for species that are metabolically inactive and functionally less important (Ostle et al., 2003). For this reason, analysis of fungal community structure by detecting rRNA genes or spacer regions in environmental DNA pools includes a strong 'historical' component. This limits the usefulness of the approach when investigating the response of communities to environmental perturbations as rRNA genes may be detected in DNA pools for species whose growth or cellular activity has declined. An alternative approach for the detection of metabolically active and functionally important species is to target fungal rRNA molecules extracted directly from environmental samples. This is commonly used in bacterial ecology and is based on the fact that metabolically active species will transcribe more rRNA for ribosome synthesis than inactive species (Prosser, 2002). In fungi, the main rRNA operon

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Table 1  
Database matches for ITS RT-PCR products generated for the fungal isolates used in this study

Fungal isolate	EMBL database match	Similarity <sup>a</sup> (%)
1. <i>Amanita rubescens</i> (AM084702)	ITS sequence– <i>A. rubescens</i> (AJ889923)	97.1
2. <i>Cenococcum geophilum</i> (AM084698)	ITS sequence– <i>C. geophilum</i> (AY394919)	100.0
3. <i>Cortinarius glaucopus</i> (AM084701)	ITS sequence– <i>C. glaucopus</i> (AY174787)	99.3
4. <i>Lactarius controversus</i> (AM084697)	ITS sequence– <i>L. controversus</i> (AJ272246)	99.4
5. <i>Paxillus involutus</i> (AM084700)	ITS sequence– <i>P. involutus</i> (AY585915)	99.7
6. ' <i>Piceirhiza bicolorata</i> ' isolate <sup>b</sup> (AM084704)	ITS sequence– <i>P. bicolorata</i> -like root tip isolate (AY579413)	98.6
7. <i>Piloderma byssinum</i> (AM084703)	ITS sequence– <i>P. byssinum</i> (AY010279)	99.1
8. <i>Pisolithus albus</i> (AM084705)	ITS sequence– <i>P. albus</i> (AF440868)	98.9
9. <i>Pisolithus microcarpus</i> (AM084706)	ITS sequence– <i>Pisolithus</i> sp. (AF270778)	100.0
10. <i>Rhizopogon roseolus</i> (AM084707)	ITS sequence– <i>R. roseolus</i> (AJ419209)	98.0
11. <i>Suillus variegatus</i> (AM084696)	ITS sequence– <i>S. variegatus</i> (L54081)	99.8
12. <i>Trametes versicolor</i> (AM084699)	ITS sequence– <i>T. versicolor</i> (AY309017)	99.3

<sup>a</sup> Similarity across the entire sequence length.

<sup>b</sup> Fungal isolate obtained from a *Piceirhiza bicolorata* ectomycorrhizal root tip.

(containing 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 25/28S rRNA) is transcribed by RNA polymerase I as a large single precursor molecule. The 5S rRNA gene is transcribed separately by RNA polymerase III. Post-transcriptional processing of the main precursor rRNA molecule removes the ITS1 and ITS2 spacer regions leaving the mature 5.8S rRNA, 18S rRNA and 25/28S rRNA genes for ribosome synthesis. The removal of ITS regions from rRNA pools limits the choice of target regions in RNA based studies to those coding structural rRNA genes. 18S rRNA has recently been used for this purpose by RT-PCR amplification from soil RNA extracts (Girvan et al., 2004; Lueders et al., 2004; Rangel-Castro et al., 2005) but the lack of taxonomic resolution between 18S rRNA genes of closely related taxa limits the usefulness of the data for species identification (Anderson and Cairney, 2004).

Hitherto it has been assumed impossible to detect ITS sequences in RNA pools (Hibbett, 1992) because they are removed after transcription of the main precursor rRNA molecule. However, we hypothesised that metabolically active fungi would constantly transcribe precursor rRNA molecules and that ITS sequences could be detected in the precursor rRNA pool prior to their removal by post-transcriptional rRNA processing events. This was based on the knowledge that precursor rRNA is the most active transcription unit in eukaryotic cells and that up to 80% of all RNA transcribed in growing eukaryotic cells at any point in time is precursor rRNA (Paule and Lofquist, 1996). Here we describe an approach for the detection of active and functionally important fungi in a mixed community by RT-PCR amplification of ITS sequences present in eukaryotic

precursor rRNA molecules. The approach was developed and validated using RNA extracted from fungi grown in pure culture, before applying the methodology to soil fungal communities.

## 2. Materials and methods

### 2.1. Fungal isolates and RNA extraction

Fungal isolates (Table 1) were maintained on nutrient agar prior to extraction of RNA from cultures that were no more than 4 weeks old. RNA was extracted from approximately 100 mg of fungal mycelium using the RNeasy Plant Mini kit, following the "Plant and Fungi" protocol, with an additional DNase digestion step (Qiagen, Crawley, United Kingdom).

### 2.2. RT-PCR amplification of fungal ITS regions from isolates

cDNA was synthesised from 1–2 µl of purified RNA (~30–50 ng) using the ITS4 primer (White et al., 1990) and Superscript II Reverse Transcriptase (Invitrogen, Paisley, United Kingdom). Control reactions containing no Superscript II were performed for each sample along with a reaction containing no RNA. Fungal ITS regions were then amplified using the primers ITS1F (Gardes and Bruns, 1993) and ITS4, with 0.5–1 µl of cDNA as template. PCR was carried out in a 50 µl reaction volume containing 20 pmol of each primer, 2 mM MgCl<sub>2</sub>, 250 µM of each dNTP, 10×NH<sub>4</sub> buffer [160 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 670 mM Tris–HCl (pH8.8 at 25 °C), 0.1% Tween 20] and 2.5 units of BIOTAQ DNA polymerase (Bioline, London, United Kingdom), on a Dyad DNA Engine thermal cycler (MJ Research Inc., Waltham, USA). Cycling parameters were 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, with a final extension of 72 °C for 10 min. An additional negative control containing no cDNA was included in the RT-PCR. All amplification products were electrophoresed in 1.5% (w/v) agarose gels, stained with ethidium bromide and visualised under UV light.

### 2.3. DNA sequencing and data analysis

RT-PCR products from the fungal isolates were purified using the Qiaquick PCR purification kit (Qiagen, Crawley, United Kingdom). Purified DNA was then sequenced with the

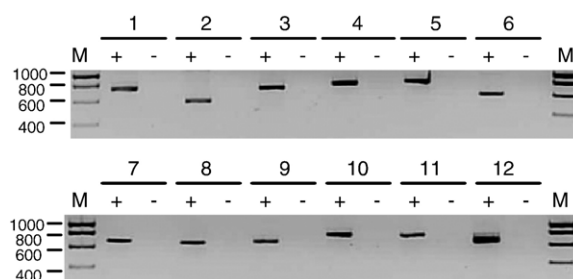


Fig. 1. ITS RT-PCR products generated for cultured fungal isolates. Numbers correspond to the fungal isolates listed in Table 1. +=ITS RT-PCR product; -=RT PCR negative control (no reverse transcriptase added in the cDNA synthesis step). M=Hyperladder I (Bioline, London, United Kingdom) molecular weight marker indicated in bp.

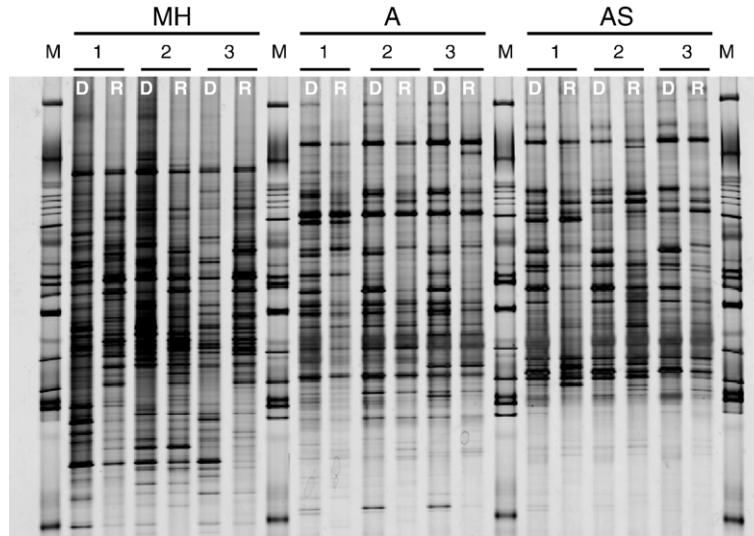


Fig. 2. Fungal ITS DGGE profiles generated for triplicate soil samples from each site. MH=montane heathland; A=agricultural field; AS=agricultural field amended with sewage sludge. D and R represent ITS-DGGE profiles generated using DNA and RNA from each sample, respectively. M=marker lanes.

primers ITS1F and ITS4 using the BigDye Terminator Cycle Sequencing Kit v3.1 on an automated DNA sequencer (ABI model 3130 × 1, Applied Biosystems, Inc., Warrington, United Kingdom). DNA sequences were manually checked and edited where necessary using the Sequencher software package (version 3.0; Gene Codes Corporation, Michigan, USA). All sequences were analysed using the FASTA 3.0 program (Pearson and Lipman, 1988) to determine the closest matches in the EMBL nucleotide database.

#### 2.4. Soil sampling

Triplicate soil samples were collected from two different field sites; a montane heathland site (MH) and an agricultural field site (A). Site MH was located on an area of prostrate *Calluna* heathland at 720 m above sea level at Invercauld, Scotland (National Grid Reference NO184985). The soil is a peaty ranker overlying diorite with a shallow (5–10 cm thick) organic horizon (pH of 3.9, 28.6% carbon and 1.1% nitrogen).

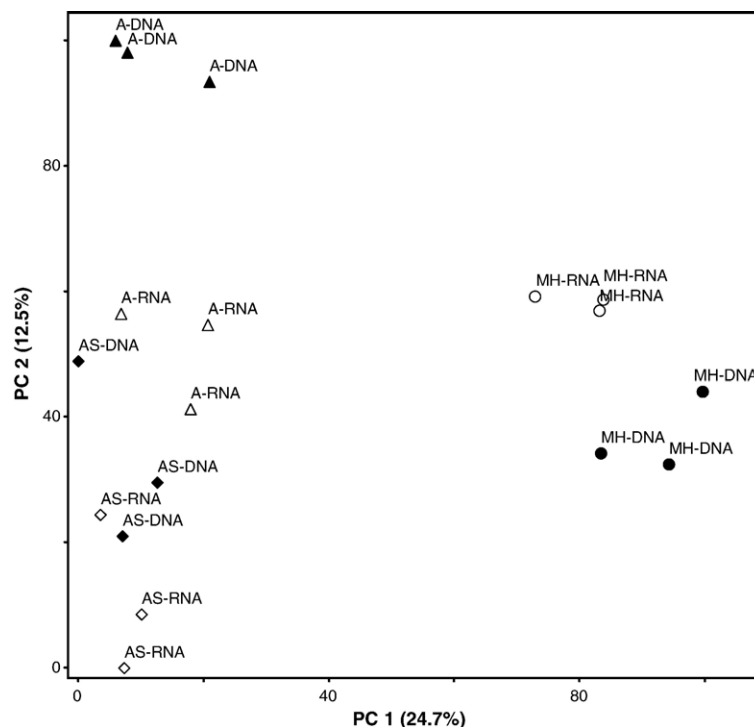


Fig. 3. Principal components analysis (PCA) of the ITS-DGGE profiles generated using DNA and RNA from triplicate soil samples from each site. MH=montane heathland; A=agricultural field; AS=agricultural field amended with sewage sludge.

Site A is situated at Hartwood Research Station, in Lanarkshire, Scotland (National Grid Reference NS855602) with soil that is a medium textured sandy clay loam (pH 5.8, 21% clay; 4.7% organic carbon) (Campbell et al., 2003). Additional samples were collected from plots at site A that had been applied with wastewater sludge 4 years earlier (AS).

### 2.5. RNA extraction and purification from soil

Nucleic acids were extracted from 0.5 g sub-samples of each soil sample in duplicate, using a previously described method (Griffiths et al., 2000). Nucleic acids from duplicate extractions were then pooled. Prior to RNA purification using the RNeasy MinElute Cleanup kit (Qiagen, Crawley, United Kingdom), a 25 µl aliquot of the nucleic acids was subjected to an additional DNase digestion step using RNase-Free DNase (Qiagen, Crawley, United Kingdom), according to the manufacturer's instructions.

### 2.6. RT-PCR amplification of fungal ITS regions from soil

cDNA was synthesised, and ITS RT-PCR products were amplified, as described above for the fungal cultures. To generate ITS PCR products for DGGE analysis, 1 µl of the RT-PCR products from soil were used as template in a nested PCR reaction with the primers ITS1F (containing a GC clamp) and ITS2 (White et al., 1990) as described in Anderson et al. (2003). ITS PCR products were also generated from the remaining genomic DNA so that ITS-DGGE profiles generated from DNA and RNA could be compared.

### 2.7. Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analyses were carried out using the DCode universal mutation detection system (Bio-Rad, Hemel Hempstead, United Kingdom). Polyacrylamide gels (8% Acrylogel 2.6 solution; BDH, Poole, United Kingdom) were prepared with a 20% [1.4 M urea–8% (vol/vol) formamide] to 60% [4.2 M urea–24% (vol/vol) formamide] vertical gradient using a gradient former (Fisher Scientific, Loughborough, United Kingdom) and a peristaltic pump with a flow rate of 5 ml min<sup>-1</sup>. Gels were poured onto the hydrophilic side of GelBond PAG film (BioWhittaker, Wokingham, United Kingdom) to facilitate handling during the staining process. Approximately 1 µl (500 ng) of each nested RT-PCR product from soil samples was loaded onto the gels and electrophoresis was performed in 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA) at 75 V and 60 °C for 16 h. Gels were silver stained as previously described (McCaig et al., 2001) and were scanned using an Epson Expression 1680 Pro scanner at a resolution of 600 dpi. DGGE gels were analysed using Gel Compar II software, version 4.01 (Applied Maths, Sint-Martens-Latem, Belgium). The profiles for each sample were normalised against the marker lanes, with a minimum profiling set at 5% to automatically search for bands. Binary matrices were produced using a band matching optimisation position tolerance of 1%. The binary data were then analysed using principal components analysis (PCA) in PC-Ord (MJM software, Oregon, USA).

### 2.8. Cloning and sequencing

ITS RT-PCR products, for a representative sample from each field site (MH-1, A-2, AS-2) were purified as stated above, and cloned using the pGEM-T Easy vector system (Promega, Southampton, United Kingdom). Ligation reactions were transformed into JM109 High Efficiency Competent Cells (Promega, Southampton, United Kingdom). White colonies (24) were picked at random from each clone library and screened for positive inserts with the primers M13 forward and M13 reverse, using the PCR conditions outlined above. RFLP analysis using *Taq*I (Promega, Southampton, United Kingdom) restriction endonuclease was performed on the positive clones according to the manufacturer's instructions. The digested amplicons were visualised by agarose gel electrophoresis and clones resulting in unique RFLP patterns were selected for sequencing. Sequencing reactions were performed as stated above.

## 3. Results and discussion

ITS RT-PCR products were successfully amplified from fungal isolate RNA extracts and they ranged in size between 600 and 800 bp depending on the isolate (Fig. 1). Database searches of the sequences generated from ITS RT-PCR products confirmed the amplified products as ITS sequences originating from

Table 2  
Database matches for sequences generated from cloned ITS RT-PCR products

Sample <sup>a</sup>	Clone	Accession number	EMBL database match	Similarity (%)
MH-1	3	AM113720	ITS sequence– <i>Capronia</i> sp. (AF284126)	98.6
MH-1	17	AM113717	ITS sequence– <i>Leucosporidium fellii</i> (AF444508)	91.5
MH-1	2	AM113719	ITS sequence– <i>Capronia villosa</i> (AF050261)	83.7
MH-1	14	AM113716	ITS sequence– <i>Botryosphaeria</i> sp. (DQ1455728)	78.2
MH-1	10	AM113715	ITS sequence– <i>Coniosporium</i> sp. (AJ971453)	77.8
MH-1	1	AM113718	ITS sequence– <i>Botryosphaeria</i> sp. (DQ14728)	73.6
A-2	1	AM113722	ITS sequence– <i>Cryptococcus terricola</i> (AF444377)	99.7
A-2	10	AM113721	ITS sequence– <i>Tetracladium furcatum</i> (AF411026)	95.8
A-2	3	AM113723	ITS sequence– <i>Pseudogymnoascus roseus</i> (AY608924)	93.9
A-2	9	AM113724	ITS sequence– <i>Rhodotorula</i> sp. (AB025982)	76.8
AS-2	18	AM113725	ITS sequence– <i>Pseudeurotium bakeri</i> (AY129287)	98.2
AS-2	5	AM113729	ITS sequence– <i>Gymnascella dakaliensis</i> (AY304514)	91.1
AS-2	2	AM113727	ITS sequence– <i>Ascomycete</i> sp. (AY354274)	86.0
AS-2	1	AM113726	ITS sequence– <i>Choiromyces echinulatus</i> (AF435825)	75.8
AS-2	4	AM113728	ITS sequence– <i>Choiromyces echinulatus</i> (AF435825)	74.6

<sup>a</sup> Sample numbers correspond to those presented in Fig. 2.



each of the respective fungal species (Table 1). The fact that no amplification product was detected in either the RT negative controls or the PCR negative controls suggests the ITS products amplified and sequenced originated from cellular precursor rRNA pools.

Fungal ITS RT-PCR products were subsequently generated from RNA extracted from two different soil types; a montane heathland soil (MH), and an agricultural soil (A). RNA was also extracted from plots at the same agricultural field site that had been treated with wastewater sludge 4 years previously (AS). DGGE analysis of the ITS PCR products revealed a complex banding pattern for each sample, regardless of the soil type or whether they originated from DNA or RNA (Fig. 2). Principal components analysis (PCA) of ITS-DGGE profiles generated using both DNA and RNA for all samples clearly separated the samples based on both location and, importantly, also on the basis of DNA and RNA (Fig. 3). Axis 1 explained 24.7% of the variation and primarily separated the samples collected from the two different field sites. Axis 2 explained a further 12.5% of the variation in the data and clearly separated the samples within a site based on whether the ITS DGGE profiles were generated using DNA or RNA. In addition, ITS DGGE profiles for agricultural soil that had been treated with wastewater sludge (AS) separated from untreated control soils (A) collected from the same site along this axis (Fig. 2). These data demonstrate that differences in indigenous soil fungal communities can be detected between those species which are present (DNA) and those which are present and metabolically active (RNA) even without perturbing the environment to influence metabolic activities. In addition, it demonstrates that the approach can be applied to soil, which is one of the most complex and difficult substrates to use in molecular analyses due to the co-extraction of contaminating compounds (e.g. humic acids) with the nucleic acid pool which can interfere with subsequent enzymatic reactions (e.g. Tebbe and Vahjen, 1993).

To check that bands in the RNA-derived DGGE profiles represented fungal ITS sequences, clones from ITS RT-PCR clone libraries for a sample from each field site were screened and sequenced (Table 2). FASTA searches of the EMBL database revealed closest matches to a diverse range of both ascomycete and basidiomycete fungal taxa (Table 2). More importantly, all sequences were identified as being an ITS sequence of fungal origin (Table 2), thus confirming that the bands present in the RNA-derived DGGE profiles represent ITS sequences of soil fungi. Because DNA was eliminated from the samples via a DNase digestion and further RNA purification procedure, and no amplification products were detected in any controls which were run independently for every sample, the RT-PCR amplified ITS products must have originated from soil precursor rRNA pools.

While the approach opens up exciting new possibilities for increasing our understanding of the ecology of these ubiquitous and ecologically important organisms, particularly compared with DNA based molecular approaches, one potential limitation must not be overlooked. RT-PCR detection of bacterial 16S rRNA molecules or fungal 18S rRNA molecules is considered to be detection of active members of the microbial community (e.g.

Girvan et al., 2004), however, this may not always be completely true. It is assumed that rRNA is rapidly degraded after decreased cellular activity or growth but we do not currently know how detection of rRNA relates to ribosome number and activity in fungi, although this is likely to be species dependent. This is important because it has been shown that while rRNA decay in some species of bacteria is rapid after nutrient starvation (Kerkhof and Kemp, 1999), it has also been shown that ribosome numbers can be higher than that required for protein synthesis in other species under starvation conditions (Flardh et al., 1992). Importantly, however, this is likely to be more of a problem in considering the relationship between rRNA detection and cellular metabolic activity when the main rRNA subunits are the target as in the 16S and 18S rRNA approaches for bacteria and fungi. This is because precursor rRNA molecules are processed after transcription, cleaving the ITS regions resulting in the mature rRNA molecules (i.e. 18S, 5.8S or 28S rRNA). Thus, the turnover rate of precursor rRNA molecules is likely to be greater than that of mature rRNA molecules. This said, rates of precursor rRNA synthesis and post-transcriptional processing is likely to vary between different fungal species.

The approach described here provides a novel way of detecting and identifying active soil fungi by exploiting the presence, and ability to detect, precursor rRNA molecules in RNA pools. The approach targets the commonly studied and taxonomic informative ITS region, and thus capitalises on the wealth of knowledge that exists on ITS sequence diversity in fungi.

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