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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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Keywords: 18S rRNA; Active fungi; Fungal diversity; Internal transcribed spacer (ITS); Precursor rRNA molecules; Soil fungi

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

[a] Similarity across the entire sequence length.
[b] 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 MgCl2, 250 μM of each dNTP, 10×NH_4 buffer [160 mM $(NH_4)_2SO_4$, 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

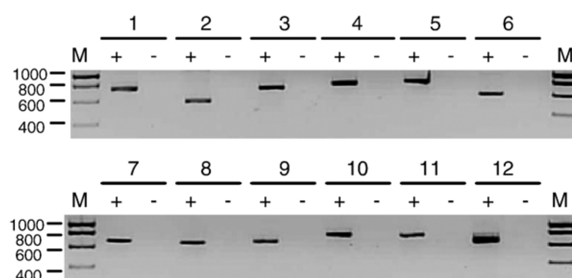

Fig. 1. ITS RT-PCR products generated for cultured fungal isolates. Numbers correspond to the fungal isolates listed in Table 1. +=ITS RT-PCR product; −=RT PCR negative control (no reverse transcriptase added in the cDNA synthesis step). M=Hyperladder I (Bioline, London, United Kingdom) molecular weight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

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