RESEARCH LETTER

Amplification of soil fungal community DNA using the ITS86F and ITS4 primers

Tony Vancov & Brad Keen

NSW Department of Primary Industries, Wollongbar, NSW, Australia

Correspondence: Tony Vancov, NSW Department of Primary Industries, 1243 Bruxner Highway, Wollongbar, NSW 2477, Australia. Tel.: +61 2 6626 1359; fax: +61 2 6628 3264; e-mail: tony.vancov@dpi.nsw.gov.au

Received 16 February 2009; accepted 6 April 2009.
Final version published online 11 May 2009.

DOI:10.1111/j.1574-6968.2009.01621.x

Editor: Richard Staples

Keywords
fungi; fungal community; fungal primers; ITS primers; soil.

Abstract

Internal transcribed spacer (ITS) 86F and ITS4 and the ITS1-F and ITS86R primer pairs were tested to specifically amplify fungal community DNA extracted from soil. Libraries were constructed from PCR-amplified fragments, sequenced and compared against sequences deposited in GenBank. The results confirmed that the ITS86F and ITS4 primer pair was selectively specific for the Ascomycetes, Basidiomycetes and Zygomycetes fungal clades. Amplified products generated by the ITS1F and ITS86R primer pair also aligned with sequences from a range of species within the Ascomycete and Basidiomycete clades but not from the Zygomycete. Both primer sets demonstrated fungal specificity and appear to be well suited for rapid PCR-based (fingerprinting) analysis of environmental fungal community DNA. This is the first reported use and assessment of the ITS86F and ITS4 and the ITS1-F and ITS86R primer pairs in amplifying fungal community DNA from soil.

Introduction

While the discipline of mycology has done much to increase our knowledge of soil fungal communities, there remains an abundance of opportunities for further discovery. Advances in our understanding of the importance, roles and functions of fungi in ecological systems require the continued evolution of methods used to study them. The limitations of culture-based methods for studying soil microbial communities are well-documented, and molecular approaches involving analysis of complex community DNA are widely recognized as having potential for overcoming these limitations (Torsvik *et al.*, 1990; Anderson & Cairney, 2004). The past decade has seen a steady evolution of microbial community DNA-profiling methods that have been utilized to assess microbial community structure and diversity in a range of environmental samples (Liu *et al.*, 1997; Dunbar *et al.*, 1999; Marsh, 1999; Smit *et al.*, 1999; Hedrick *et al.*, 2000; Hill *et al.*, 2000; Kowalchuk *et al.*, 2003; Manter & Vivanco, 2007). These methods have the capacity to provide descriptive and quantitative information, discriminate between soil microbial communities in comparative studies and identify the presence of uncultivable and undescribed microorganisms (Anderson & Cairney, 2004).

Primer design and selection plays an important role in the successful application of these methods. Oligonucleotide primers selected for use in microbial community DNA-profiling methods need to be specific to, but universal within, the target group. For analysis of bacterial communities, primers that amplify DNA from the small subunit (SSU) 16S rRNA gene region are frequently used. The main reasons for this are that the 16S rRNA gene is found universally in bacteria and contains both highly conserved and variable sequence regions (Hill *et al.*, 2000). Therefore, primers that target regions within the 16S rRNA gene are likely to amplify a broad range of bacteria. In fungi, the 18S, 5.8S and 28S rRNA genes, which are traversed by the hypervariable internal transcribed spacer (ITS) 1 and ITS2 regions (Fig. 1), are recognized as being useful for the same reasons (Hill *et al.*, 2000).

Fungal-specific primer pairs that have been tested for analysis of fungal community DNA extracted from environmental samples include: ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990); 2234C and 3126T (Sequerra *et al.*, 1997); EF4 and EF3 (Smit *et al.*, 1999); EF4 and fung5 (Smit *et al.*, 1999); nu-SSU-0817-5′ and nu-SSU-1536-3′; nu-SSU-0817-5′ and nu-SSU-1196-3′ (Borneman & Hartin, 2000); and several others reviewed by Anderson & Cairney (2004).

Turenne *et al.* (1999) designed the ITS86F primer and coupled this with the ITS4 reverse primer (White *et al.*, 1990) for use in screening human blood for medically important fungal pathogens and maybe useful in fungal isolates. This primer pair amplifies the hypervariable ITS2 region, which straddles the 5.8S and 28S genes (Fig. 1). Turenne *et al.* (1999) and others have reported that this primer pair has fungal specificity. However, to date the ITS86F and ITS4 primer pair has only been applied in clinical medicine. The utility of this primer pair for amplifying fungal community DNA isolated from environmental samples is yet to be assessed. In the following study, we examined the capacity for the ITS86F and ITS4 primer pair to amplify fungal community DNA extracted from soil through sequencing amplified PCR products. We also tested a primer pair consisting of ITS1F (Gardes & Bruns, 1993) coupled with ITS86R, the reverse complement of the ITS86F primer. One of the criteria for selecting these primer pairs for use in fungal community F-ARISA was that the primers produce amplicons that could be accurately sized by automated capillary electrophoresis. Size variability of amplicons produced with ITS1F and ITS4 are poorly detected and/or resolved by agarose gel electrophoresis (Turenne *et al.*, 1999). Additionally, length variation of the ITS2 region could potentially be used in identifying soil fungal isolates.

Materials and methods

Soil DNA extraction

Microbial community DNA was extracted from a medium clay–loam red-ferrosol soil sampled from an avocado orchard located on the north coast of NSW, Australia. DNA extractions were carried out using the method described by Bell *et al.* (2006) with the exception that an additional clean-up step was performed by passing the DNA sample through a polyvinylpolypyrrolidone spin column (Berthelet *et al.*, 1996).

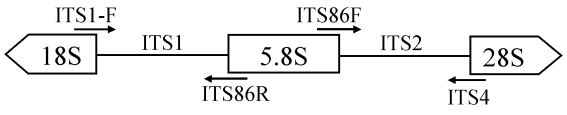

Fig. 1. Schematic representation of fungal ribosomal genes bridged by ITS regions targeted by the two primer pairs that were assessed in this study.

PCR amplification

PCR amplification was carried out using the following primer sets: (1) ITS1F and ITS86R and (2) ITS86F and ITS4 (Table 1). The first primer pair (subsequently referred to as the ITS1 primer set) targets the ITS1 region between the 18S rRNA gene and the 5.8S rRNA gene. The second prime pair (subsequently referred to as the ITS2 primer set) targets the ITS2 region between the 5.8S rRNA gene and the 28S rRNA genes (Fig. 1). All primers were synthesized by Invitrogen.

PCR amplification reaction mixtures contained 20 ng of soil community DNA, 0.2 mM premixed deoxynucleoside triphosphates (Astral Scientific), 150 μg DNAse- and RNAse-free bovine serum albumin (Amersham Biosciences, UK), 0.4 μM forward primer, 0.4 μM reverse primer, 1.25 U of HotMaster^{TM} Taq DNA polymerase (Eppendorf, GmbH) and 1 × HotMaster^{TM} Taq DNA polymerase buffer in a final volume of 50 μL. PCR amplification was carried out in a PE 9600 thermocycler (Perkin-Elmer) using an initial denaturation step at 94 °C for 3 min, followed by 30 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 2 min, with a final extension of 72 °C for 7 min. Successful PCR amplification was confirmed by visualizing 5 μL of the products following electrophoresis on a 1.5% agarose TAE gel and subsequent staining in ethidium bromide for 15 min. To remove unincorporated nucleotides and primers, PCR products were purified by polyethylene glycol (PEG)/NaCl (20%/1.6 M) precipitation (Sambrook *et al.*, 1989) and resuspended in a volume of 40 μL of sterile DNAse-free milli-Q water.

Cloning, sequencing and analysis

Two separate clone libraries were constructed from PCR amplification products resulting from the ITS1 and ITS2 primer sets. Following PEG precipitation, PCR products were cloned into pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen) and electroporated into One Shot® electrocompetent *Escherichia coli* cells using a Micropulser Electroporation Apparatus (BioRad).

Fifty randomly selected transformants from each library (ITS1 and ITS2 clone libraries) were PCR amplified with the

Table 1. Oligonucleotide sequences for the two primer sets that were tested

Target genomic region	Primers	Sequences (5′–3′)	Approximate size PCR product (bp)	References
ITS1	ITS1-F	CTTGGTCATTTAGAGGAAGTA	350	Gardes & Bruns (1993)
	ITS86R	TTCAAAGATTCGATGATTCAG		This study
ITS2	ITS86F	GTGAATCATCGAATCTTTGAA	400	Turenne *et al.* (1999)
	ITS4	TCCTCCGCTTATTGATATGC		White *et al.* (1990)

universal M13 forward and M13 reverse primers (supplied with TOPO TA cloning kit), purified by PEG precipitation as described above and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing kit (version 3.1) and an ABI 3730 Sequencer (Applied Biosystems) according to the manufacturer's instructions.

After editing (primer and vector sequences removed), the sequences were initially screened for chimeric fragments with the CHIMERA_CHECK program version 2.7 (Cole *et al.*, 2003), and then compared with reference sequences contained in the GenBank nucleotide sequence database using the BLAST algorithm (Altschul *et al.*, 1990). The closest matching sequences were retrieved from the database, aligned with the relevant cloned sequence and percentage homology was determined using pairwise alignment functions in BIOEDIT (Tom Hall, Ibis Therapeutics: http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequence data was deposited in GenBank under accession numbers FJ386853–FJ386954.

Results

Both primer sets successfully amplified DNA extracted from soil with PCR products from ITS1 and ITS2 primer sets appearing as intense bands on agarose gels concentrated at *c.* 400 and *c.* 350 bp, respectively (data not shown). Sequencing of clone libraries indicated the presence of fragments up to 602 bp within the ITS1 library and 403 bp within the ITS2 library. Both libraries contained a number of clones represented by the same fragment sequence lengths, but with different sequences, that aligned with fungal species either within the same genus or with distantly related genera (Tables 2 and 3).

When compared against nucleotide sequences in the GenBank database, sequences from the ITS1 clone library (Table 2) were found to most closely align to fungi in the Ascomycota and Basidiomycota clades. Within the Ascomycota group there were 30 clonal sequences distributed between 22 species with six clonal sequences of 346 bp being most closely related (84–96%) to a *Lecythophora* sp. (GenBank accession number: AY219880). There were 16 clones distributed between 15 Basidiomycete species; however, most of the clones displayed a relatively lower degree of homology with their closest match. Three of the sequenced isolates revealed a closer association to (1) an unknown ectomycorrhizal fungal species, (2) *Sphaerothecum destruens* nov. gen., nov. sp. – a *Dermocystida* (unique group at evolutionary stage of fungal–animal transition), and (3) *Cyphomyrmex rimosus* Spinola – a subterranean ant species that cultivates fungi (Murakami & Higashi, 1997).

Sequences from the ITS2 clone library most closely aligned with sequences acquired from the three major fungal clades (Table 3). None of the ITS2 clone sequences aligned with prokaryotic DNA sequences or eukaryotic sequences from organisms other than fungi. Thirty-nine ITS2 sequences were most closely related to 22 fungal species within the Ascomycota clade. There were 14 repeat fragments with a sequence length of 396 bp that had 88% sequence similarity with a *Verticillium* sp. (GenBank accession number AY172097). The remaining sequences were distributed among 21 Ascomycete species. Six sequences uniquely aligned with Basidiomycete species whereas the remaining six sequences aligned with four Zygomycete species.

Discussion

Both primer pairs ITS2 (ITS86F and ITS4) and ITS1 (ITS1-F and ITS86R) successfully amplified DNA extracted from soil. Sequence analysis showed that the ITS1 primers amplified DNA from two fungal clades (Ascomycota and Basidiomycota). However, within the ITS1 clone library two sequences were found to align with nonfungal DNA. The first was a 606-bp fragment with 96% homology to *S. destruens*, which lives in aquatic habitats and is a pathogen of fish (Arkush *et al.*, 2003). Interestingly, this organism is a member of the *Dermocystida* order, a unique phylogenetic group at an evolutionary point of fungal–animal transition (Mendoza *et al.*, 2002). The second, a 440-bp sequence, aligned with an arthropod *C. rimosus* from South America (Murakami & Higashi, 1997). Given that this mycophagous ant species cultivates fungi, this raises some doubt about the origin of the sequence published for *C. rimosus*. It may in fact correspond to DNA from a fungal contaminant.

There was no uncertainty surrounding the fungal specificity of the ITS2 primer set, which yielded sequences that aligned with only fungal species. This supports the findings of Turenne *et al.* (1999), which indicate that the ITS86F and ITS4 primer pair is fungal specific. Among the three fungal clades that were represented by ITS2 sequences, the Ascomycota had the highest representation, but there were also a number of sequences that aligned most closely with fungi from the Basidiomycota and Zygomycota. This indicates that either the Ascomycota may have been more dominant in the mixed DNA sample or that this result was an artefact of amplification biases during the PCR. The latter is an issue that is commonly observed during PCR of mixed template DNA and is an issue for which a solution is yet to be attained (Manter & Vivanco, 2007).

With the exception of the 396-bp fragment, which shared 88% homology with a *Verticillium* sp., there was generally a low incidence of repeat sequences. More comprehensive sequencing of the ITS2 PCR library would likely have yielded additional unique sequences. Similarly, the ITS1 primer set demonstrated a similar capacity to potentially detect a range of fungal species beyond those that were sequenced. As such, both primer pairs may be suitable for

Table 2. Closest matches in the GenBank database to sequences from the ITS1 clone library

Clades	Number of clones	Sequence length (bp)	Closest match	Accession number	Homology (%)
Ascomycota	1	284	*Myrothecium* sp.	AJ301998	88
	1	287	*Volutella ciliata*	AJ301966	96
	2	300	Unidentified fungal endophyte	AY433809	95
	1	303	Leaf litter Ascomycete	AF502734	49
	1	308	*Chaetomium funicola*	AJ279450	98
	1	310	*Verticillium dahliae*	AF104926	89
	1	316	*Muscodor albus*	AF324336	73
	1	318	*Metarhizium anisopliae*	AB099510	99
	1	318	*Fusarium oxysporum*	AY667486	98
	1	322	*Mycosphaerella nubilosa*	AF449096	76
	2	323	*Scedosporium prolificans*	AY228124	93
	1	328	*Emericella nidulans*	AY452983	99
	1	333	*Gaeumannomyces graminis*	AJ246150	75
	1	341	*Penicillium* sp.	AY513955	98
	1	344	*Penicillium* sp.	AY513955	98
	1	345	*Penicillium thomii*	AY373934	99
	1	346	*Penicillium* sp.	AY513955	98
	6	346	*Lecythophora* sp.	AY219880	84–96
	1	347	*Fusarium oxysporum*	AY667486	80
	1	349	*Arthrographis cuboidea*	AY557369	95
	1	356	*Neurospora crassa*	M13906	82
	1	363	*Neurospora crassa*	M13906	97
	1	366	*Cladophialophora boppii*	AB109182	85
Basidiomycota	1	301	*Exidia recisa*	AF291276	52
	1	310	*Polyporus varius*	AF516580	66
	2	336	*Tricholoma flavovirens*	AF458456	68
	1	336	*Bullera arundinariae*	AF547662	70
	1	353	*Rhodotorula* sp.	AF444638	70
	1	365	*Pisolithus* sp.	AY179747	72
	1	399	*Tyromyces chioneus*	AJ006676	72
	1	414	*Coprinus* aff. *phlyctidosporus*	AB071789	91
	1	421	*Gomphidius smithii*	AY077471	62
	1	435	*Bullera* sp.	AY313027	69
	1	436	*Chrysomphalina chrysophylla*	U66430	63
	1	437	*Bullera* sp.	AY313027	68
	1	442	*Schizophyllum commune*	AF350925	62
	1	482	*Thelephoraceae* sp.	U83476	52
	1	590	*Polyporus tuberaster*	AF516599	54
Unknown	1	294	Ectomycorrhizal root tip fungus	AF481369	86
Dermocystida	1	602	*Sphaerothecum destruens*	AY388645	96
Arthropoda: *Insecta*	1	440	*Cyphomyrmex rimosus* Note: fungi-cultivating ant species from South America	AF079693	78

use in studies that aim to characterize structure and diversity within fungal communities.

Assessments of microbial community structure and diversity by the method of amplifying, cloning and sequencing isolated DNA is laborious and expensive, especially where a number of samples require analysis. Several molecular approaches for comparing the structural characteristics and estimating diversity of microbial communities have been developed. These include TGGE and DGGE (Muyzer, 1999), T-RFLP (Marsh, 1999), A-RISA (Ranjard *et al.*, 2001) and LH-PCR (Suzuki *et al.*, 1998) and LH-qPCR (Manter & Vivanco, 2007). A-RISA and LH-PCR methods are based on measurements of natural variation in sequence lengths and, therefore, the amplicons produced by the PCR must be within a range that can be resolved using the available amplicon separation instrument (usually < 1000 bp). As was observed by Turenne *et al.* (1999), the ITS86F and ITS4 primer set produced amplicons of < 500 bp. As such this

Table 3. Closest matches in the GenBank database to sequences from the ITS2 clone library

Clades	Number of clones	Sequence length (bp)	Closest match	Accession number	Homology (%)
Ascomycota	1	276	*Massarina corticola*	AF383957	88
	1	282	*Chaetomium* sp.	AY533556	91
	1	285	*Ellurema* sp.	AY148442.1	100
	3	286	*Lecythophora* sp.	AY219880	96
	1	286	*Chrysosporium lobatum*	AJ131688.1	97
	1	287	*Discostroma tricellulare*	AF377285.1	95
	1	292	Leaf litter Ascomycete strain ITS-295 isolate	AF502810.1	96
	1	294	*Penicillium brevicompactum*	AY373898.1	94
	1	294	*Penicillium brevicompactum*	AF521657.1	97
	1	294	*Nectria vilior*	U57673	90
	1	297	*Vertexicola caudatus*	AF177151	79
	1	298	*Microdochium* sp.	AF455402.1	100
	1	301	*Cladosporium oxysporum*	AY391832	78
	1	303	*Myrothecium verrucaria*	AJ301999	91
	2	306	*Williopsis californica*	Z93883	100
	1	308	*Botryosphaeria dothidea*	AY259092	79
	1	309	*Metarhizium anisopliae* var. *anisopliae*	AY635451	100
	1	363	*Verticillium coccosporum*	AF108469	77
	1	380	*Verticillium rexianum*	AF108482	92
	2	385	*Verticillium coccosporum*	AF110531	96
	14	396	*Verticillium* sp.	AY172097	88
	1	397	*Verticillium coccosporum*	AF110531	92
Basidiomycota	1	315	*Bullera unica*	AF444441	85
	1	331	*Lepiota clypeolaria*	AY176361.1	85
	1	345	*Fomitopsis pinicola*	AY089731	67
	1	374	*Scleroderma bovista*	AB099901	80
	1	397	*Amanita vaginata*	AB015693.1	62
	1	403	*Lepiota pyrochroa*	AY176477	59
Zygomycota	3	382	*Mortierella alpina*	AY310443	97
	1	383	*Mortierella alpina*	AY310443	100
	1	384	*Mortierella alpina*	AY310443	99
	2	391	*Mortierella* sp.	AJ541798.1	95
Unknown	1	366	Salal-associated fungal clone	AY112929	58

primer pair may be suitable for use in length-heterogeneity analysis of fungal community DNA isolated from soil and other environmental samples. The ITS1F and ITS86R primer set may also prove useful in the analysis of fungal community DNA, but the uncertainty surrounding the fungal specificity of this primer pair requires resolution.

Acknowledgement

This work was funded by an Australian Research Council, Australian Postgraduate Award through the University of Western Sydney with in-kind support from the NSW Department of Primary Industries.

References

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Anderson IC & Cairney JWG (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environ Microbiol* **6**: 769–779.

Arkush KD, Mendoza L, Adkison MA & Hedrick RP (2003) Observations on the life stages of *Sphaerothecum destruens* n. g., n. sp., a mesomycetozoean fish pathogen formally referred to as the rosette agent. *J Eukaryot Microbiol* **50**: 430–438.

Bell M, Seymour N, Stirling GR, Stirling AM, Van Zwieten L, Vancov T, Sutton G & Moody P (2006) Impacts of management on soil biota in vertosols supporting the broadacre grains industry in northern Australia. *Aust J Soil Res* **44**: 433–451.

Berthelet M, Whyte LG & Greer CW (1996) Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. *FEMS Microbiol Lett* **138**: 17–22.

Borneman J & Hartin RJ (2000) PCR primers that amplify fungal rRNA genes from environmental samples. *Appl Environ Microb* **66**: 4356–4360.

Cole JR, Chai B, Marsh TL *et al*. (2003) The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**: 442–443.

Dunbar J, Takala S, Barns SM, Davis JA & Kuske CR (1999) Levels of bacterial community diversity in four arid soils compared by cultivation and 16s rRNA gene cloning. *Appl Environ Microb* **65**: 1662–1669.

Gardes M & Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhiza and rusts. *Mol Ecol* **2**: 113–118.

Hedrick DB, Peacock A, Stephen JR, Macnaughton SJ, Bruggemann J & White DC (2000) Measuring soil microbial community diversity using polar lipid fatty acid and denaturing gradient gel electrophoresis data. *J Microbiol Meth* **41**: 235–248.

Hill GT, Mitkowski NA, Aldrich-Wolfe L, Emele LR, Jurkonie DD, Ficke A, Maldonado-Ramirez S, Lynch ST & Nelson EB (2000) Methods for assessing the composition and diversity of soil microbial communities. *Appl Soil Ecol* **15**: 25–36.

Kowalchuk GA, Os GJ, Aartrijk J & Veen JA (2003) Microbial community responses to disease management soil treatments used in flower bulb cultivation. *Biol Fert Soils* **37**: 55–63.

Liu WT, Marsh TL, Cheng H & Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16s rRNA. *Appl Environ Microb* **63**: 4516–4522.

Manter DK & Vivanco JM (2007) Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by QPCR and length heterogeneity analysis. *J Microbiol Meth* **71**: 7–14.

Marsh TL (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr Opin Microbiol* **2**: 323–327.

Mendoza L, Taylor JW & Ajello L (2002) The class *Mesomycetozoea*: a heterogeneous group of microorganisms at the animal–fungal boundary. *Annu Rev Microbiol* **56**: 315–344.

Murakami T & Higashi S (1997) Social organization in two primitive attine ants, *Cyphomyrmex rimosus* and *Myrmicocrypta ednaella*, with reference to their fungus substrates and food sources. *J Ethol* **15**: 17–25.

Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr Opin Microbiol* **2**: 317–322.

Ranjard L, Poly F, Lata JC, Mougel C, Thioulouse J & Nazaret S (2001) Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Appl Environ Microb* **67**: 4479–4487.

Sambrook J, Fritsch EF, Maniatis T & Laboratory CSH (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York.

Sequerra J, Marmeisse R, Valla G, Normand P, Capellano A & Moiroud A (1997) Taxonomic position and intraspecific variability of the nodule forming *Penicillium nodositatum* inferred from rflp analysis of the ribosomal intergenic spacer and random amplified polymorphic DNA. *Mycol Res* **101**: 465–472.

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

Suzuki M, Rappe MS & Giovannoni SJ (1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. *Appl Environ Microb* **64**: 4522–4529.

Torsvik V, Goksoyr J & Daae FL (1990) High diversity in DNA of soil bacteria. *Appl Environ Microb* **56**: 782–787.

Turenne CY, Sanche SE, Hoban DJ, Karlowsky JA & Kabani AM (1999) Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *J Clin Microbiol* **37**: 1846–1851.

White T, Bruns T, Lee S & Taylor J (1990) *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics*. Academic Press, New York.