

Chapter 2

Analysis of Rhizosphere Fungal Communities Using rRNA and rDNA

Ari Jumpponen

2.1 Introduction

Soils and soil-borne microbial communities are probably the greatest source of organismal diversity on Earth. In this soil matrix, plant roots and the soil impacted by those roots – the rhizosphere – host a great diversity of fungi. Fungal community composition in soil and rhizosphere has been assessed by growing fungi from environmental samples in different pure culture media. These studies have provided some impressive and comprehensive volumes of fungi (Domsch et al. 1980; Rambelli et al. 1983). However, such culture-based studies may suffer from some serious limitations because many soil-borne organisms can be difficult to bring into pure culture, or because the diversity is shadowed by few fast-growing organisms that hamper detection of others. The application of molecular techniques has resulted in great improvements in our understanding of the rhizosphere fungal ecology and revolutionized the tools available for exploring environmental fungal communities (Horton and Bruns 2001). A majority of these fungal community studies use PCR-based tools that specifically target the fungal ribosomal RNA (rRNA)-encoding genes (rDNA) or the non-coding regions of the rRNA gene repeats. The rDNA is a convenient target as the fungal rDNA is arranged in tandem repeats in multiple copies that contain both coding regions for primary rRNAs and non-coding regions with various levels of sequence conservation.

The rRNA genes and their spacer regions are extremely useful for detecting and identifying the fungi in complex environmental samples. However, rDNA may persist in the environmental DNA pools for organisms that maintain no metabolic activity and are not necessarily participating in the ecosystem functions at the time of assessment. This results in a fundamental difficulty of interpreting rDNA assays: the environmental DNA pools can maintain a large component of organisms that

A. Jumpponen

Division of Biology, Kansas State University, Manhattan, KS 66506, USA
e-mail: ari@ksu.edu

were once active in the environment but which presently remain dormant and inactive community components or are present as residual naked DNA detectable via PCR-based approaches. Use of rRNA molecules extracted directly from the environment presents an exciting alternative which specifically targets those communities that are active at the time of sampling (Girvan et al. 2004). The underlying assumptions are that rRNA is less stable than rDNA in the environment and that the metabolically active organisms maintain higher numbers of rRNA, or transcribe and process the rRNA precursors at a higher rate (Anderson and Parkin 2007; Prosser 2002).

This chapter describes a relatively rapid, kit-based application of rRNA-based community analysis from *Andropogon gerardii* rhizosphere in a tallgrass prairie ecosystem. Although one should be careful in extrapolating rRNA-based community assessment into enumeration of active cells in the environment, rRNA assays do provide a different view of the community when compared to rDNA-based assays (Duineveld et al. 2001). In the effort described here, the extracted rRNAs were reverse-transcribed, PCR-amplified, cloned and sequenced to assay the rhizosphere community composition at one snapshot in this environment. To test congruence among the rRNA- and rDNA-inferred community compositions, rDNA was also extracted from the same samples and subjected to community analyses through PCR-amplicon sequencing. The primary hypotheses included: (1) rRNA serves as a convenient target for molecular fungal community analysis and (2) fungal communities inferred from rRNA data include taxa that are a subset of those observed in the fungal communities inferred from rDNA. A further inference from the latter hypothesis implies that the species richness in the rDNA-assayed communities is higher than that in the rRNA-assayed ones.

2.2 Materials

2.2.1 Equipment

Liquid nitrogen vapor cryoshipper
FastPrep instrument
Microcentrifuge (refrigerated and nonrefrigerated)
Rotating shaker
Micropipettors
–80°C Ultrafreezer
Thermocycler
Micropipettors
Power supply
Horizontal gel electrophoresis apparatus
UV illuminator
NanoDrop Spectrophotometer
42°C Water bath
37°C Incubator with a shaker

2.2.2 Materials

FastRNA pro soil-direct kit
FastDNA spin kit for soil
Nuclease-free water
Thermoscript RT-PCR two-step system
Primer for reverse transcription (NS8)
RNase inhibitor (RNaseOUT)
10× PCR buffer
25 mM MgCl₂
2 mM dNTPs
Forward (nu-SSU-0817-5') and reverse (nu-SSU-1536-3') fungus-specific primers
Taq polymerase
TOPO-TA cloning kit for sequencing with chemically competent *E. coli*
Luriani Broth medium with 60 µg ml⁻¹ ampicillin (LBA)
60% Glycerol
Liquid N₂

2.2.3 Procedure

2.2.3.1 Sampling of the Rhizosphere Tissues

The plant materials were collected at the Konza Prairie Biological Station (KPBS, 39°05' N, 96°35' W), a Long-Term Ecological Research (LTER) site that represents a native tallgrass prairie in the Flint Hills of eastern Kansas, USA. The vegetation is dominated by big blue stem (*Andropogon gerardii* Vitman), indian grass [*Sorghastrum nutans* (L.) Nash.], little bluestem [*Schizachyrium scoparium* (Michx.) Nash.], and switch grass (*Panicum virgatum* L). The soil parent material is chert-bearing limestone with the soil bulk density of 1.0 g cm⁻³. January mean temperature is -3°C (range -9°C to 3°C) and the July mean temperature is 27°C (range 20–33°C). Annual precipitation averages 835 mm, 75% of which falls in the growing season.

A total of three intact *A. gerardii* plants with their roots attached were excavated in early growing season (May) from an annually burned watershed (1D) at the KPBS. The focal watershed is a part of the regular LTER program that aims to study the impacts of fire frequency on ecosystem structure and dynamics. The watershed represents tallgrass prairies under a frequent fire cycle. The plant roots were shaken free of loose soil and ca. 10 cm of the root material still attached to the identifiable *A. gerardii* aboveground tissues were transferred to the Lysing Matrix E of the FastRNA Pro Soil-Direct kit (Qbiogene, Carlsbad, CA, USA). The tubes with the Lysing Matrix and the *A. gerardii* tissues were immediately flash-frozen in a liquid nitrogen cryoshipper (Mini-Moover Vapor Shipper, Chart Biomedical, Marietta,

GA, USA) to avoid degradation of the RNA. The samples were maintained frozen in the cryoshipper until the nucleic acid extraction.

2.2.3.2 Nucleic Acid Extraction

The *A. gerardii* roots and the soil adhering to the roots were homogenized in the FastPrep instrument (Q-BioGene, Irvine, CA, USA) at setting 6 for 40 s in 1 ml RNapro Soil Lysis Solution and total RNA extracted using FastRNA Pro Soil-Direct kit as outlined by the kit manufacturer. Note that use of nuclease-free plastic disposables, cleaning the work surfaces with nuclease eliminators and keeping the extracts on ice will minimize the total RNA loss during the extractions. To obtain the genomic or total environmental DNA from the same samples, the nucleic acids that were eluted in nuclease-free water after the phenol-chloroform clean-up and the ethanol precipitation included in the FastRNA Pro Soil-Direct protocol were divided into two 200 μ l aliquots. One of the two aliquots was cleaned up as instructed by the manufacturer of the FastRNA Pro Soil-Direct kit and eluted in 100 μ l nuclease-free water with 100 units of RNaseOUT (Invitrogen, Carlsbad, CA, USA) to inhibit RNA degradation. The second aliquot was stored on ice until DNA was isolated using components of the FastDNA Spin Kit for Soil (Q-BioGene, Carlsbad, CA, USA). The DNA aliquot was combined with 978 μ l of the Sodium Phosphate Buffer and 122 μ l of the MP Buffer, mixed with 250 μ l of the PPS reagent by frequent vortexing over a period of 10 min. After pelleting the precipitant at 14,000 g for 5 min, the supernatant was treated as instructed by the FastDNA Spin Kit manufacturer and eluted in 100 μ l of nuclease-free water. Both nucleic acid extracts were stored on ice or in -80°C until further processed.

2.2.3.3 Reverse Transcription of the rRNA

The extracted rRNAs were reverse-transcribed using ThermoScript RT-PCR two-step system (Invitrogen, Carlsbad, CA, USA). To acquire a near full-length cDNA of the small subunit (SSU) of the rRNA, NS8 primer (White et al. 1990) located near the 3'-end of the SSU was chosen. This primer choice for reverse transcription also permits use of a number of nested priming sites within the SSU. The rRNAs were denatured prior to the first strand cDNA synthesis. For denaturing, 2 μ l of each RNA template was combined with 1 μ l of nuclease-free 10 μM NS8 primer, 2 μ l of the 10 mM dNTP mix and 7 μ l of nuclease free H_2O and incubated at 65°C for 5 min in an Eppendorf Mastercycler (Hamburg, Germany). The denatured RNAs were transferred to ice and combined with 1 μ l of 0.1 M DTT, 1 μ l of RNaseOUT (40 U μl^{-1} ; Invitrogen), 1 μ l nuclease-free H_2O , and 1 μ l ThermoScript Reverse Transcriptase (Invitrogen). Reverse transcription was carried out in an Eppendorf

Mastercycler at 55°C for 60 min and the synthesized cDNAs returned to ice until PCR amplification. Note that the relatively temperature-stable reverse transcriptase used here allows higher reverse transcription temperatures and thus provides more stringent reaction conditions for target-specific primers during the reverse transcription.

2.2.3.4 PCR Amplification

The reverse-transcribed cDNAs and the environmental DNAs extracted from the *A. gerardii* rhizospheres were PCR-amplified with Platinum *Taq* polymerase (Invitrogen) using primers targeting an approximately 760bp region within the SSU of the fungal rRNA gene (Borneman and Hartin 2000). For each 50 μ l PCR reaction, 5 μ l 10 \times PCR buffer, 5 μ l 25 mM MgCl₂, 5 μ l 2 mM dNTPs, 1 μ l of 10 μ M forward and reverse primers, 0.4 μ l polymerase (5 U μ l⁻¹) and 2 μ l of the cDNA or environmental DNA plus 30.6 μ l of nuclease-free water were combined. PCR reactions were carried out with initial 3 min denaturation at 93°C followed by 30 cycles of 1 min at 93°C, 1 min at 56°C, 2 min at 72°C and a terminal elongation at 72°C for 8 min. Longer extension steps may be necessary for long amplicons in complex environmental samples to minimize or eliminate the generation of artifactual PCR products (Jumpponen 2007). Presence of the target-sized amplicons was confirmed by visualizing the products by horizontal gel electrophoresis using 1.5% agarose gels in 0.5 \times TBE. The PCR products were quantitated using an ND-1,000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) in a 1 μ l volume.

2.2.3.5 Control Reactions

To account for contaminating DNA in the samples, three controls were included. First, to account for DNA contamination from the extraction system, a blank extraction without a sample was carried through the extraction protocol. Second, to account for PCR reagent-borne contaminants, a PCR control without template cDNA or DNA was included in the PCR. Third, to account for DNA carry-over through the RNA extraction, a control where Thermoscript reverse transcriptase was replaced with Platinum *Taq* polymerase was included. All these controls remained free of contaminants and yielded no visible PCR amplicons, indicating absence of DNA carry-through in the RNA extraction, absence of contaminating DNA in the DNA/RNA extraction, and absence of contaminating DNA in the PCR reagents. Note that many rRNA extraction protocols may fail to exclude DNA, and DNA removal with RNase-free DNase may be necessary.

2.2.3.6 Cloning, Sequencing, and Analysis of the cDNA and Environmental DNA Amplicons

The mixed populations of PCR products were cloned using TOPO-TA cloning system (Invitrogen). A total of 40 ng of each PCR product from cDNA or environmental DNA template was ligated into 10 ng of linearized pCR4 vector (Invitrogen) and the circularized plasmids were transformed into competent TOP10 cells (Invitrogen) by a 30 s heat shock in a 42°C water bath following the protocol for TOPO-TA cloning. Bacteria were grown for an hour at 37°C under 200 rpm agitation in 250 µl of SOC medium. A total of 10 and 100 µl of the incubated bacteria were plated on LB agar with 60 µg ml⁻¹ ampicillin and grown at 37°C overnight to confirm putative positive transformants. A random sample of the transformants was screened for presence of an insert by PCR in 15 µl reaction volumes, using conditions and cycling parameters outlined above, and the products visualized on 1.5% TBE agarose gels with EtBr on a UV illuminator. The clone libraries were combined with an equal volume of 60% glycerol, flash-frozen in liquid N₂ and shipped for plasmid prep and sequencing at University of Washington High Throughput Genomics Unit (Seattle, WA, USA). A total of 96 clones from each of the six clone libraries were randomly sampled and sequenced. Vector contamination was removed using the automated vector trimming function in Sequencher (Version 4.6, GeneCodes, Ann Arbor, MI, USA). The similarities to existing rDNA sequences in the GenBank database were determined using Blast at the National Center for Biotechnology Information (Altschul et al. 1997).

2.3 Results

2.3.1 *Nucleic Acid Extraction and Reverse Transcription from the *Andropogon gerardii* Rhizosphere*

This chapter describes simple, kit-based protocols for use of rRNA in fungal community analysis. The RNA extraction and cDNA synthesis should be completed expediently following the environmental sampling. Otherwise, the preservation and storage of RNA from environmental samples require special caution. The approach taken in this work included immediate sample preservation in an N₂-vapor cryoshipper that should ensure sample maintenance with minimal RNA degradation. Furthermore, the addition of RNase inhibitors in the final elutes aims to minimize RNA template loss. Finally, use of extraction kits expedites the protocol and maximizes the RNA yields, as well as usually improves RNA quality for downstream applications.

The simple FastPrep-kit method successfully isolated reverse-transcribable and PCR-amplifiable nucleic acids from *A. gerardii* rhizosphere. DNA contamination is a serious concern in PCR-based applications – especially if cDNA is used as a

primary template. Should DNA carry-through occur, the extracted total RNAs must be further cleaned with RNase-free DNase. The three included controls behaved as expected: (1) The controls with the reverse-transcriptase substituted with a polymerase failed to produce visible PCR amplicons, indicating absence of contaminating DNA in the rRNA samples. (2) The extraction controls without the environmental samples remained clean of PCR amplicons regardless of whether or not the reverse-transcription step was included. (3) Finally, the regular PCR controls indicated that no false PCR amplicons were generated in absence of the template.

Universal primer NS8 was chosen for reverse transcription. NS8 provides a near full-length SSU cDNA and thus allows use of a number of priming sites for downstream PCR-applications. This work targeted the fungal community as a whole by use of fungus-specific primers that have been shown to amplify fungal targets across various fungal phyla (Borneman and Hartin 2000; Jumpponen 2003). Other sets of SSU-targeting primers would similarly be compatible with the use of a universal reverse transcription primer. For example, primer sets with strong bias toward Ascomycota and Basidiomycota (Smit et al. 1999) as well as toward Glomeromycota (Helgason et al. 1998) are readily available and should allow target-specific amplification using the same cDNAs.

2.3.2 Community Assessment Using Reverse-Transcribed cDNAs and Environmental rDNAs

The communities observed in the *A. gerardii* rhizosphere contained fungi from three phyla: Ascomycota, Basidiomycota and Glomeromycota (Table 2.1). The abundances of the phyla varied among the libraries. While one of the three cDNA libraries was comprised nearly exclusively of Glomeromycotan sequences (closest affinity to *Glomus mosseae*), two additional cDNA libraries had a minimal Glomeromycotan component and were dominated by various ascomycetes. Similar results on a phylum level were also observed in the rDNA libraries: only one of the three libraries was dominated by various Glomeromycotan sequences (*Glomus proliferum* and an unknown *Glomus* species). The low frequency of Glomeromycotan sequences as well as incongruences among the libraries are most likely attributable to temporal and spatial dynamics in the tallgrass prairie ecosystem. Ongoing, yet unpublished, results indicate that *A. gerardii* roots maintain low levels of Glomeromycotan colonization early during the growing season, whereas other, septate fungi are more common (Mandyam and Jumpponen, unpublished). These results corroborate observations of mycorrhizal colonization made by others at the KPBS (Bentivenga and Hetrick 1992).

The initial hypothesis in assessing the two different nucleic acid pools in the rhizosphere was that the cDNA community should be a subset of the rDNA community. Contrary to this hypothesis, the cDNA and rDNA clone libraries

Table 2.1 Fungi detected in the *Andropogon gerardii* roots and rhizosphere by cloning and sequencing the PCR-amplified reverse transcribed ribosomal RNAs (cDNAs) and the PCR-amplified environmental DNAs. The totals refer to the total number of sequenced clones in each of the six clone libraries, *SR* indicates the species richness observed in each of the six clone libraries. Sequences from cDNA and DNA libraries that bin together at 98% similarity using Sequencher are highlighted in italics and bold

Species (accession)	Order (Phylum)	Similarity (%)	cDNAs (SR)	DNAs (SR)
Sample 1				
<i>Ceratomyrium linnaeae</i> (AF022715)	Incertae Sedis (Asc)	99	19	
<i>Coniosporium</i> sp. (AJ972863)	Incertae Sedis (Asc)	97	8	
<i>Cordyceps konnoana</i> (AB031192)	Hypocreales (Asc)	99		26
<i>Exidiopsis calcea</i> (AY293130)	Auriculariales (Bas)	93	30	
<i>Exidiopsis calcea</i> (AY293130)	Auriculariales (Bas)	93	1	
<i>Fusarium oxysporum</i> (DQ916150)	Hypocreales (Asc)	98		10
<i>Fusarium oxysporum</i> (DQ916150)	Hypocreales (Asc)	98		2
<i>Glomus intraradices</i> (AY635831)	Glomales (Glo)	99		12
<i>Glomus intraradices</i> (AY635831)	Glomales (Glo)	99	1	
<i>Helicodendron paradoxum</i> (AY856945)	Helotiales (Asc)	99	4	
<i>Lithothelium septemseptatum</i> (AY584662)	Pyrenulales (Asc)	99	25	
<i>Lithothelium septemseptatum</i> (AY584662)	Pyrenulales (Asc)	99	1	
<i>Tubeufia pezizula</i> (AY856950)	Incertae Sedis (Asc)	98		45
<i>Zopfia rhizophila</i> (L76622)	Incertae Sedis (Asc)	95	7	
Total			96 (9)	95 (5)
Sample 2				
<i>Campanella</i> sp. (AY916675)	Agaricales (Bas)	98	8	15
<i>Campanella</i> sp. (AY916675)	Agaricales (Bas)	93		1
<i>Campanella</i> sp. (AY916675)	Agaricales (Bas)	94		1
<i>Campanella</i> sp. (AY916675)	Agaricales (Bas)	89		1

(continued)

Table 2.1 (continued)

Species (accession)	Order (Phylum)	Similarity (%)	cDNAs (SR)	DNAs (SR)
<i>Glomus mosseae</i> (AY635833)	Glomales (Glo)	88	86	
<i>Glomus proliferum</i> (AF213462)	Glomales (Glo)	99		33
<i>Glomus proliferum</i> (AF213462)	Glomales (Glo)	98		1
<i>Glomus proliferum</i> (AF213462)	Glomales (Glo)	98		1
<i>Glomus</i> sp. (AF480155)	Glomales (Glo)	99	1	41
Total			95 (3)	94 (8)
Sample 3				
<i>Asteromassaria olivaceohirta</i> (AY313953)	Pleosporales (Asc)	99	7	
<i>Coniosporium</i> sp. (AJ972863)	Incertae Sedis (Asc)	99	7	
<i>Endoxyla cirrhosa</i> (AY761089)	Boliniales (Asc)	99	46	
<i>Endoxyla cirrhosa</i> (AY761089)	Boliniales (Asc)	98	12	
<i>Endoxyla cirrhosa</i> (AY761089)	Boliniales (Asc)	99	1	
<i>Helicodendron paradoxum</i> (AY856945)	Helotiales (Asc)	98	5	27
<i>Helicodendron paradoxum</i> (AY856945)	Helotiales (Asc)	99	4	8
<i>Helicodendron paradoxum</i> (AY856945)	Helotiales (Asc)	92	1	
<i>Thanatephorus cucumeris</i> (DQ917659)	Ceratobasidiales (Bas)	88		5
Total			86 (8)	40 (3)

contained discrete taxa and the overlapping similar sequences were rare. To exemplify, while a single sequence type (*Glomus mosseae*) dominated one of the cDNA libraries, the rDNA library from that sample was dominated by two different Glomeromycotan sequences (one most similar to *Glomus proliferum*, and another most similar to an unidentified *Glomus* sp.). The reasons for this disparity remain unclear and open to speculation. It is possible that sampling of 96 cloned PCR products is inadequate, represents a gross undersampling for complex and diverse fungal communities, and thus fails to detect copies that are present in soil and rhizosphere in different numbers among the two nucleic acid extracts. The organisms that are present in high numbers in rRNA-derived communities should also be present in the rDNA-derived communities. Greater sampling intensity that would saturate the diversity in a soil environment should provide a more reliable estimate

of the community structure. It is also possible that the methods to isolate the different nucleic acids result in serious biases (von Wintzingerode et al. 1997): the template stability and the rate of template acquisition may differ among targets due to different cell resistance to homogenization or different nucleic acid folding structures that may preferably maintain some templates through the extraction protocols whereas others may be lost. Finally, in addition to these biases, reverse transcription and cDNA synthesis add steps and sources of possible incongruence to the data acquisition for community analysis. The cDNA synthesis in addition to the RNA isolation may be a source of bias (Kowalchuk et al. 2006) and, as such, a possible explanation for the observed differences among the cDNA- and rDNA-inferred fungal communities.

The analyses of reverse-transcribed and PCR-amplified rRNAs and PCR-amplified rDNAs indicated presence of relatively low diversity in the *A. gerardii* rhizosphere. On average, the rRNA-derived cDNA clone libraries had 6.7 taxa, while the rDNA-derived clone libraries had slightly lower species richness with 5.7 taxa. Both approaches provided comparable estimates of the diversity and evenness (Shannon's diversity index $H = 1.17$ and 1.15 and evenness $E_H = 0.59$ and 0.71 ; Simpson's diversity index $D = 2.86$ and 2.67 and evenness $E_D = 0.41$ and 0.51 for the cDNA and rDNA libraries, respectively). Note that none of these differences is significant at $\alpha = 0.05$ (Student's *t*-test). Again, contrary to the initial hypotheses, the rDNA libraries did not contain a higher species richness or diversity than the cDNA libraries.

2.4 Conclusions

The kit-based extraction method proved successful in producing reverse-transcribable and PCR-amplifiable rRNA templates. A major drawback of the procedure described here is that FastRNA extraction systems require relatively costly equipment (FastPrep instrument). However, other, alternative nucleic acid purification systems can be adopted for environmental samples (e.g., AllPrep RNA and DNA purification system from QiaGen, Valencia, CA, USA or PowerSoil RNA system from MoBio, Carlsbad, CA, USA). In adopting the former product, one must solve issues of tissue homogenization.

Although RNA isolation from environmental samples is relatively simple, the sample maintenance and inhibition of RNA degradation can be problematic. During sampling, the samples need to be maintained frozen or specific RNA stabilization reagents (e.g., RNeasy from QiaGen) may be necessary to minimize RNA degradation. Both approaches may have their caveats. While the immediate flash-freezing in a cryoshipper proved successful here, the transport of a bulky container with residual liquid N_2 can become problematic if travel by air or by foot is necessary during the sampling. Use of RNase inhibitors (such as RNeasy) only extends the longevity of the environmental RNA to a limited extent, and this time is temperature-dependent.

The rRNA provides a target that may allow an improved understanding of the microbial communities (Poulsen et al. 1993). However, one should be cautious in considering the obtained data as a true reflection of active organisms in the environment. Many soil-inhabiting organisms may be able to maintain high ribosome levels through dormancy in order to be able to regain activity when favorable environmental conditions emerge (Kowalchuk et al. 2006). Furthermore, while the use of rRNA may circumvent the problems of using rDNA (non-active cells, naked DNA in soil), the reverse transcription necessitates additional steps in the data acquisition that may introduce additional biases that complicate community analyses. It remains unclear what is the impact of use of the universal primer for the reverse transcription, and how that choice impacts observed species-richness estimators.

Finally, the reasons for the observed incongruences among the cDNA- and rDNA-derived communities remain unclear. However, one should keep in mind that both community compositions may be equally true. While the cDNA community may provide a snapshot of the organisms that are present in the current active and/or dormant pools, the rDNA community carries a historical trace, and thus provides a view of that community and of those present in the recent past. As pointed out by Duineveld et al. (2001), the two views of the same community may differ. One just needs to be cautious about the interpretation of those results.

Acknowledgements This material is based upon work supported by the National Science Foundation under Grants 0344838 and 0516456. Stacie Kageyama assisted in sampling *Andropogon gerardii* roots. Konza Prairie Biological Station and its personnel maintain the research site, and are funded partly through the National Science Foundation Long Term Ecological Research program.

References

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl Acids Res* 25:3389–3402
- Anderson IC, Parkin PI (2007) Detection of active soil fungi by RT-PCR amplification of precursor rRNA molecules. *J Microbiol Meth* 68:248–253
- Bentivenga SP, Hetrick BAD (1992) Seasonal and temperature effects on mycorrhizal activity and dependence of cool- and warm-season tallgrass prairie grasses. *Can J Bot* 70:1596–1602
- Borneman J, Hartin RJ (2000) PCR primers that amplify fungal rRNA genes from environmental samples. *Appl Environ Microbiol* 66:4356–4360
- Domsch KH, Gams W, Anderson T-H (1980) *Compendium of soil fungi*. Academic, New York
- Duineveld BM, Kowalchuk GA, Keijzer A, van Elsas JD, van Veen JA (2001) Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Appl Environ Microbiol* 67:172–178

- Girvan MS, Bullimore J, Ball AS, Pretty JN, Osborn AM (2004) Responses of active bacterial and fungal communities in soils under winter wheat to different fertilizer and pesticide regimens. *Appl Environ Microbiol* 70:2692–2701
- Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? *Nature* 394:431
- Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Mol Ecol* 10:1855–1871
- Jumpponen A (2003) Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. *New Phytol* 158:569–578
- Jumpponen A (2007) Soil fungal communities underneath willow canopies on a primary successional glacier forefront: rDNA sequence results can be affected by primer selection and chimeric data. *Microbial Ecol* 53:233–246
- Kowalchuk GA, Drigo B, Yergeau E, van Veen JA (2006) Assessing bacterial and fungal community structure in soil using ribosomal RNA and other structural gene markers. In: Nannipieri P, Smalla K (eds) *Nucleic acids and proteins in soil*, vol 7. Springer, Berlin, pp 159–188
- Poulsen LK, Ballard G, Stahl DA (1993) Use of ribosomal-RNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. *Appl Environ Microbiol* 59:1354–1360
- Prosser JI (2002) Molecular and functional diversity in soil microorganisms. *Plant Soil* 244:9–17
- Rambelli A, Persiani AM, Maggi O, Lunghini D, Onofri S, Riess S, Dowgiallo G, Puppi G (1983) Comparative studies on microfungi in tropical ecosystems. UNESCO, Rome
- 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 Microbiol* 65:2614–2621
- von Wintzingerode F, Göbel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* 21:213–229
- White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR protocols: a guide to methods and applications*. Academic, New York, pp 315–322