Fungal Diversity in Soils as Assessed by Direct Culture and Molecular Techniques

Jon K. Magnuson and Linda L. Lasure
Pacific Northwest National Laboratory
Richland, WA

It has been consistently reported that bacterial diversity in a given environment as determined by culture techniques represents a small fraction of the total bacterial diversity detected by molecular methods. This may be attributable to a variety of related factors, such as: the limitations of the culture techniques employed; complex interdependencies between microorganisms in soil that cannot be replicated in the laboratory; and the presence of bacteria in dormant or "viable but non-culturable" states. We hypothesize that a similar situation exists with regard to soil fungi. Traditional culture methods were used to cultivate fungi from different soil samples. In brief, 10% (w/v) soil was dispersed in phosphate buffered saline containing 0.02 % sodium dodecylsulfate, agitated for ten minutes and the resulting liquid phase was used to prepare dilutions for cultivation of fungi. Concurrently, the DNA from each soil was extracted using the Qbiogene Soil DNA Extraction Kit and a portion of the rRNA gene was amplified by the polymerase chain reaction (PCR) using fungal specific primers. The PCR products were ligated into the pCR4Blunt-TOPO vector (Invitrogen) and cloned into *E. coli*. Individual clones were examined by restriction fragment length polymorphism (RFLP) analysis to identify unique patterns. The number of fungal species that could be cultured from various soils ranged from three to fifteen. However, the total fungal diversity of the sample, as assessed by the number of unique RFLP patterns, varied significantly from the number obtained by direct culture.

INTRODUCTION

In the past decade, a large number of studies of bacterial diversity in soils and other environments have been performed using molecular techniques, such as ARDRA (amplified ribosomal DNA restriction analysis) (Vaneechoutte et al, 1992). These studies have revealed that bacterial diversity is approximately one hundred times greater than indicated by direct culture methods. This discrepancy has been ascribed to the presence of bacteria in viable but non-culturable states or the existence of complex nutritional interdependencies precluding the isolation of pure cultures (the latter point has recently been addressed by Kaeberlein et al., 2002). In contrast to the large amount of data regarding bacterial communities, we are aware of only one study examining total fungal diversity in soils (Viaud et al. 2000).

Fungi are an important component of the soil microbiota typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions (Ainsworth & Bisby, 1995). Many important plant pathogens (e.g. smuts and rusts) and plant growth promoting microorganisms (e.g., ecto- and endo-mycorrhizae) are fungi. The saprobic fungi represent the largest proportion of fungal species in soil and they perform a crucial role in the decomposition of plant structural polymers, such as, cellulose, hemicellulose, and lignin, thus contributing to the maintenance of the global carbon cycle. In addition, these catabolic activities enable fungi to grow on inexpensive substrates. This property, coupled with their ability to produce commercially interesting organic molecules and enzymes explains the significant (but quiet) interest in the biotechnological utilization of filamentous fungi.

Like bacteria, the number of cultured fungi (~100,000) is believed to be a small fraction of the total number of extant species. The advent of major taxonomy projects, such as, the fungal

specific endeavor known as Deep Hypha (http://ocid.NACSE.ORG/research/deephyphae/), and the Tree of Life (http://tolweb.org/tree/phylogeny.html) contribute to the need for an estimate of the total diversity of the fungal kingdom and the proportion of fungi that will be difficult to obtain in pure culture. These estimates are also important for assessing the potential to discover novel fungi of value to the biotechnology industry.

The goals of this study were to utilize direct culture and molecular methods to estimate fungal diversity and the percentage of culturable fungi in various soil and compost environments. Two long-term goals of the continuing study are to determine if the information provided by the molecular analysis can identify novel species and facilitate the isolation of additional fastidious fungi from the original samples.

MATERIALS & METHODS

Soil and Compost Samples

All soil and compost samples were collected in late October to early November, 2001 and stored in sterile bottles at 4^{o}C until used for culturing fungi (no greater than 72 hours). Portions of the samples were stored at –80^{o}C pending extraction of community genomic DNA. Two compost samples were collected from different sites: SL1, a thermal compost; and FC, a cool residential compost. Two soil samples were collected from an agricultural field and a riverine environment in the same geographic area (south-central Washington State).

Fungal Culture

For extraction of fungi, a 10% (w/v) soil suspension was prepared in 10 mM potassium phosphate, pH 7, 150 mM NaCl, 0.02% SDS (PBS-SDS). Agitated at 150 rpm for 15 minutes in 250 ml baffled flasks, allowed soil particles to settle for 15 minutes and decanted liquid. The decantate was diluted to 1:100 and 1:1000 in PBS. The diluted soil extracts (100 µl) were spread on multiple rose bengal agar plates containing tetracycline (30 µg/ml) and incubated at 22°C, 30°C and 42°C (SL1 only) for 3-6 days.

Extraction of Genomic DNA from Soils and Fungal Isolates

A bead beating procedure, FastDNA® Spin Kit for Soil (Qbiogene), was used to extract DNA from 500 mg samples of soil and compost according to the manufacturers protocol. For extraction of genomic DNA from fungal isolates, two different procedures were used. In both procedures, spores and/or mycelia were collected from liquid cultures in YM broth or by scraping the surface of agar cultures flooded with PBS-0.1% Tween 80 buffer. The mycelia and spores were harvested by centrifugation for 10 minutes at 21,000 x g. The first procedure involved grinding the fungal cells in liquid nitrogen, suspending the powder in the lysis buffer supplied with the DNeasy® Plant Mini Kit (Qiagen) and proceeding with the manufacturers protocol. In the second procedure, fungal cell mass was suspended in the phosphate buffer supplied with the Qbiogene soil kit and added to the tube containing glass beads. Lysis and DNA extraction was carried out following the manufacturers protocol.

ARDRA (Amplified rDNA Restriction Analysis)

The fungal specific 18S and 28S primers FS38F (NS1; 5'-GTAGTCATATGCTTGTCTC-3') and FL2590R (ITS4; 5'-TCCTCCGCTTATTGATATGC-3') (*S. cerevisiae* numbering) were

used to amplify most of the 18S rRNA gene and the entire ITS1/5.8S/ITS2 segment of the rRNA operon (Bruns et al., 1991). A proofreading DNA polymerase (ThermalAce™, Invitrogen) was used with dilute soil gDNA (1-10% (v/v) in TE buffer), as template. The PCR reactions were performed in a DNA Engine Tetrad thermal cycler (MJ Research) as follows: 3 minutes at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 52°C, 240 seconds at 74°C, with a final extension of 10 minutes at 74°C. The PCR amplicons were subjected to electrophoresis in 0.7% agarose gels (1 x TAE buffer), the 2.6 kb bands were excised and the DNA was purified using the QIAquick® Gel Extraction Kit (Qiagen). PCR amplicons were ligated to the pCR4Blunt-TOPO® vector (Invitrogen) and transformed into *E. coli*. After overnight incubation at 37°C on LB/kanamycin (50 µg/ml) agar plates, 96 colonies were picked, transferred to 1.5 ml LB/kan broth in a 96 x 2 ml square-well microplate and incubated 20 hours at 37°C, 325 rpm. 100 µl of the culture was transferred to a standard 96 well microplate containing 100 µl of LB broth plus 20% glycerol and stored at –80°C (Vergin et al., 2001). 1-2 µl of culture was transferred to a 50 µl PCR reaction containing Taq DNA polymerase, primers FS214F and FL2590R, and subjected to the following thermal cycling program: 10 minutes at 94°C to lyse E. coli and inactivate nucleases followed by 35 cycles of 94°C for 15 seconds, 50°C for 15 seconds, 72°C for 150 seconds, with a final extension time of 10 minutes at 72°C. 15 µl of the PCR reaction was digested with the tetrameric restriction enzyme HhaI and the DNA fragments were separated on a 3% agarose gel (NuSieve® 3:1 agarose, BioWhittaker) in 1 x TBE at 50 V for 4 hours. Analysis of ARDRA patterns was performed by an ocular sensor interfaced to a biomolecular computer with natural intelligence software. The dendrogram for the SL1 compost ARDRA patterns was generated with Dendron 2.4 software (Solltech).

DNA Sequencing

E. coli clones containing pTOPO plasmids with the fungal 18S/ITS insert were grown overnight and the plasmid DNA isolated by the alkaline-lysis mini-prep procedure. The plasmid was separated by gel electrophoresis, excised from the gel and extracted with the QIAquick® Gel Extraction Kit. The plasmids along with the primers FL2590R and FS1766F (ITS5, 5'-GGAAGTAAAAGTCGTAACAAGG-3') were submitted to the Iowa State DNA Sequencing Facility (Ames, IA) for cycle sequencing.

RESULTS & DISCUSSION

PCF: Agricultural Soil

The PCF sample consisted of the top 10 cm of a loamy-sand soil from a tilled and irrigated agricultural field located at 46° 16' N latitude, 119° 10'W longitude in Pasco, WA. Sandy soils are often thought to be low in diversity, especially fungal diversity. However, the largest number of unique cultures were obtained from this sample. The ARDRA patterns of 94 clones were examined, revealing 52 unique patterns only 13 of which occurred more than once.

YR: Riverine Soil

The source of the YR sample was the top 5 cm of a sandy-loam soil supporting a riverine meadow dominated by a single grass species. The site was located approximately one meter above river level near the confluence of the Yakima and Columbia Rivers at 46° 15' N latitude, 119° 15'W longitude. ARDRA indicated that 65 of 84 clones (77%) had unique patterns, with

only eight patterns represented by multiple clones. This indicated that the YR site had the highest relative fungal diversity of any of the sites examined.

FC: Residential Compost

A cool compost sample was collected from a residential site located in a wet temperate environment (Fall City, WA). It consisted of a wide variety of plant-derived organic matter at various stages of decomposition. The temperature of the compost at the time of sampling was 13ºC. Dilution plating led to the isolation of seven unique fungi. Four of the fungal isolates were identified as either Zygomycetes belonging to the genera *Rhizopus* and *Mucor*, or Ascomycetes from the genera *Aspergillus* (condidiophore in background of Fig.2) and *Penicillium*. Analysis of 91 clones by ARDRA indicated a high level of diversity as represented by 58 unique patterns. Twelve of these patterns corresponded to two or more clones.

SL1: High Temperature Cellulose Compost

The source of sample SL1 was a large industrial composting operation consisting of waste cellulose fibers from a paper mill inoculated with paunch (stomach contents of cattle from a nearby slaughterhouse). The compost is piled into 1.5 m high windrows that are turned intermittently. At the time of collection, the center of the windrow was 55ºC (ambient temperature was 2ºC) but operators of the site typically observe temperatures of 65ºC during the compost cycle. Dilution plating and incubation at 30ºC or 42ºC gave rise to a large number of dark green dusty colonies (about 95% of total) tentatively identified as the known thermophilic fungus, *Aspergillus fumigatus*. These fungi grew faster (larger colony diameter) at the higher temperature, consistent with their thermophilic designation.

ARDRA of 65 clones indicated only twelve unique patterns, the lowest diversity of any of the samples. This was to be expected given the relative rarity of thermophilic fungi in comparison to mesophilic fungi. One of the twelve patterns was associated with 67% of the clones. Even with the biases inherent in PCR based analyses (e.g., differential lysis) this percentage indicates that this pattern is associated with one of the most common fungi in this sample. Plasmid DNA was purified from the twelve clones that gave unique ARDRA patterns and the ITS region was sequenced (Table II). BLAST analysis of the sequence indicated close relation to *Scytalidium thermophilum*, a thermophilic cellulose-degrading fungus. The closest relative of seven of the twelve clones sequenced was *S. thermophilum*, even though they displayed unique ARDRA patterns. Three sequences closely related to basidiomycetes were also identified.

The absence of sequences with similarity to the genus *Aspergillus* was surprising, given the predominance of *Aspergillus fumigatus* in culture. Part of the explanation may lie in the difficulty we encountered in lysis of *Aspergillus* spores and subsequently obtaining DNA suitable for amplification in PCR. DNA was purified at low yield from spores of the *Aspergillus fumigatus* isolate using the bead beating technique (Qbiogene). The DNA was unsuitable for PCR amplification of the 18S/ITS region regardless of dilution. After precipitation with ammonium acetate-ethanol and dilution in TE buffer a solution of DNA was obtained that resulted in a PCR product. Similar problems were encountered with other *Aspergillus* isolates. The identities of the substances interfering with the PCR are unknown. However, it was observed that the ammonium acetate-ethanol precipitation eliminated the greenish tinge of the original DNA solution and ammonium acetate is the preferred salt for eliminating co-precipitation of polysaccharides, thus suggesting the possibility that pigments or polysaccharides inhibited the

PCR. The ARDRA pattern of the *Aspergillus fumigatus* isolate did not correspond to any of the twelve patterns observed in the clones obtained using soil DNA. Spores of this fungus were obviously plentiful in the soil based on the culturing results but they were as difficult to lyse in the soil as in pure form using the bead-beating method. Thus, the most common fungus detected by ARDRA was a species related to *Scytalidium thermophilum* but the most common fungus detected in culture was *Aspergillus fumigatus*, emphasizing that caution must be exercised when making quantitative conclusions based on analysis of soil community DNA.

Table I. Summary of molecular and culture based studies of soil and compost samples.

Sample	Clones Examined	Unique RFLP Patterns	# of Fungi Cultured	% Cultured*
SL1	65	12	4	33
PCF	94	52	11	21
FC	91	58	7	12
YR	84	65	6	9

*(Cultured Fungi) ÷ (Unique RFLP Patterns) · 100%.

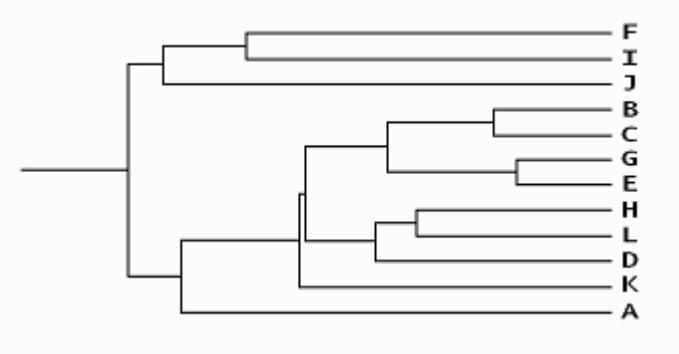

Figure 1. Dendrogram for thermal cellulose compost fungi (SL1).
The rooted tree was constructed on the basis of differences in band positions (molecular weights) in the ARDRA gels. This example indicates that one must use caution in constructing and interpreting "phylogenetic" trees based on DNA characteristics rather that actual sequence data.

Table II. Sequence based identification of species in the thermal compost, SL1.

SL1 Strain	Closest Relative	% Similarity	Phylum
A	*Amanita sp.*	90	Basidiomycota
B	*Scytalidium thermophilum*	98	Ascomycota
C	*Scytalidium thermophilum*	99	Ascomycota
D	*Scytalidium thermophilum*	99	Ascomycota
E	*Cercophora appalachianensis*	95	Ascomycota
F	*Coprinus bisporus*	97	Basidiomycota
G	*Scytalidium thermophilum*	99	Ascomycota
H	*Scytalidium thermophilum*	99	Ascomycota
I	*?*		
J	*Coprinus bisporus*	96	Basidiomycota
K	*Scytalidium thermophilum*	99	Ascomycota

L	*Scytalidium thermophilum*	99	Ascomycota

CONCLUSIONS

The majority of ARDRA patterns were represented by a single clone suggesting that the total diversity of each of the samples is higher than the analysis of 65-95 clones revealed. This would suggest that the percentage of culturable fungi is over-estimated. On the other hand, a more rigorous approach to culturing fungi than the use of a single media formulation might result in the isolation of additional fungi. Nevertheless, the data clearly indicate that a large percentage of the fungi in a given soil sample are fastidious or unculturable. The percentages of culturable fungi we found in this survey of four samples is consistent with the estimate of 17% proposed by Hawksworth (1991). If 80-90 % of soil fungi are unculturable, this would imply that 0.5-1 million fungal species exist. Clearly, a large unexploited diversity of fungi awaits discovery.

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