ELSEVIER

FEMS Microbiology Ecology 42 (2002) 327–337

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: comparison of 18S and ITS ribosomal regions

N.S. Lord, C.W. Kaplan, P. Shank, C.L. Kitts, S.L. Elrod *

Environmental Biotechnology Institute, Biological Sciences Department, California Polytechnic State University, San Luis Obispo, CA 93407, USA

Received 27 February 2002; received in revised form 30 May 2002; accepted 3 June 2002

First published online 16 September 2002

Abstract

Assessment of fungal diversity in environmental samples is currently a challenge. Several recently developed molecular methods offer new avenues for determining the presence and diversity of fungi in complex microbial communities. Terminal restriction fragment (TRF) pattern analysis was tested as a method for assessing the fungal molecular diversity of a terrestrial microbial community. Community DNA was isolated from sand samples taken from a pilot-scale petroleum-contaminated land treatment unit. PCR amplification was carried out using primers, one of which was fluorescently labeled, designed to hybridize to conserved sequences in the fungal ribosomal small subunit (18S) or the internal transcribed spacer ITS1–5.8S–ITS2 (ITS) ribosomal region. Amplicons were then digested separately with *Hpa*II or *Hae*III; fluorescently labeled TRFs were detected by capillary gel electrophoresis. ITS region TRF patterns were predicted and observed to generate a greater richness than 18S TRF patterns. Unique TRF patterns were also observed for each community examined. Finally, the ITS region showed a higher degree of specificity in matching observed TRF profiles to those generated from GenBank sequence data for species identification. These data suggest that ITS rDNA TRF pattern analysis has great potential as a rapid and specific method for fungal community analysis and species identification.
© 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Ribosomal DNA; 18S ribosomal gene; Internal transcribed spacer; Terminal restriction fragment length polymorphism; Fungal ribosomal DNA database; Fungal community

1. Introduction

Fungi play important roles in terrestrial and aquatic ecosystems. Accurately measuring their presence, abundance and diversity is a challenging task. It has been estimated that only 5% of fungal species have been accurately described due to culture limitations, unexplored habitats and misidentifications in culture collections [1]. Recently, a molecular method has been developed for the assessment of bacterial diversity in complex microbial communities, terminal restriction fragment (TRF) pattern or terminal restriction fragment length polymorphism (T-RFLP) analysis (for reviews, see [2,3]), that may prove useful for the analysis of fungal communities. This method begins with PCR amplification of small subunit ribosomal genes from a community DNA sample using conserved primers, one of which is fluorescently labeled. After amplification, independent restriction digestion with various enzymes is carried out followed by electrophoresis to resolve labeled TRFs present in the population of amplicons. In this way, different community members may be observed as different TRF lengths in a pattern. Patterns can be compared from different communities over time or under different environmental or treatment conditions (for examples, see [4–8]). In addition, TRF profiles or ribotypes (ribosomal genotypes, in this case characterized by combinations of TRF lengths across different enzymes for the same analyzed sample) may be used to identify specific community members [4,7,9].

Efforts to characterize the fungal members of a microbial community using other molecular techniques have been made by PCR amplification of either the small subunit rDNA or internal transcribed spacer (ITS) region

* Corresponding author. Tel.: +1 (805) 756-2875; Fax: +1 (805) 756-1419.
E-mail address: selrod@calpoly.edu (S.L. Elrod).

0168-6496/02/$22.00 © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0168-6496(02)00301-X

followed by sequencing of cloned amplicons, temperature or denaturing gradient gel electrophoresis (TGGE or DGGE, respectively), or analysis of PCR amplicon length or restriction length polymorphisms [10–19]. While effective, methods such as sequencing libraries of rDNA amplicons can be more laborious and costly than TRF analysis. In addition, the TRF method appears to be more direct and offers better resolution than temperature and gradient gel electrophoresis methods [2,3]. Only one published study has applied the TRF method to a eukaryotic microbial community; Marsh et al. [20] employed 18S PCR primers to analyze activated sludge samples taken from a water treatment plant. This TRF analysis revealed the presence of a variety of primarily protozoan eukaryotic microorganisms in this community; detection of fungi was not reported.

The primary goals of this work were to investigate (1) which ribosomal region, the 18S or ITS1–5.8S–ITS2 (ITS), would be best suited for application of the TRF method to fungal communities, (2) if the TRF method could be used to evaluate fungal diversity, and (3) whether TRF profiles could be used to identify a particular fungus. GenBank database analyses were performed on fungal rDNA sequences in order to predict potential TRF results. In addition, soil DNA and pure isolate fungal DNA were analyzed to test the predictions using both the ITS and 18S regions.

2. Materials and methods

2.1. Bioinformatics

Searches for fungal ribosomal DNA sequences in GenBank returned sequences that were used for predicting TRF lengths. Sequences were downloaded on April 4, 2001 using the search string: '(fungal OR fungi OR fungus) AND (ribosomal OR ribosome OR 18S OR ITS OR 28s OR 25s OR 5.8 OR 'internal transcribed spacer') NOT 'partial genome' NOT 'complete genome' NOT 'complete mitochondria genome' NOT 'cosmid' NOT 'chromosome'.' An Excel macro was designed to first locate specific forward and reverse PCR primer sequences within the downloaded sequences, then search for various four-base-cutter restriction enzyme recognition sequences. Primer matching was accomplished using an algorithm to compute a hybridization potential (HP) for a given primer across the entire sequence and choosing a position where the HP was greatest and over a certain threshold [21]. Restriction enzyme cut sites were tabulated and a spreadsheet of predicted TRFs was generated. When matching observed TRF peaks to predicted TRFs a range of −2 to +2 bases was allowed to account for variation in electrophoretic mobility [3,4]. The range was also adjusted for differences in mobility induced by the addition of the fluorescent dye on the ABI 310 Genetic Analyzer. The CEQ 2000XL DNA analysis system software has an internal size correction to compensate for the electrophoretic shift associated with the fluorescent label (Beckman-Coulter, Fullerton, CA, USA).

2.2. Soil sample collection and storage

Sand was collected weekly from each treatment cell at the former Guadalupe oil field land treatment unit (LTU) west of Santa Maria, CA, USA. Treatment test cells of the LTU differed based on nutrient amendments: cell one, the control, was not amended with additional carbon or organic nitrogen; cell two was amended with 0.2% (v/v) corn steep liquor; cell three was amended with 0.1% (w/v) dextrose. Ten samples were taken from each treatment cell sample site at each collection time, combined and stored at −80°C. The combined sample was used for DNA extraction. All samples were taken 9 inches below the surface. Samples collected on the 14th, 49th, and 98th days of LTU operation were analyzed in this study.

2.3. Microbial community DNA extraction

Community DNA was extracted from the soil samples using a modified Ultraclean MoBio Soil DNA kit (MoBio, Solana Beach, CA, USA) protocol. Five replicates of 1 g soil were added to MoBio lysis tubes with S1 solution. The tubes were processed on the Fast Prep bead beater (Bio 101, Vista CA) at 5.0 m s^{-1} for 45 s. Samples were spun down at 12 000 × *g* for 2 min and 450 μl of supernatant was transferred to a new tube with 250 μl of S2 solution. Samples were incubated at −20°C for 5 min, then centrifuged at 12 000 × *g* for 1 min. An aliquot (450 μl) of supernatant was then added to a new tube with 900 μl of S3 solution and vortexed. S3 solution and DNA mix were added to a spin filter and centrifuged at 10 000 × *g* for 1 min. The filtrate was discarded and the spin filter was washed with 300 μl of S4 solution then centrifuged at 10 000 × *g* for 2 min. The spin filter was placed in a new tube and DNA was eluted in 50 μl of S5 solution or sterile distilled water; replicates from each sample were combined for PCR.

2.4. 18S and ITS primers and community PCR

Primers that amplify a ~500-bp fragment of the 18S rDNA, Fun18S1 5′-CCATGCATGTCTAAGTWTAA-3′ and Fun18S2 5′-GCTGGCACCAGACTTGCCCTCC-3′, or a ~650-bp region of the ITS rDNA, EF3RCNL 5′-CAAACTTGGTCATTTAGAGGA-3′ (reverse complement of EF3 from [12]) and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ [22], were used for PCR. The forward primers, Fun18S1 and EF3RCNL, were 5′-labeled with FAM®, a fluorescent sequencing dye (Perkin-Elmer) for use when analyzing samples on the ABI 310 Genetic Analyzer (Applied Biosystems); EF3RCNL was 5′-labeled

with D4-PA, a different fluorescent dye for analyzing samples using the CEQ 2000XL DNA analysis system (Beckman-Coulter, Fullerton, CA, USA). Reactions were performed in 50 μl with 1×*Taq* buffer B (Fisher Scientific International), 0.6 μM dNTP, 8 μg ml^{-1} bovine serum albumin, 2 μM $MgCl_2$, 2 U of *Taq* DNA polymerase (Fisher Scientific International), 0.2 μM of each primer and 10 ng template DNA. Reaction temperatures and cycling were: 94°C for 2 min, 35 cycles of 94°C for 1 min, 46.5°C (18S primers) or 53°C (ITS primers) for 1 min, 72°C for 2 min, followed by 72°C for 10 min. PCR products were detected by agarose gel electrophoresis.

2.5. Primer removal and PCR product concentration

For each sample, five replicate PCR reactions were cleaned up and combined using a MoBio PCR Clean Up kit according to the manufacturer's instructions with the following modifications. Five volumes of SpinBind solution were added to combined reactions. The solution was then concentrated on the spin filter supplied with the kit. Spin Clean Buffer was then added to the spin filter and centrifuged for 30 s at 10 000×*g*; filtrate was discarded. Excess fluid was removed by centrifugation for 1 min at 10 000×*g*. The filter was then transferred to a clean 2-ml tube and DNA was eluted with 50 μl of sterile distilled water.

2.6. PCR product digestion

Digestion reactions consisted of 0.4 μg of PCR product, 8 U of enzyme in the manufacturer's suggested reaction buffer. The reactions were incubated for 2 h at 37°C followed by enzyme denaturation at 65°C for 20 min. A labeled digest standard was included with samples to ensure that digests were complete; HEX-labeled DNA was used for samples run on the ABI 310 Genetic Analyzer and D4-PA-labeled DNA was used for samples run on the CEQ 2000XL DNA analysis system (see Section 2.7). Samples were ethanol-precipitated by incubating at −20°C in 50 μl of 100% ethanol for 30 min and then centrifuged at 14 000×*g* for 10 min. Ethanol was removed and samples were centrifuged with 50 μl of 70% ethanol at 14 000×*g* for 10 min. The remaining ethanol was removed and samples were dried for 20 min in a vacuum centrifuge.

2.7. Capillary gel electrophoresis

TRFs from LTU community DNA and isolate GLF10 were obtained using the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) as follows. Digested DNA samples were resuspended in 10 μl of formamide (Bio-Rad, Benecia, CA, USA) and 0.5 μl of Rox500® size standard (Perkin-Elmer) and 0.5 μl of 550–700 Rox size standard (BioVentures, Murfreesboro, TN, USA). The samples were denatured at 95°C for 5 min and cooled immediately on ice. Capillary gel electrophoresis was performed with an ABI 310 Genetic Analyzer using Performance Optimized Polymer 6 and a 61-cm capillary. The samples were injected for 7 s at 15 kV, separated for 2 h in a 15-kV field at 60°C. TRFs between 35 and 700 nucleotides were sized by GeneScan software (Perkin-Elmer). TRFs from all other isolates were obtained using the CEQ 2000XL DNA analysis system (Beckman-Coulter, Fullerton, CA, USA) as follows. Digested isolate DNA was resuspended in 20 μl of SLS solution (Beckman-Coulter, Fullerton, CA, USA) containing 0.25 μl of DNA size standard-600 (Beckman-Coulter, Fullerton, CA, USA). Samples were injected for 5 s at 5 kV, and separated in separation gel-LPAI (Beckman-Coulter, Fullerton, CA, USA) for 65 min at 5 kV with a capillary temperature of 60°C. TRFs were analyzed by the Beckman-Coulter DNA analysis system software version 4.2.0.

2.8. Pattern normalization, analysis and comparison

TRF samples were normalized as in Kaplan [4]. TRF patterns from all samples were analyzed by counting the number of TRF peaks as an index of richness.

2.9. Fungal cultures

GLF (Guadalupe LTU fungi) were isolated from LTU sand samples by direct spreading or overlaying onto MYPD plus antibiotics (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1% dextrose, 2% agar, 100 μg ml^{-1} streptomycin, 60 μg ml^{-1} penicillin). Cultures were spore-purified three times by passage to fresh MYPD plates and identified based on ITS sequence information (using BLAST), as well as colony and spore morphology.

2.10. Fungal genomic DNA extraction

Fungal isolate genomic DNA was extracted from fungi plated from the site as previously described by Wahleithner [23] or as follows: pure cultures were grown on MYPD plates for 120 h then 6 ml of 0.002% Tween 20 was used to collect spores. The spores were pelleted by microcentrifuging at 10 000×*g* for 1 min. The spore pellets were resuspended in 150 μl of sterile water. DNA was extracted from these spores using the Ultraclean MoBio Soil DNA kit as described in Section 2.3, except that the spore suspension replaced the 1 g of soil.

2.11. DNA sequencing

Samples were sequenced from PCR reactions with unlabeled ITS primers (EF3RCNL and ITS4). Sequencing reactions were carried out using Big Dye Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instruction. Samples were

then ethanol-precipitated and analyzed on an ABI Prism 377 (Applied Biosystems, Foster City, CA, USA) automated sequencer as recommended by the manufacturer. Sequences were assembled using the SeqMan software program (DNASTAR, Madison, WI, USA).

3. Results

3.1. Primers

In order to determine if TRF pattern analysis could be applied to fungal members of a terrestrial environmental community, appropriate rDNA PCR primers were identified. Since the small subunit of rDNA has been successfully employed for bacterial TRF pattern analysis and there is a large database of fungal small subunit rDNA sequences available, primers complementary to fungal small subunit (18S) rDNA sequences were designed. Fun18S1 and Fun18S2 (Fig. 1) were designed by identifying conserved regions in fungal 18S rDNA sequences that flanked an approximately 500 ± 20-bp region of divergence in the 5′ region of the gene. BLAST® searches demonstrated that the Fun18S1 primer is specific for eukaryotic sequences, with fungi representing 25% of the 500 returned sequences; 37% of the fungal taxa were from ascomycetes, 26% basidiomycetes, 1.6% zygomycetes, and 0% chytridomycetes. The remaining 75% of the 500 BLAST results were sequences from 10 other eukaryotic taxa. For the Fun18S2 primer, 17.4% of 493 BLAST returns matched sequences from fungi; of the fungal sequences returned, 74.4% were from ascomycetes, 23.3% basidiomycetes, 11% chytridomycetes, and 0.4% zygomycetes. The remaining 82.6% were matches to non-fungal eukaryotic species that were dominated by metazoan sequences. Neither BLAST search returned prokaryotic sequences. Because the ITS region is used for analyzing species-level fungal phylogenetic relationships [22], primers corresponding to the ITS region (spanning ITS1, 5.8S, and ITS2) were also identified (Fig. 1). The upstream primer, EF3RCNL, is the reverse complement of the fungal-specific 18S primer EF3 [12]. Primer EF3 is complementary to a region at the 3′ end of the fungal 18S gene and has been demonstrated by Smit and co-workers [12] to specifically amplify fungal rDNA sequences across the major phyla. ITS4, which is complementary to the 5′ end of the eukaryotic 28S gene [22], was used as the downstream ITS region primer. Amplification with EF3RCNL and ITS4 primers was predicted to yield fragments averaging 650 bp in size (Fig. 1).

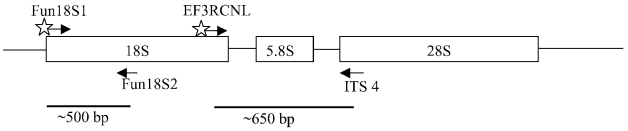

Fig. 1. Primer locations. The locations of primers used to amplify the 18S region, Fun18S1 and Fun18S2, and the ITS region, EF3RCNL and ITS4, are shown on this illustration of the ribosomal DNA. Stars indicate fluorescently labeled primers. Not to scale.

3.2. Database predictions

An analysis of known ITS and 18S sequences was performed in order to determine which rDNA region would yield the greatest fungal diversity (i.e. richness) of TRF lengths. A total of 26 163 fungal ribosomal DNA sequences were downloaded from GenBank and analyzed for predicted TRF lengths from the DNA sequences. Approximately 7.5% of these sequences (1958) contained both the Fun18S1 and Fun18S2 primer sites and 1.2% (328) contained both the EF3RCNL and ITS4 primer sequences.

Use of the ITS region for TRF analysis was predicted to result in greater richness and specificity compared to the 18S region. The average number of different TRF lengths per genus predicted from the 18S region was 4.0 ± 2.9 for *Hae*III, 4.4 ± 3.1 for *Hpa*II, and 4.3 ± 3.5 for *Dpn*II (Table 1). The average number of different TRF lengths per genus predicted from the ITS region averaged 7.5 ± 4.9, 6.2 ± 3.7, and 1.5 ± 0.5 for the same enzymes, respectively (Table 1). To verify that these trends were not limited to these enzymes, three additional enzymes were evaluated for predicted TRF richness. Average predicted TRF numbers for the enzymes *Rsa*I, *Hha*I, and *Bsh*1263I supported the trend for the 18S with 3.5 ± 2.4, 4.4 ± 2.8, and 4.2 ± 3.0, as well as for the ITS, with 8.8 ± 4.0, 7.1 ± 3.8, and 8.1 ± 4.9 for each enzyme, respectively.

In addition to providing a richer assessment of fungal diversity, the ITS region may allow for identification of community members to the species, and possibly the strain level. The number of different predicted TRF lengths from the ITS equaled or slightly exceeded the number of species for 60% (6/10) of the genera analyzed (Table 1, in bold). In contrast, only 10% (3/31) of the genera analyzed from the 18S region had the same or greater numbers of different TRF lengths predicted (Table 1, in bold). Many genera in the 18S analysis showed predicted TRF numbers that were much lower than the number of species in the sample.

3.3. Fungal TRF analysis of soil samples

In order to test these predictions on environmental samples, community DNA was isolated from soil samples collected at different time points from the three treatment cells of the LTU. DNA was then amplified separately using the 18S or ITS primers. Resulting amplicons were digested with *Hpa*II or *Hae*III and fluorescently labeled TRFs were detected using capillary gel electrophoresis. The ITS region produced a greater number of TRFs for both *Hpa*II and *Hae*III than the 18S using the same enzymes (Fig. 2). The increased numbers of observed ITS TRFs are consistent with the predictions shown in Table 1. Actual TRF peak lengths obtained from cell three, day

Table 1
Number of database predicted TRF patterns

rDNA region	Genus[a]	Number of sequences/genus	Number of species/genus	Number of TRFs predicted[b]		
				*Dpn*II	*Hpa*II	*Hae*III
18S	*Hypocrea*	8	5	2	2	2
	Leptsphaeria	8	4	1	2	1
	Mucor	8	7	1	4	3
	Rhizopus	8	4	2	3	3
	Saccharomyces	8	4	1	1	1
	Tilletiopsis	8	8	7	4	4
	Trichaptum	8	4	1	2	1
	Kluyveromyces	9	4	3	2	2
	Paraphaeosphaeria	9	9	2	2	3
	Acaulospora	**10**	**3**	**4**	**3**	**4**
	Arixmzyma	**10**	**1**	**1**	**1**	**1**
	Metschnikowia	11	9	5	6	4
	Ophioceras	11	8	3	4	1
	Fellomyces	12	11	4	2	1
	Ophiostoma	12	9	3	3	2
	Rhodotorula	13	8	3	5	5
	Graphium	14	7	5	4	4
	Myrothecium	14	6	3	3	3
	Bensingtonia	15	11	8	10	7
	Alternaria	16	12	1	3	3
	Pichia	17	12	8	8	9
	Taphrina	17	15	8	4	5
	Bullera	20	20	3	6	4
	Rhizoctonia	**22**	**2**	**1**	**2**	**2**
	Trichosporon	22	22	3	5	8
	Cordyceps	24	20	5	5	3
	Sporobolomyces	24	24	13	11	11
	Glomus	26	14	8	7	8
	Smittium	31	23	15	14	11
	Aspergillus	39	21	2	2	2
	Cryptococcus	59	34	6	7	6
	Average[c]			4.3 ± 3.5	4.4 ± 3.1	4.0 ± 2.9
ITS						
	Athelia	8	7	1	6	4
	Cladosporium	**9**	**6**	**2**	**4**	**6**
	Pseudozyma	**9**	**4**	**2**	**7**	**5**
	Arthroderma	10	3	1	1	1
	Monacrosporium	**10**	**8**	**2**	**9**	**7**
	Tricholoma	**13**	**11**	**2**	**10**	**12**
	Myrothecium	**14**	**6**	**1**	**6**	**9**
	Cordyceps	18	16	1	13	13
	Trichophyton	24	12	2	2	2
	Stachybotrys	**30**	**14**	**1**	**4**	**16**
	Average[c]			1.5 ± 0.5	6.2 ± 3.7	7.5 ± 4.9

[a]Genera included only if they were represented by > 5 sequences from the results of predicted TRF analysis. Genera in bold show TRF numbers greater than or equal to the number of species in the genus sample.
[b]Number of different TRF lengths predicted for each genus.
[c]Average number of different TRF lengths ± standard deviation.

14 for both the ITS and 18S regions using the *Hae*III enzyme are shown in Fig. 3. The 18S pattern contains fewer peaks, many of which have a high fluorescence intensity, whereas the ITS pattern contains a greater number of peaks generally showing lower fluorescence intensity. The larger 18S peaks most likely contain multiple fungal community members that are resolved into smaller, distinct peaks in the ITS pattern. One of the most concentrated areas of fluorescence intensity in the 18S pattern is in the 200–230-bp range. When predicted TRF data for the 18S were examined, 77% (1510/1927) of all species in the database showed predicted TRF lengths ranging from 201 to 234 bp in size.

A limited number of LTU samples were processed for TRF analysis using both the 18S and ITS regions using the *Dpn*II enzyme; these results were also consistent with those predicted in Table 1 (data not shown). That is, the number of *Dpn*II TRFs from the ITS region was much lower than those observed from digestion with *Hpa*II and *Hae*III, while the number of *Dpn*II TRFs from the

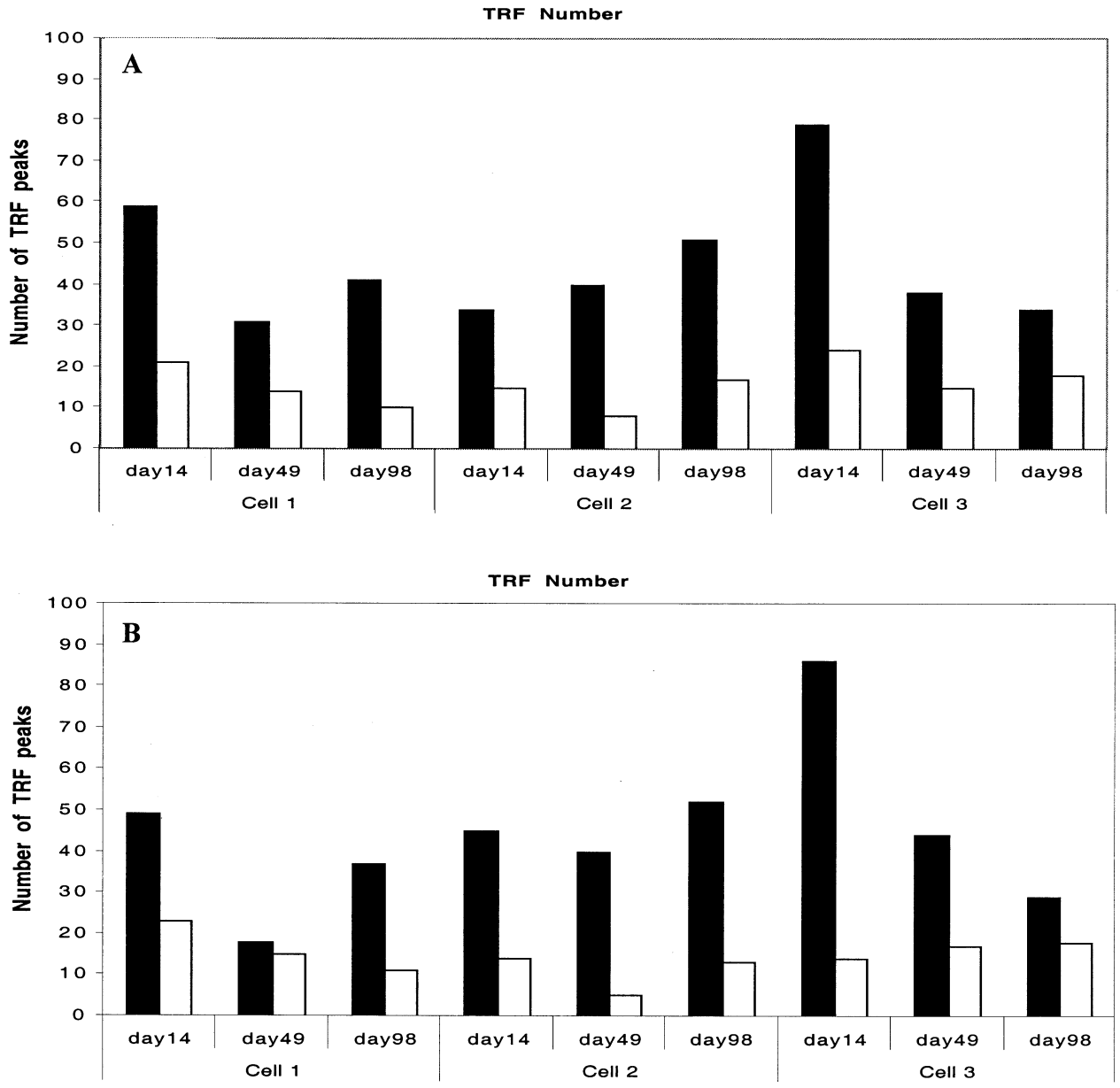

Fig. 2. Comparison of 18S and ITS TRF analysis of soil community DNA with *Hpa*II and *Hae*III enzymes. Numbers of different 18S (white bars) and ITS (black bars) TRF lengths generated with the enzyme *Hpa*II (A) or *Hae*III (B) for LTU cells 1, 2 and 3 for three different time points, days 14, 49 and 98, are shown. The ITS region shows a larger number of peaks across all cells for each day and enzyme used.

18S region was consistent with those observed from digestion with *Hpa*II and *Hae*III.

3.4. Identification of fungi using TRF patterns

One advantage of using the TRF method to analyze complex microbial communities is that individual community members may be identified based on the presence of individual peaks in the TRF pattern [4,7,9]. In order to investigate whether the ITS or 18S regions would better facilitate this kind of analysis, observed TRF profiles (both 18S and ITS) from four pure fungal isolates were compared against those predicted from DNA sequence database information. In this analysis, a TRF profile consisted of TRF lengths from all of the enzymes combined, e.g. the observed ITS TRF profile for GLF7 was 139 bp – *Hpa*II and 473 bp – *Hae*III (Table 2). The predicted TRFs (from the previously discussed computer database analysis) were sorted sequentially by enzyme TRF length to identify a subset of fungal sequences (i.e. species) with predicted profiles matching those observed. For each individual isolate tested, ITS TRF profiles were more accurate predictors of identity than 18S TRF profiles. Isolates GLF7, GLF19 and GLF41 were each identified as members of the genus *Trichoderma* based on BLAST searching with ITS DNA sequence data; the top scoring BLAST

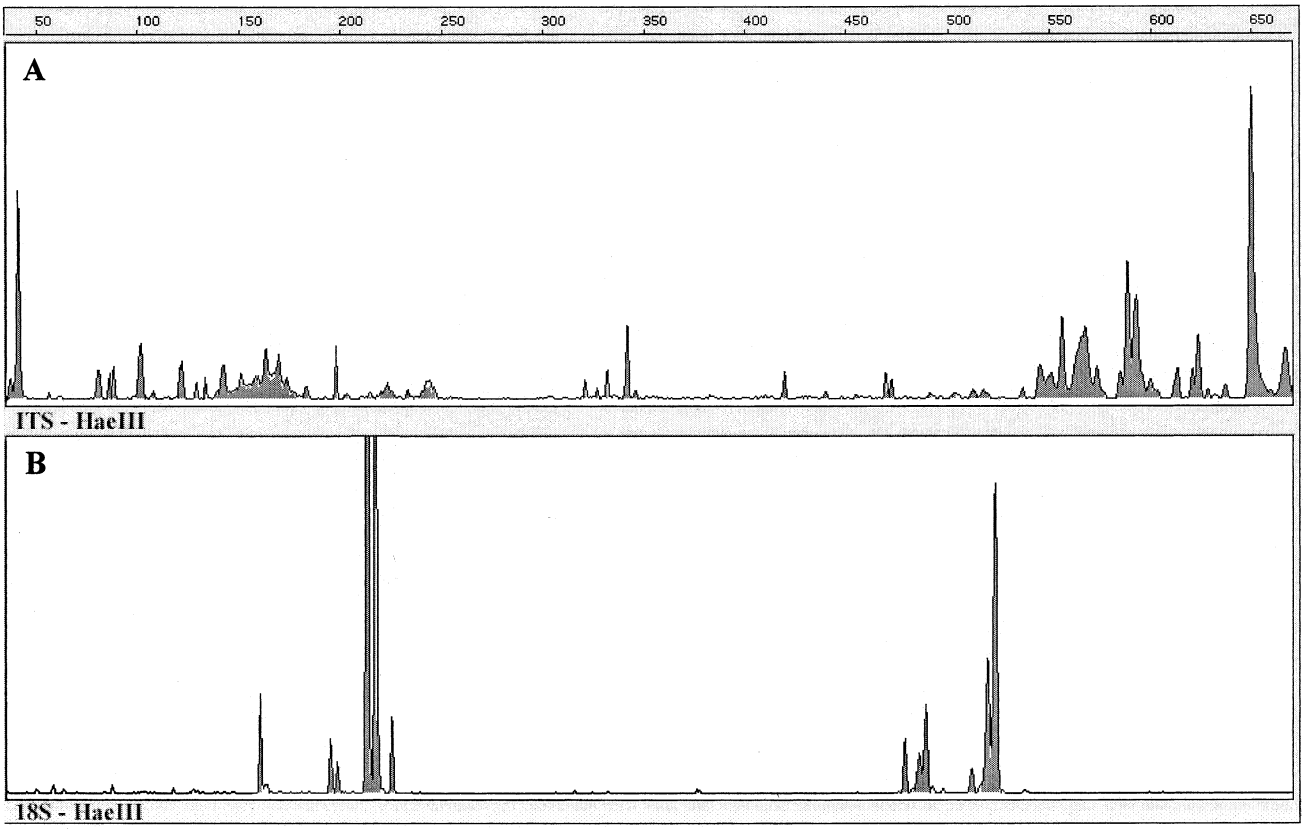

Fig. 3. TRF data from an environmental sample. TRF peak lengths obtained using *Hae*III for the ITS region (A) and the 18S region (B) for cell 3, day 14. Peak lengths are shown in base pairs (scale across top) and heights are given in relative fluorescence. Length and fluorescence scales for both electropherograms are the same.

results for GLF7 were sequences from *Trichoderma asperellum*, *T. atroviride*, *T. viride*, *T. hamatum*, *T. koningii*, *Hypocrea koningii*, *H. vinosa*, and two unnamed *Trichoderma* species, and the top scoring BLAST results for both GLF19 and 41 were sequences from *Trichoderma harzianum*, *T. inhamatum* and *T. aureoviride*. The observed 18S TRF profile for GLF7 matched sequences from various *Trichoderma* and *Hypocrea* species as well as 349 other genera. The observed ITS TRF profiles for these isolates specifically matched profiles predicted from *Hypocrea lutea*, *H. koningii*, *T. koningii* and two unidentified species of *Trichoderma*. (Table 2). The two unidentified species of *Trichoderma* were the same two that were in the highest scores from the BLAST results discussed above. Predicted ITS TRFs from *T. asperellum*, *T. atroviride*, *T. hamatum*, *T. harzianum*, *T. inhamatum*, *T. aureoviride* and *H. vinosa* were not in the TRF database and *Trichoderma* is an anamorph of *Hypocrea*, so the matches shown in Table 2 are consistent with the BLAST results. Isolate GLF10 was identified as a member of the genus *Penicillium* based on BLAST searching with ITS DNA sequence data; the top scoring BLAST results were sequences from *Penicillium commune*, *P. allii*, *P. camemberti*, *P. venatum*, *P. melanoconidium*, *P. viridicatum*, *P. neoechinulatum*, *P. expansum* and two unnamed *Penicillium* species. The observed 18S TRF profile for GLF10 matched the above stated *Penicillium* species as well as sequences from 337 other genera. The observed ITS TRF profile for GLF10 matched profiles within the expected drift range of four sequences from at least three species: *P. expansum*, *P. commune*, *P. allii* and an unnamed *Penicillium* species (Table 2). The unnamed species identified in the TRF profile search was from the same unnamed sequence returned in the BLAST results. It is possible that the use of a third enzyme may aid in narrowing the matches for more accurate identification using ITS TRF profiles. For example, the enzyme *Mse*I was found in the predicted TRF database to distinguish *P. allii* from other *Penicillium* species.

An additional three GLF isolates were analyzed using only the ITS region and observed TRF profiles were used to mine the predicted TRF database for matches. GLF12 and GLF26 were identified as *Geotrichum* and GLF13 was identified as *Fusarium* based on BLAST searching with ITS DNA sequence data (Table 2). The highest scoring BLAST results for GLF12 and 26 were *Geotrichum candidum*, *G. klebahnii* and *Dipodascus australiensis* sequences and the highest scoring BLAST results for GLF13 were *Fusarium oxysporum* sequences. Observed TRF profiles for GLF12 and GLF26 matched only *G. candidum* (Table 2). This match was 1 bp outside of the expected drift range, however no other fungal sequences in the predicted TRF database had profiles near the observed TRF profiles. The

Table 2
TRF profile matches

Isolate and genus identification[a]	Observed TRF peaks (bp)		Matching TRF peaks from database (bp)[b]		Matching species from database[c]
	*Hpa*II	*Hae*III	*Hpa*II	*Hae*III	
GLF7 *Trichoderma*	139	473	138	472	*Hypocrea lutea*
GLF19 *Trichoderma*	139	471	137	475	*Hypocrea koningii*
GLF41 *Trichoderma*	139	472	137	475	*Trichoderma koningii*
			137	475	*Trichoderma* sp.
GLF10 *Penicillium*	151	76	153	81	*Penicillium expansum*
			154	81	*Penicillium communae*
			154	81	*Penicillium allii*
			154	81	*Penicillium* sp.
GLF12 *Geotrichum*	416	416	413	413	*Geotrichum candidum*
GLF26 *Geotrichum*	416	416	413	413	*Geotrichum candidum*
GLF13 *Fusarium*	138	154	137	152	*Fusarium oxysporum*
			138	152	*Cordyceps coccidiicola*

[a]ITS regions were sequenced and resulting sequences were BLAST-searched using the nucleotide database at NCBI (www.ncbi.nlm.nih.gov). Only the lowest *E*-value and highest bit score matches were used to determine fungal genera.
[b]Matching profiles were found by comparing observed TRF profiles consisting of fragment lengths from *Hpa*II and *Hae*III TRF results to the predicted TRF database. The GLF10 TRF patterns were obtained using the ABI 310 machine, so were corrected for mobility drift (see Section 2) in order to find predicted TRF pattern matches in the database.
[c]All species with matching TRF profiles (column three) from the predicted TRF database are shown. The *Trichoderma* sp. match was to two different GenBank records (gi numbers 6706326 and 6706328) and the *Penicillium* sp. match was from a GenBank record (gi 6723934); none of these records named a particular species.

GLF13 observed TRF profile matched two sequences from two different genera, *F. oxysporum* and *Cordyceps coccidiicola*. These two genera are in the same order, Hypocreales (for reference, see [24]), and may be distinguished using an additional enzyme; the enzymes *Rsa*I, *Hha*I, and *Bsh*1236I are predicted to produce distinct TRF profiles for these two genera. In each case, the observed TRF profile was successfully used to specifically mine the predicted TRF database to produce a limited number of matches that were consistent with, yet more specific than, the BLAST database search results.

4. Discussion

Primers to both the 18S and ITS regions were designed or identified and successfully used to generate TRF patterns from DNA isolated from soil community samples as well as individual fungal isolates. The ITS region provided a more accurate and extensive assessment of community richness and species identity than did the 18S region. The utility of the ITS region was specifically demonstrated by the greater number (i.e. richness) of predicted and observed ITS TRF peaks and the decreased redundancy when matching observed TRF profiles to predicted TRF profiles.

For all enzymes examined, except *Dpn*II, sequence database predictions suggested that the ITS region would produce a greater number of different TRF lengths per genus than the 18S region (Table 1). The reason that *Dpn*II gave such a low number of ITS TRF fragments compared to 18S could be due to a highly conserved cut site 64 bp downstream from the forward primer. This type of conserved cut site makes the examination of predicted TRF lengths from a variety of enzymes helpful in identifying the most useful enzymes for TRF analysis [25]. The ITS region was also predicted to provide a more species-specific indication of community richness; the majority of genera examined showed that the number of different ITS TRF lengths predicted equaled or exceeded the number of species represented by the sequences (Table 1). Overall, these data suggest that TRF analysis using the ITS region can produce a more species-specific diversity index than the 18S region. Furthermore, the ITS region is widely used for fungal phylogenetics and studying fungal diversity using other molecular methods [17,26,27].

The prediction that ITS TRFs would be greater in number than 18S TRFs was confirmed by experimental observations for *Hpa*II and *Hae*III; that is, higher numbers of TRF fragments were observed when the ITS primers were used (Fig. 2). These data suggest that the ITS TRF method has the potential to be a useful tool for assessing differences in fungal community structure. Analysis of DNA from other soil communities will be useful in determining the utility of this method for providing further insight into fungal community dynamics.

Primer region and sequence is a key element of TRF analysis; primers that are too broad (e.g. amplify eukaryotic sequences) could over estimate diversity, while primers that are too narrow (e.g. amplify only ascomycete sequences) may underestimate diversity. Although this study did not exhaustively examine the various regions of the 18S gene for TRF analysis, this region is still expected to yield a more narrow view of fungal diversity than the ITS region. Primers used to amplify the 18S region in this study were determined to be only 17–25% fungal-specific using

BLAST data; the remaining matches were eukaryotes other than fungi. Thus, these primers may have amplified other eukaryotic members of the community. Despite this fact, the numbers of observed TRF peaks in each community pattern were lower for these primers than for the ITS primers (Fig. 2). This may be due to a lack of sequence diversity in this region of the 18S gene. Fungal primers have been developed for other regions of the 18S gene [28]; these may be explored by interested researchers for development of the TRF method. The upstream primer used to amplify the ITS region, EF3RCNL primer, is the reverse complement of a primer experimentally established by Smit et al. [12] to be fungal-specific. It was paired with a universal eukaryotic ITS primer, ITS4, assuming that the fungal-specific primer would limit PCR amplification to primarily fungal sequences.

The third goal of this work was to investigate whether the identification of specific fungi to the genus and/or species level could be done based on observed TRF profiles from either ribosomal region. This type of analysis proved more successful when the ITS region was used. The observed 18S TRF profiles were very redundant, matching hundreds of genera (and species), while observed ITS TRF profiles were found to match a very small number of sequences that included the highest scoring BLAST results for the isolate (Table 2). The matching of observed TRF profiles to predicted profiles may prove to be a more rapid method for identification of fungal community members, especially when compared to other methods such as generating sequence data. For example, a BLAST search usually returns dozens of sequence matches that must be sorted through and analyzed using phylogenetics, while a TRF profile search, as demonstrated in this study, returns a limited number of sequence matches. The accuracy of the TRF profile matching will also be greatly enhanced by using a third or possibly a fourth enzyme to generate the TRF profiles. While only a limited number of isolates were tested in this study, analysis of additional isolates and environmental samples will help determine (1) whether identifications can be made using TRF profiles obtained from environmental samples and (2) the taxonomic level of fungal diversity that can be obtained from ITS TRF analysis.

The TRF profile-matching data presented in this study suggest that the rapid identification of individual community members from an environmental sample may be possible. In fact, visual inspection of the observed ITS LTU TRF data allowed detection of *Penicillium*, *Fusarium*, *Geotrichum*, and *Trichoderma* TRF profiles in several of the treatment cells (unpublished observations). Interestingly, *Penicillium*, *Trichoderma*, and *Fusarium* have been identified as hydrocarbon degrading genera (for review, see [29]). Matching observed TRF profiles from community samples to individuals in a database will be the most challenging aspect of this work.

In the current analysis of fungal isolates, all seven showed single TRF lengths for each enzyme tested (Table 2). These data suggest that in each fungal strain the copies of ITS rDNA present are similar in sequence, at least at the level detected by TRF pattern analysis. However, the presence of polymorphisms in the multiple copies of the ITS region in other fungal species, heterokaryotic fungi, or spores is not well characterized. For example, in one species, *Scutellospora castanea*, rDNA polymorphisms have been detected [30], however a subsequent analysis concluded that most of the sequences were of ascomycete origin [31] suggesting the polymorphisms may not be as numerous as initially thought. Clearly, additional analysis is needed to address this question. TRF analysis of pure cultures or spores may be able to determine the degree to which multiple copies of ITS rDNA are similar or different in various fungi.

Ultimately, identification of fungal community members from TRF profiles will depend largely on the fidelity and completeness of the available fungal rDNA sequence information. A relatively low return of sequences with both the EF3RCNL and ITS4 primer sites (1.2%) was observed from processing fungal rDNA sequences available in GenBank. Two hypotheses regarding this low return were considered: (1) a high degree of sequence ambiguity (i.e. number of unidentified nucleotides/total sequence length) leading to inefficient primer matching, (2) low number of sequence entries long enough to contain the required sequence information (e.g. both primer sites). The median percent nucleotide ambiguity of the sequences used for this analysis was 0.0 ± 2.0, with 95% of sequences having $< 2\%$ ambiguity. These data suggest that the sequence information is relatively robust, with a limited number of exceptions. The median sequence length was found to be 580 ± 861, with only 33% of sequences being longer than 700 bp. Our projected ITS amplicon size ranges from 400 to 700 bp, so shortness of available rDNA sequences may explain the low return of ITS sequences containing both primer sites. It is likely that many ITS sequences deposited in GenBank are generated using primers in locations very near or overlapping with EF3RCNL and ITS4. The paucity of complete fungal ribosomal sequence information is in sharp contrast to the availability of prokaryotic rDNA sequence information. In a recent database search conducted in this laboratory, $\sim 52\,000$ prokaryotic small subunit rDNA sequences were downloaded from GenBank and $\sim 12\,000$ were identified as containing a conserved bacterial primer set used for TRF analysis. It is also worthwhile to note that sequences available in the public databases represent only a small portion of the fungi that are known to exist in nature. Thus, augmentation of current databases with additional and more complete ITS rDNA sequences will help pave the way for identification of fungi from TRF pattern data.

In conclusion, TRF analysis shows great promise for the study of fungi in complex microbial communities, in particular when primers that amplify the ITS region are

used. Other regions of the ribosomal gene complex, including the large subunit, may also be useful in TRF analysis of fungi. In addition, the TRF method may be broadly applied when using primers designed to include the major taxa, or more specifically applied by using primers that target the rDNA region of a particular group. TRF analysis may even be applied to other genes of interest within a community; for example, degradative enzyme genes may be targeted. Further work is needed to determine the degree to which TRF analysis of fungal communities will be useful for characterizing changes in community structure and identifying particular community members. In addition, TRF analysis of individual fungi may help shed light on the level of polymorphism within the ribosomal gene complex of fungal genera, species and individual strains.

Acknowledgements

The authors would like to thank Unocal and CSUPERB (California State University Program for Education and Research in Biotechnology) for financial support of this project, Bob Pease of BFJ Services for sampling at the dunes and L. Martinez, E. Mui, K. Palmer, C. Reimers and T. Stine for technical assistance.

References

[1] Hawksworth, D.L. and Rossman, A.Y. (1997) Where are all the undescribed fungi? Phytopathology 87, 888–891.
[2] Marsh, T.L. (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.
[3] Kitts, C.L. (2001) Terminal restriction fragment patterns: A tool for comparing microbial communities and assessing community dynamics. Curr. Issues Intest. Microbiol. 2, 14–25.
[4] Kaplan, C.W., Astaire, J.C., Sanders, M.E., Reddy, B.S. and Kitts, C.L. (2001) 16S Ribosomal DNA terminal restriction fragment pattern analysis of bacteria communities in feces of rats fed *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. 67, 1935–1939.
[5] Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63, 4516–4522.
[6] Liu, W.T., Marsh, T.L. and Forney, L.J. (1998) Determination of the microbial diversity of anaerobic–aerobic activated sludge by a novel molecular biological technique. Water Sci. Technol. 37, 417–422.
[7] Dunbar, J., Ticknor, L.O. and Kuske, C.R. (2000) Assessment of microbial diversity in four Southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl. Environ. Microbiol. 66, 2943–2950.
[8] Clement, B.G., Kehl, L.E., DeBord, K.L. and Kitts, C.L. (1998) Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. J. Microbiol. Methods 31, 135–142.
[9] Avaniss-Aghajani, E., Jones, K., Holtzman, A., Aronson, T., Glover, N., Boian, M., Froman, S. and Brunk, C.F. (1996) Molecular technique for rapid identification of Mycobacteria. J. Clin. Microbiol. 34, 98–102.
[10] van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A. and Gianinazzi-Pearson, V. (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. 7, 879–887.
[11] Borneman, J., Skroch, P.W., O'Sullivan, K.M., Palus, J.A., Rumjanek, N.G., Jansen, J.L., Nienhuis, J. and Triplett, E.W. (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. 62, 1935–1943.
[12] Smit, E., Leeflang, P., Glandorf, B., van Elsas, J.D. and 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.
[13] Kowalchuk, G.A., Gerards, S. and Woldendorp, J.W. (1997) Detection and characterization of fungal infections of *Ammophila arenaria* (Marram Grass) roots by denaturing gel electrophoresis of specifically amplified 18S rDNA. Appl. Environ. Microbiol. 63, 3858–3865.
[14] Pennanen, T., Paavolainen, L. and Hantula, J. (2001) Rapid PCR-based method for the direct analysis of fungal communities in complex environmental samples. Soil Biol. Biochem. 33, 697–699.
[15] van Elsas, J.D., Duarte, G.F., Keijzer-Wolters, A. and Smit, E. (2000) Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. J. Microbiol. Methods 43, 133–151.
[16] Taylor, J.W. and Bruns, T.D. (1999) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol. Ecol. 8, 1837–1850.
[17] Turenne, C.Y., Sanche, S.E., Hoban, D.J., Karlwasky, J.A. and Kabani, A.M. (1999) Rapid identification of Fungi using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. J. Clin. Microbiol. 37, 1846–1851.
[18] Peter, M., Ayer, F. and Egli, S. (2001) Nitrogen addition in a Norway spruce stand altered macromycete sporocarp production and below-ground ectomycorrhizal species composition. New Phytol. 149, 311–325.
[19] Redecker, D., Thierfelder, H., Walker, C. and Werner, D. (1997) Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the Order Glomales. Appl. Environ. Microbiol. 63, 1756–1761.
[20] Marsh, T.L., Liu, W., Forney, L.J. and Cheng, H. (1998) Beginning a molecular analysis of the eukaryal community in activated sludge. Water Sci. Technol. 37, 455–460.
[21] Brunk, C.F., Avaniss-Aghajani, E. and Brunk, C.A. (1996) A Computer analysis of primer and probe hybridization potential with bacterial small-subunit rRNA sequences. Appl. Environ. Microbiol. 62, 872–879.
[22] White, T.J., Burns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White., T.J., Eds.), pp. 315–322. Academic Press, San Diego, CA.
[23] Wahleithner, J.A., Xu, F., Brown, K.M., Brown, S.H., Golightly, E.J., Halkier, T., Kauppinen, S. and Pederson, A. (1996) The identification and characterization of four laccases from the plant pathogenic fungus Rhizoctonia. Curr. Genet. 29, 395–403.
[24] Alexopoloulos, C.J., Mims, C.W. and Blackwell, M. (1996) Phylum ascomycota. In: Introductory Mycology, 4th edn., pp. 323–375. John Wiley and Sons, New York.
[25] Marsh, T.L., Saxman, P., Cole, J. and Tiedje, J. (2000) Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Appl. Environ. Microbiol. 66, 3616–3620.
[26] Jackson, C.J., Barton, R.C. and Evans, E.G.V. (1999) Species iden-

tification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J. Clin. Microbiol. 37, 931–936.

[27] Makimura, K., Tamura, Y., Mockizuki, T., Hasegawa, A., Tajiri, Y., Hanazawa, R., Uchida, K., Saito, H. and Yamaguchi, H. (1999) Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J. Clin. Microbiol. 37, 920–924.

[28] Borneman, J. and Hartin, R.J. (2000) PCR primers that amplify rRNA genes from environmental samples. Appl. Environ. Microbiol. 66, 4356–4360.

[29] Atlas, R.M. and Cerniglia, C.E. (1995) Bioremediation of petroleum pollutants: Diversity and environmental aspects of hydrocarbon biodegradation. BioScience 45, 332–338.

[30] Hosny, M., Hijiri, M., Passerieux, E. and Dulieu, H. (1999) rDNA units are highly polymorphic in *Scutellospora castanea* (Glomales, Zygomycetes). Gene 226, 61–71.

[31] Redecker, D., Hijri, M., Dulieu, H. and Sanders, I.R. (1999) Phlogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of ascomycete origin. Fungal Genet. Biol. 28, 238–244.