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Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis

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Abstract

Molecular-based approaches to assess microbial biomass and diversity from soil and other ecosystems are rapidly becoming the standard methodology for analysis. While these techniques are advantageous, because they do not rely on the need to culture organisms, each technique may have its own biases and/or limitations when used to assess fungal diversity from mixed-template samples. In this study, we analyzed PCR specificity and efficiency of the ITS primers (ITS1F and ITS4) in a series of single- and mixed-template samples using a combined quantitative PCR–length heterogeneity analysis (LH-qPCR) approach. As expected, these primers successfully amplified all higher fungal species tested (10 ascomycetes, 6 basidiomycetes, and 4 zygomycetes) and no members of the oomycetes. Based on our results, and a search of the GenBank database, amplicons of the ITS1F and ITS4 primer set exhibit considerable variability (420 to 825 bp), but due to similarities in amplicon sizes of some fungal species, actual species diversity in environmental samples may be underestimated approximately two-fold. The addition of an initial qPCR step allowed for the accurate quantitation of total fungal DNA in mixed-template samples over five orders of magnitude (10^{-1} to 10^{3} pg μl^{-1}). PCR biases between individuals in mixed-templates rendered it impossible to determine the absolute quantity of any individual within a population from its individual peak height. However, relative changes in individuals within a mixed-template sample could be determined due to a constant proportionality between peak heights and starting template concentration. Variability associated with the individual steps of the LH-qPCR analysis was also determined from environmental samples.

Keywords: Molecular ecology; Fungi; Diversity; Soil; DNA

1. Introduction

A wide variety of molecular-based approaches for microbial communities have recently been developed to overcome some of the limitations associated with traditional culture-based techniques. For example, it has been estimated that only ca. 1% of soil bacteria can be isolated and cultured using traditional methods (Torsvik et al., 1998). Similarly, many fungi cannot be cultured using standard culturing techniques (Thorn, 1997; van Elsas et al., 2000) leading to the development of many new culture-independent techniques. Many of these techniques rely on an initial polymerase chain reaction (PCR) step to amplify genes of interest, such as single-strand conformation polymorphism (SSCP) (Borneman and Hartin, 2000; Hurek et al., 1997; Smit et al., 1999), restriction fragment length polymorphism (RFLP) (Dunbar et al., 2000; Liu et al., 1997; Lukow et al., 2000), terminal-RFLP (Marsh, 1999; Kitts, 2001), thermal and denaturing gradient gel electrophoresis (TGGE and DGGE) (Harris, 1994), amplified rRNA intergenic spacer analysis (ARISA) (Fischer and Triplett, 1999; Ranjard et al., 2000, 2001; Cardinale et al., 2004) and length heterogeneity PCR (LH-PCR) analysis (Suzuki et al., 2003; Ritchie et al., 2000). While these methods differ in the PCR amplicon characteristics analyzed (*i.e.*, DNA secondary structure, thermal or chemical denaturation, or size), each requires an initial PCR amplification to generate sufficient sample for analysis and depending on the

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specificity of the primers employed, various taxonomic or functional groups within the community can be selected.

Length heterogeneity analysis (LH-PCR and ARISA) rely on the detection of different organisms based on the natural variation in amplicon size. When combined with capillary electrophoresis, each peak represents a unique PCR amplicon, and the total number of peaks is an index of the diversity in that sample. The degree to which this genetic, or length heterogeneity, diversity truly represents the total microbial diversity, and at what taxonomic level (*e.g.*, strain, species, genera, etc.), is dependent upon the primer set utilized. Furthermore, the abundance (peak height or area) of each fragment in the profile has been suggested to be directly proportional to the abundance of that template in the sample (Clement and Kitts, 2000; Dunbar et al., 2000; Polz and Cavanagh, 1998). However, PCR biases within the target community (Reysenbach et al., 1992; Brunk and Eis, 1998; Polz and Cavanagh, 1998) may cause this proportionality factor to vary from one organism to another due to a variety of factors, *e.g.*, differences in gene copy number (Rooney and Ward, 2005).

While the above methods are typically used to determine the abundance and diversity of individuals within a community, it may also be desirable to determine the abundance of the entire target population within a community. Previous studies have shown that qPCR can be used to determine the abundance of specific phylogenetic groups of microorganisms in soil (Kabir et al., 2003; Kolb et al., 2003; Smits et al., 2004; Fierer et al., 2005) or other environmental samples (Winton et al., 2003; Gachon et al., 2004). qPCR was developed to overcome the basic weakness of classical PCR technology, which cannot directly quantify the amount of amplicon and measure low amounts of DNA (Freeman et al., 1999; Bustin, 2000, 2002). Unlike classical end-point PCR, qPCR measures the fluorescence of a reporter molecule (*e.g.,* SYBR green) as the amount of PCR product increases during each amplification cycle of the PCR reaction, allowing for the comparison of samples during the exponential phase, prior to saturation of the reaction, resulting in greater accuracy of starting template quantities. Due to the mixed-template nature of environmental samples a non-specific reporter molecule like SYBR green is the cheapest and best candidate, unlike Taqman or other sequence-specific probes. However, the suitability of using SYBR green as the reporter molecule should be empirically determined for each assay since the precise mechanism of SYBR green binding to DNA, and the resulting fluorescence intensity, is not fully understood and includes both intercalary and minor groove binding, and may be dependent upon amplicon size and GC content (Giglio et al., 2003).

A variety of primer sets have been designed to be specific for the 18S and ITS regions of fungi (for review see Anderson and Cairney, 2004). The ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) primers appear to have specificity for the ascomycetous, basidiomycetous, and zygomycetous fungi (Gardes and Bruns, 1993; Dickie et al., 2002; Klamer et al., 2002; Anderson et al., 2003a,b; Izzo et al., 2005). This primer set has been used to specifically amplify fungi from mixed-template samples (Chen and Cairney, 2002; Dickie et al., 2002; Lord et al., 2002; Anderson et al., 2003a,b) but PCR biases, or differences in PCR efficiency, within the target population have not been examined.

In this study, we analyzed the potential utility of a combined qPCR–length heterogeneity (LH-qPCR) approach using the conserved rRNA primers, ITS1F and ITS4, to characterize fungal communities. The specific objectives were to test the ITS1F and ITS4 primer set for: (i) differences in PCR efficiency and amplicon lengths of 28 different fungal species obtained from pure cultures, (ii) conduct an *in-silico* analysis of the theoretical ITS1F and ITS4 amplicon lengths deposited in the GenBank database, (iii) the ability of qPCR to determine total fungal abundance in mixed-template samples, (iv) potential PCR biases that may result in preferential amplification of some members of a mixed-template sample, and (v) technique reproducibility from various environmental samples.

2. Materials and methods

2.1. DNA extraction and template preparation

DNA was extracted from mycelium (1 g) of five-day-old pure fungal cultures grown in 2% potato dextrose broth using the UltraClean microbial kit (Mo Bio, Carlsbad CA). The total amount of genomic DNA extracted was quantified using a spectrophotometer outfitted with a 5 μl cell (Biospec Mini, Shimadzu, Columbia, MD). *Phytophthora* spp. genomic DNA was kindly provided by Dr. Everett Hansen (Oregon State University, Corvallis) and isolates of *Pythium* spp. and *Ophiosphaerella* spp. by Dr. Ned Tisserat (Colorado State University, Fort Collins). All other fungal cultures (see Table 1) were isolated by direct plating of field-collected soil (Colorado soil described below) on 2% malt agar plates.

2.2. Preparation of fungal DNA standards

Serial dilutions of fungal DNA (see Section 2.1) obtained from five different fungal isolates (*Fusarium equiseti*, *Alternaria solani*, *Verticillium* sp., *Rhizoctonia solani*, *Scletorina sclerotium*) were analyzed individually and used to generate a single standard curve for estimating DNA concentrations in unknown samples by qPCR or LH. For qPCR, DNA concentrations in unknown samples were calculated by interpolation using measured cycle threshold (Ct) values and a regression equation between Ct and DNA concentration. For LH, DNA concentrations in unknown samples were calculated by interpolation using a regression equation between amplicon peak height and starting DNA concentration.

2.3. Quantitative PCR and length heterogeneity analysis (LH-qPCR)

Fungal DNA was amplified using the highly conserved fungal rRNA gene primers (ITS1F and ITS4) previously described (White et al., 1990; Gardes and Bruns, 1993). Quantification of total fungal DNA in a sample was determined by SYBR green fluorescence (iCycler iQ, Biorad, Hercules,

Table 1
One-tube PCR efficiency and amplicon fragment size for various fungal cultures using the rRNA primers ITS1F and ITS4

Fungus	PCR efficiency (%) [a]		Fragment size (bp) [b]	
	Mean	SD	Mean	SD
Ascomycota				
Alternaria solani — 1 [c]	98.8	3.9	608.2	0.2
Alternaria solani — 2	112.8	5.9	608.5	0.2
Alternaria solani — 3	101.1	3.9	608.3	0.1
Botrytis cineri	103.6	4.3	575.1	0.1
Cercophora sp.	103.1	2.9	603.7	0.1
Cladosporium sp.	99.5	2.8	580.3	0.3
Fusarium equiseti — 1	97.4	3.1	574.1	0.1
Fusarium equiseti — 2	101.8	4.2	574.2	0.1
Fusarium sp.	103.1	6.6	576.2	0.2
Ophiosphaerella korrae	98.7	3.6	602.5	0.2
Ophiosphaerella narmari	99.0	5.4	602.3	0.1
Trichoderma virens	111.0	5.7	632.4	0.3
Verticillium sp. — 1	98.2	4.2	675.9	0.1
Verticillium sp. — 2	96.3	3.6	678.8	0.2
Verticillium sp. — 3	100.3	3.4	676.0	0.1
Basidiomycetes				
Rhizoctonia solani	105.1	1.6	776.1	0.1
Sclerotina rolfsii	101.1	7.3	723.7	0.1
Sclerotina sclerotium	112.0	10.1	576.4	0.2
Unidentified — 1 [d]	108.5	4.7	664.8	0.1
Unidentified — 2	97.3	2.9	707.0	0.3
Unidentified — 3	114.4	3.2	750.9	0.2
Zygomycete				
Mortierella alpina — 1	102.9	3.8	716.6	0.2
Mortierella alpina — 2	110.0	8.1	716.5	0.1
Mortierella gamsii — 1	96.1	1.5	682.9	0.2
Mortierella gamsii — 2	104.5	7.1	682.7	0.3
Mortierella sp.	104.6	2.0	654.7	0.1
Mucor hiemalis — 1	104.5	3.1	787.5	0.2
Mucor hiemalis — 2	96.7	2.1	787.7	0.1
Oomycetes				
Phytophthora cryptogea	–	–	–	–
Phytophthora erythroseptica	–	–	–	–
Phytophthora megasperma	–	–	–	–
Phytophthora ramorum	–	–	–	–
Phytophthora sojae	–	–	–	–
Pythium arrhenhomanes	–	–	–	–
Pythium intermedium	–	–	–	–
Pythium irregulare	–	–	–	–

[a] One-tube PCR efficiency was calculated according to the methods of Rutledge (2004). Reported values are the mean and standard deviation (SD) of three replicate samples.
[b] Fragment size (bp) was determined using the Genescan 2500 (TAMRA) size standard and local southern method (Genemapper Vers. 4).
[c] Numbers denote different isolates.
[d] Cultures were tentatively identified as basidiomycetes based on the presence of hyphal clamps.

CA) using an external standard curve. PCR reactions (20 µl) contained 5 µl of template DNA, 1× SYBR green jumpstart reaction mix (Sigma, St. Louis, MO), 3 mM $MgCl_2$, 1 nM fluorescein, 100 nM of ITS1F primer, and 500 nM ITS4 primer. The PCR thermal protocol consisted of an initial 5 min denaturation step at 95 °C, 32 amplification cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension step of 72 °C for 10 min. All reactions were done in triplicate.

Length heterogeneity of the PCR amplicons was detected by capillary electrophoresis (ABI Prism 310, Applied Biosystems) and a 5′ 6-carboxyfluorescein (FAM)-labeled ITS1F primer. PCR reactions were diluted with 80 µl of distilled water and 2 µl was added to 13 µl of a loading buffer (12 µl formamide, 0.5 µl 0.3 M NaOH, and 0.5 µl Genescan 2500 (TAMRA) size standard) and analyzed directly by capillary electrophoresis without further modification, *i.e.*, denaturation heating. Electrophoresis conditions were as follows: 47 cm capillary, Genescan POP4 polymer, 15 s injection for 15 kV, and 65 min electrophoresis at 10 kV. Scoring of amplicons into unique two-bp bins (or fungal species) was performed using Genemapper software (Vers. 4). Species richness was determined as the number of unique fragments present in a sample. Shannon's species diversity index and evenness (Magurran, 2004) and Bray–Curtis [Sorenson] similarity (McCune and Grace, 2002) were calculated using peak heights as a measure of abundance.

2.4. Reproducibility from soil samples

Length heterogeneity analysis was performed on 10 g soil samples (0–3 cm depth) collected from each of six plots (>20 m apart) located in a potato field in San Luis Valley, Colorado. Briefly, genomic DNA was extracted from each plot's 1 g sub-samples ($n=6$) using the UltraClean soil DNA kit (Mo Bio). The total amount of extracted DNA was quantified spectrophotometrically and diluted to a concentration of 10 ng μl^{-1} prior to LH-qPCR analysis as described above. Variability associated with each level of the procedure (plot or within field, DNA extraction or sub-sample, and PCR reaction) was examined by comparison of six replicates from each level.

2.5. GenBank database analysis

The ability of length heterogeneity analysis to identify individual fungal taxa was analyzed on 261 sequences contained in GenBank (National Center for Biotechnology Information) that exhibited a 100% match to both primers which were examined for predicted amplicon size. Length heterogeneity in amplicon length was determined on all unique amplicons, defined as amplicons associated with differing species, unique isolates or strains identified within a species, or amplicons of different lengths within a species. Duplicate sequences of the same length from a single fungal species were removed unless they were labeled as a unique strain.

3. Results and discussion

3.1. PCR efficiency and amplicon length of pure fungal cultures

Single-tube PCR efficiency and amplicon size using the ITS1F and ITS4 primer pair was tested using genomic DNA from 36 fungal isolates representing 28 different species (10 ascomycetes, 6 basidiomycetes, 4 zygomycetes, and 8 oomycetes). Fungi from all taxonomic classes, except the oomycetes, were successfully amplified with an average PCR efficiency of 102.9±5.3 when used as the sole PCR template (Table 1).

Amplicons ranged in size from 587.1±0.1 bp to 782.7±0.1 bp, and none of the isolates tested resulted in an amplicon of the same length.

3.2. In-silico analysis

A total of 261 DNA sequences from 83 genera and 185 fungal species were obtained from the GenBank database that had 100% homology to the ITS1F and ITS4 primer pair. Across all samples, predicted amplicon sizes averaged 641+40 bp and ranged from 420 to 825 bp with 135 unique fragments. The range in fragment size was similar across all three fungal classes (ascomycete: 420–806, $n=155$; basidiomycete: 503–825, $n=78$; and zygomycete: 653–788, $n=28$), although the mean fragment size was smaller for the ascomycetes (612±40 bp) compared to the basidiomycetes (697±55 bp) and zygomycetes (698±33 bp). On average, each amplicon size contained 1.8 unique fungal species with 79 amplicon sizes unique to a single fungal species and 56 amplicon sizes common to two or more species. As a result, species diversity determined from length heterogeneity will be underestimated on average two-fold. Conversely, species diversity determined from length heterogeneity may be overestimated if strains from the same species have amplicons of differing sizes. In the compiled database, 22 different fungal species were included by two or more identifiable strains. In 18 of the 22 multi-strain species taxa, each strain resulted in a unique amplicon. Based on the above, length heterogeneity analysis may best represent fungal strain diversity, not species diversity, and due to similar amplicon lengths from unrelated fungal species this diversity will be underestimated approximately two-fold. In addition, fungal diversity estimates using this technique would be expected to consist solely of the higher fungi. A GenBank search showed that while many oomycetes contain an ITS4 binding site with 100% homology, the 21 bp ITS1F binding site matches only at bp 7–19. Lowering the annealing temperature, similar to the methods of Larena et al. (1999), may result in the amplification of some oomycete species; however, the reduced stringency can result in false products in some of the higher fungi (data not shown).

Ranjard et al. (2001) also analyzed the GenBank database for length heterogeneity in fungal species using a different set of conserved rRNA primers (2234C and 3126T). Similar to the ITS1F and ITS4 primers, a significant overlap between fungal taxonomic groups was noted, with a size range of 390 bp to 1065 bp based on a sample of 251 species from 104 genera. However, unlike the ITS1F and ITS4 primers, members of the Chytridiomycota, Oomycota, and Plasmodiophoromycota contained conserved primer sequences and should be amplified with the 2234C and 3126T primers (Ranjard et al., 2001).

3.3. Quantification of total fungal abundance in mixed-template samples

Starting template concentrations were estimated by both the qPCR-cycle threshold and capillary electrophoresis-peak height techniques using serially diluted genomic DNA from five fungal species (*F. equiseti*, *A. solani*, *Verticillium* sp., *R. solani*, and *S. sclerotium*). As shown in Fig. 1, the qPCR technique was superior, resulting in a linear relationship between cycle threshold and the log of starting template concentration over five orders of magnitude in DNA concentration (10^{-1} to 10^{3} pg μl^{-1}); whereas, the end-product-based technique (capillary electrophoresis peak height) could not detect template concentrations greater than ca. 40 pg μl^{-1}. For both techniques, similar standard curves were observed for all fungal species, and the average regression line of all five species was used for estimation of starting template concentrations in further studies.

The relative ability of the two techniques to estimate starting fungal DNA concentration was also analyzed using mixtures of up to five different fungal species. Similar to the single template results, the qPCR method was the superior method, successfully

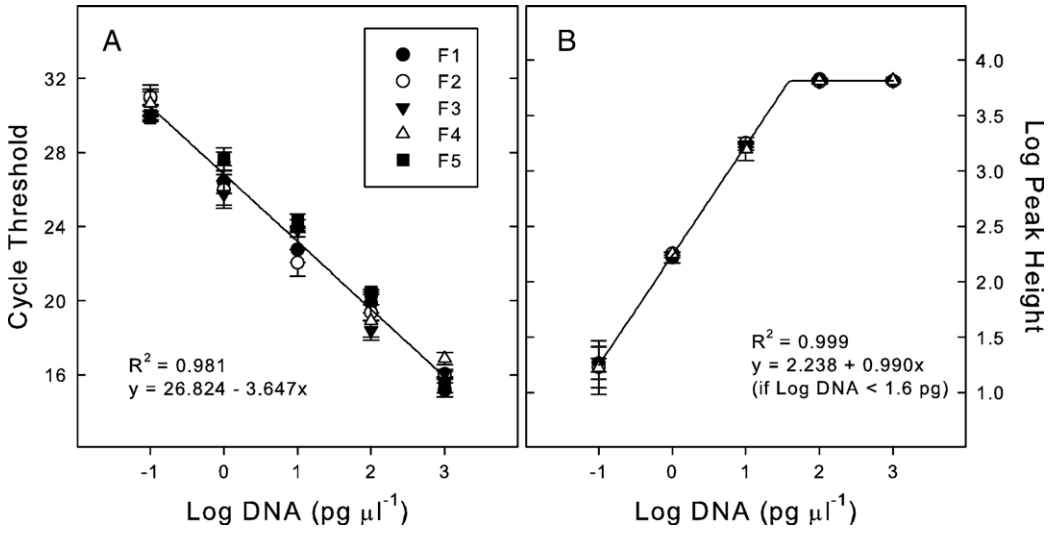

Fig. 1. Relationship between starting template concentration and cycle threshold (panel A) or peak height (panel B). DNA was isolated from pure cultures of five different species (F1 — *Fusarium equiseti*, F2 — *Alternaria solani*, F3 — *Verticillium* sp., F4 — *Rhizoctonia solani*, F5 — *Scletorina sclerotium*).

Table 2
Effect of template mixtures on total fungal DNA (pg) quantitation

Mixture	DNA template in mixture (pg μl^{-1})[a]					Total DNA (pg μl^{-1})[b]		
	F1	F2	F3	F4	F5	Actual	qPCR	LH
1	200.0	200.0	200.0	200.0	200.0	1000.0	983.9 (1.4)	40.9 (1.3)
2	20.0	20.0	20.0	20.0	20.0	100.0	117.4 (1.3)	40.6 (0.5)
3	2.0	2.0	2.0	2.0	2.0	10.0	14.3 (1.2)	11.5 (0.1)
4	0.2	0.2	0.2	0.2	0.2	1.0	0.6 (1.6)	0.8 (0.1)
5	200.0	0.2	0.2	0.2	0.2	200.8	213.5 (1.2)	39.6 (2.1)
6	20.0	0.2	0.2	0.2	0.2	20.8	23.5 (1.2)	17.4 (1.3)
7	2.0	0.2	0.2	0.2	0.2	2.8	3.0 (1.4)	3.8 (0.1)
8	50.0	50.0	0.0	0.0	0.0	100.0	103.7 (1.3)	39.6 (0.4)
9	50.0	0.0	50.0	0.0	0.0	100.0	97.4 (1.1)	39.8 (0.6)
10	50.0	0.0	0.0	50.0	0.0	100.0	99.5 (1.2)	39.4 (0.9)
11	50.0	0.0	0.0	0.0	50.0	100.0	101.5 (1.1)	38.9 (0.8)

[a] DNA from five representative fungal species were added to each PCR reaction at the specified amount (F1 — *Fusarium equiseti*, F2 — *Alternaria solani*, F3 — *Verticillium* sp., F4 — *Rhizoctonia solani*, F5 — *Sclerotina sclerotium*).

[b] Actual is the total amount of DNA (pg) added to PCR reaction mixture. qPCR is the total amount of DNA (pg) in the reaction mixture estimated by qPCR using the standard curve shown in Fig. 1A. LH is the total amount of DNA in the reaction mixture estimated by capillary electrophoresis (sum of all peak heights) using the standard curve shown in Fig. 1B.

estimating starting template concentration, typically within 10%, over the entire range of template concentrations (Table 2); whereas, capillary electrophoresis could not detect template concentrations above 40 pg μl^{-1}.

We made no effort to control for differences in gene copy number or genome sizes in the development of our standard curves; *i.e.*, serial dilutions were carried out on total genomic DNA quantified spectrophotometrically and expressed in mass units. Although the number of gene copies per genome, or per unit mass of DNA, can potentially influence qPCR sensitivity and quantitation this did not appear to be a problem with the five species chosen for analysis. Corradi et al. (2007) showed that a 2- to 4-fold difference in rRNA copies can influence Ct values by ~1 to 2 cycles. Although rRNA copies per genome may range from 50 to 100 in filamentous fungi (Rooney and Ward, 2005) and ascomycetes often have relatively smaller genome sizes (Kullman et al., 2005) the resultant number of rRNA copies per unit mass appears to vary little between the chosen fungal isolates and/or has little impact on our observed standard curves.

Differences in SYBR green binding, and resultant fluorescence intensity, between various amplicons could also lead to different standard curves and estimation of starting template quantities between fungal isolates. Although SYBR green binding to DNA can be dependent upon both amplicon size and/or GC content (Giglio et al., 2003) this did not appear to be a significant problem since all five standards, with amplicons ranging in size from 574 bp (*F. equiseti*) to 776 bp (*R. solani*), all showed similar standard curves.

3.4. Individual PCR bias in mixed-template samples

Despite the relative ability of the qPCR technique to quantify total starting template concentration of template mixtures, differential amplification of various individuals was observed. For example, mixtures of all five templates at equal ratios resulted in large differences in peak heights (Fig. 2). In all four cases, amplification was much greater for the ascomycetes as compared to the basidiomycetes. To further explore this class-based PCR bias, 1:1 mixtures of all 25 species of fungi were analyzed resulting in a consistent class-based PCR bias. For example, 1:1 ascomycete mixtures resulted in an average peak height ratio of 0.92±0.15, 1:1 ascomycete:basidiomycete mixtures resulted in an average peak height ratio of 0.32±0.22, and ascomycete:zygomycete mixtures resulted in an average peak height ratio of 0.86±0.17.

The apparent class-based bias needs to be further examined as only a limited set of fungal cultures were included in this analysis. However, PCR bias in mixed-template samples is a known problem that may arise from differences in gene copy numbers and/or genome sizes (Farrelly et al., 1995; Fogel et al., 1999; Klappenbach et al., 2000), the effect of GC content on template dissociation (Reysenbach et al., 1992), template reannealing (Suzuki and Giovannoni, 1996), and/or primer mismatches (Polz and Cavanagh, 1998). Of these factors, we suggest that gene copy numbers and/or genome size plays only a minor role in the preferential amplification of ascomycetes, since similar standard curves were observed for both ascomycetous and basidiomycetous fungi using DNA extracted from pure cultures.

One of the major goals of PCR-based techniques has been to analyze community profiles for species diversity and individual abundance. Based on the PCR biases discussed above community measures (*e.g.*, species diversity and evenness) that are dependent upon abundance characteristics will be the most affected. Analysis of various template mixtures showed that species richness estimates were robust (Table 3), unless the mixtures were extremely unequal, particularly in favor of the more easily amplified ascomycete species. Despite the effects of PCR bias on abundance-based measures, relative differences between treatments may still be identifiable unless the

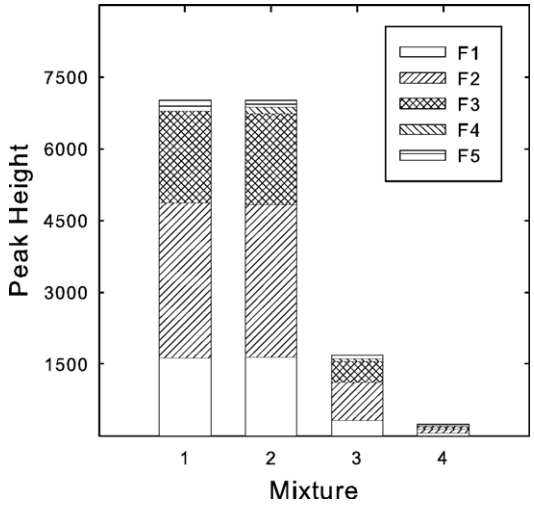

Fig. 2. Observed peak heights of five different fungal species (F1 — *Fusarium equiseti*, F2 — *Alternaria solani*, F3 — *Verticillium* sp., F4 — *Rhizoctonia solani*, F5 — *Sclerotina sclerotium*) from mixed-template PCR reactions. Mixture compositions are shown in Table 2.

Table 3
Relative ability of length heterogeneity to detect species presence and abundance in DNA template mixtures

Mixture[a]	Species richness (S)[b]		Species diversity (H)		Species evenness (H_E)	
	Actual	Observed	Actual	Observed	Actual	Observed
1	5	4.3 (0.6)	1.61	1.16 (0.05)	1.00	0.84 (0.03)
2	5	5.0 (0.0)	1.61	1.13 (0.04)	1.00	0.70 (0.05)
3	5	5.0 (0.0)	1.61	1.15 (0.04)	1.00	0.71 (0.07)
4	5	5.0 (0.0)	1.61	1.22 (0.08)	1.00	0.76 (0.05)
5	5	1.0 (0.0)	0.03	0.00 (0.00)	0.02	–
6	5	1.3 (0.6)	0.22	0.00 (0.00)	0.13	–
7	5	3.3 (0.6)	0.99	0.31 (0.03)	0.62	0.28 (0.02)
8	2	2.0 (0.0)	0.69	0.65 (0.05)	1.00	0.94 (0.04)
9	2	2.0 (0.0)	0.69	0.69 (0.05)	1.00	0.99 (0.06)
10	2	2.0 (0.0)	0.69	0.17 (0.03)	1.00	0.24 (0.02)
11	2	2.0 (0.0)	0.69	0.67 (0.05)	1.00	0.96 (0.03)

[a] DNA from five representative fungal species were added to each PCR reaction in amounts (F1 — *Fusarium equiseti*, F2 — *Alternaria solani*, F3 — *Verticillium* sp., F4 — *Rhizoctonia solani*, F5 — *Scletorina sclerotium*) specified in Table 1.

[b] Species richness was determined as the number of unique fragments present in a sample. Shannon's species diversity index and evenness (Magurran, 2004) were calculated using peak heights as a measure of abundance.

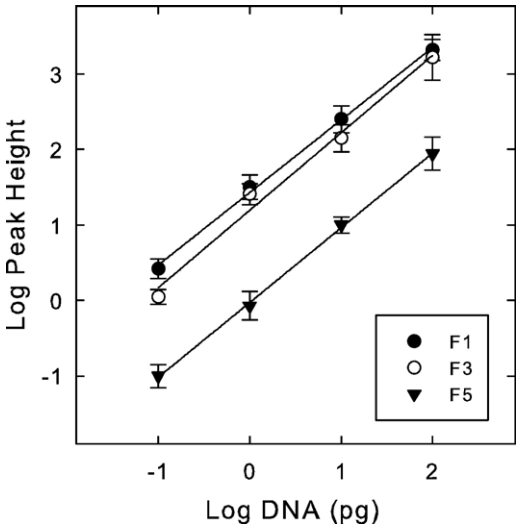

Fig. 3. Relationship between peak height and starting DNA template concentration for three pure fungal cultures. (F1 — *Fusarium equiseti*: $y=1.430+0.960x$, $R^2=0.998$; F3 — *Verticillium* sp.: $y=1.195+1.025x$, $R^2=0.988$; F5 — *Scletorina sclerotium*: $y=-0.0270+0.989x$, $R^2=0.999$).

composition of the community changes drastically (*i.e.*, shifts from species of high to low PCR efficiency). For example, various amounts of DNA were added to a mixed-template DNA sample (SLV soil sample). As predicted by the PCR bias estimates, peak heights were greatest for two ascomycetes compared to a basidiomycete. However, regardless of the template a linear relationship was observed between the peak height and amount of DNA added. Thus, although peak heights may not be useful to detect absolute values of any given individual (without a specific standard curve for that individual–template combination), it should be possible to identify relative differences between species samples within a larger soil sample. For example, all three species tested had a similar slope (Fig. 3) but different peak heights due to differences in PCR efficiency.

3.5. Assessment of technique reproducibility

To assess the variability associated with each step of the LH-qPCR analysis, 10 g soil samples (0–5 cm depth) were collected from three plots (>50 m apart) in a potato field in San Luis Valley, CO. From each plot DNA was extracted from three sub-samples and each sub-sample was subjected to three individual PCR reactions and analyzed for total fungal DNA (ng g^{-1} DW) by qPCR and capillary electrophoresis. The reproducibility of total fungal biomass estimates for both the qPCR and LH methods is shown in Table 4. As expected, the greatest variability was associated with field sampling (*e.g.*, between plots and sub-samples) but was highly reproducible between PCR reactions (CV 5.2 and 7.2% for the qPCR and LH methods, respectively). Similarly, efforts to estimate the relative abundance of any given fungal phylotype (*e.g.*, peak height of an individual fragment) showed a high degree of reproducibility between reactions (Table 4). Various fungal community attributes (Bray–Curtis [Sorenson] similarity, C_S; species richness, S; Shannon's diversity index, H; and species evenness, H_E) were also determined for the field collected samples (Table 5). Like fungal biomass estimates, levels of variability were most associated with field sampling, not individual PCR reactions. Similar to other studies (Fischer and Triplett, 1999; Ranjard et al., 2001), community profile differences between runs of the same sample were highly reproducible ($C_S=0.96+0.03$) and mainly due to differences in peak heights; whereas, both the abundance (*i.e.*, peak heights) and number of phylotypes present contributed to the differences in samples at the sub-sample and plot level (Table 5).

3.6. Conclusions

As suggested by others (Fischer and Triplett, 1999; Ranjard et al., 2001), length heterogeneity analysis of the rRNA genes provides a rapid and reproducible method for estimating the

Table 4
Reproducibility of total and relative fungal DNA estimates from soil samples

Source	Total fungal DNA (ng g^{-1} DW)[a]				Relative fungal DNA (%)[b]					
	qPCR		LH		Fragment 1 (525 bp)		Fragment 2 (581 bp)		Fragment 3 (622 bp)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PCR rxn	10.9	0.57	4.3	0.31	25.8	1.5	18.2	0.9	5.8	0.4
Sub-sample	11.4	1.40	4.4	0.63	22.7	1.9	17.9	1.2	4.5	0.3
Plot	12.8	3.11	4.7	1.30	20.9	2.1	17.1	1.6	3.2	0.6

[a] qPCR is the total amount of DNA (pg) in the reaction mixture estimated by qPCR using the standard curve shown in Fig. 1A. LH is the total amount of DNA in the reaction mixture estimated by capillary electrophoresis (sum of all peak heights) using the standard curve shown in Fig. 1B.

[b] Relative fungal DNA is the individual peak height for the fragment of interest divided by the sum of all peak heights for that sample.

Table 5
Reproducibility of fungal community attributes from soil samples

Source	Species richness (S) [a]		Shannon's diversity index (H)		Species evenness (H_E)		Bray–Curtis [Sorenson] similarity (C_S)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PCR rxn	8.4	0.31	1.9	0.06	0.88	0.04	0.96	0.03
Sub-sample	10.2	1.80	2.2	0.09	0.84	0.03	0.85	0.10
Plot	12.3	3.73	2.4	0.11	0.83	0.03	0.78	0.19

[a] Species richness was determined as the number of unique fragments present in a sample. Shannon's species diversity index and evenness (Magurran, 2004) and Bray–Curtis [Sorenson] similarity (McCune and Grace, 2002) were calculated using peak heights as a measure of abundance.

diversity and composition of microbial communities. In this study, we analyzed the potential PCR biases and utility of the ITS1F and ITS4 primer set (Gardes and Bruns, 1993). While these primers amplified all higher fungi when tested individually, significant PCR bias was observed in mixed templates, rendering it impossible to determine the absolute quantity of any individual within a population. However, based on the commonality in the slope of various standard curves, this technique appears suitable for determination of the relative abundance, or relative change between samples, for individuals within the community. Furthermore, we also showed that the substitution of the initial PCR with a quantitative real-time PCR appears to be a robust and accurate method to determine total fungal biomass in a mixed-template sample.

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