Mycorrhiza (2007) 18:15–22
DOI 10.1007/s00572-007-0148-z

ORIGINAL PAPER

Intra-specific and intra-sporocarp ITS variation of ectomycorrhizal fungi as assessed by rDNA sequencing of sporocarps and pooled ectomycorrhizal roots from a *Quercus* woodland

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Received: 19 June 2007 / Accepted: 19 July 2007 / Published online: 21 August 2007
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Abstract The Internal Transcribed Spacer (ITS) regions of ribosomal DNA are widely used as markers for phylogenetic analyses and environmental sampling from a variety of organisms including fungi, plants, and animals. In theory, concerted evolution homogenizes multicopy genes so that little or no variation exists within populations or individuals. However, contrary to theory, ITS variation has been confirmed in populations and individuals from a diverse range of eukaryotes. The presence of intraspecific and intra-individual variation in multicopy genes has important implications for ecological and phylogenetic studies, yet relatively little is known about natural variation of these genes, particularly at the community level. In this study, we examined intraspecific and intra-sporocarp ITS variation by DNA sequencing from sporocarps and pooled roots from 68 species of ectomycorrhizal fungi collected at a single site in a *Quercus* woodland. We detected ITS variation in 27 species, roughly 40% of the taxa examined. Although intraspecific ITS variation was generally low (0.16–2.85%, mean = 0.74%), it was widespread within this fungal community. We detected ITS variation in both sporocarps and ectomycorrhizal roots, and variation was present within species of Ascomycota and Basidiomycota, two distantly related lineages within the Fungi. We discuss the implications of such widespread ITS variability with special reference to DNA-based environmental sampling from diverse fungal communities.

Keywords Cloning · Ectomycorrhiza · Environmental sampling · Fungi · ITS variation · Ribosomal DNA

Electronic supplementary material The online version of this article (doi:10.1007/s00572-007-0148-z) contains supplementary material, which is available to authorized users.

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Introduction

The internal transcribed spacer (ITS) regions of ribosomal DNA, including both ITS1 and ITS2, have been used extensively for phylogenetic analyses and environmental sampling for a variety of organisms including fungi, plants, and animals (e.g., Campbell et al. 1993; Downie et al. 2000; Madani et al. 2005; O'Brien et al. 2005). ITS is a convenient marker for environmental sampling because several taxonomic group-specific primer sets exist for this gene region (e.g., Gardes and Bruns 1993; Martin et al. 2004), and it is easily polymerase chain reaction (PCR)-amplified from many substrates (Bruns and Shefferson 2004). DNA-based detection techniques are particularly well suited for fungi and other microorganisms because these techniques can identify microbes that exhibit few morphological characters or grow embedded within opaque substrates (Bruns and Shefferson 2004; O'Brien et al. 2005). Furthermore, DNA-based techniques have the

potential to avoid some of the biases associated with community sampling based on reproductive structures or culture-based assays (Bidartondo and Gardes 2005). The ITS has been the region of choice for molecular analysis of fungal communities since Gardes and Bruns (1996) used restriction digests [restriction fragment length polymorphism (RFLP)] of this region to differentiate species of ectomycorrhizal fungi colonizing individual roots. Recently, derivative fingerprinting techniques, in combination with DNA sequencing of the ITS, have been used to examine diverse fungal communities from roots, soil, air, wood, and animal tissues (Jasalavich et al. 2000; Buchan et al. 2002; Dickie et al. 2002; Redecker 2002; Allmer et al. 2006; Scupham et al. 2006). Such studies have revealed a wide array of cryptic, uncultured, and previously unrecognized microbes not readily detected by other means (Bidartondo and Gardes 2005). In addition to being routinely used for community analyses and phylogenetics, the ITS region has become a popular target region for molecular diagnosis of important human and animal diseases caused by fungi and other eukaryotes (e.g. Reiss et al. 1998; Bensoussan et al. 2006; Petti 2007).

Multicopy genes, such as the ITS, are used extensively as approximately species-specific markers, yet relatively little is known about natural variation of these genes within populations or individuals (Avis et al. 2006). Theoretically, concerted evolution homogenizes multicopy genes, such as ITS, over time, resulting in low or nonexistent variation within individuals and species (Elder and Turner 1995; Liao 1999). However, the paradigm of concerted evolution does not always apply (e.g., Rooney and Ward 2005), and ITS variation has been noted within species and individuals from a wide variety of organisms including crustaceans (Harris and Crandall 2000; Gandolfi et al. 2001), angiosperms (Buckler et al. 1997; Feliner et al. 2004), insects (Fairley et al. 2005), nematodes (Hugall et al. 1999), marine sponges (Worheide et al. 2004), and fungi (Kåren et al. 1997; O’Donnell et al. 1998; Aanen et al. 2001; Horton 2002).

The presence of intraspecific and intra-individual variation within multicopy genes, such as the ITS, has widespread implications for phylogenetics, microbial ecology, and DNA-based diagnostics of diseases and disease vectors. Within the realm of fungal ecology, high levels of intra-specific ITS variation make it difficult to accurately group taxa at the species level, affect our ability to detect disease agents of plants and animals, and obscure relationships between reproductive structures and hyphae from environmental samples (Horton 2002). The ITS region usually correlates with the phylogenies of other genes (e.g. Douhan and Rizzo 2005; Geml et al. 2006), but in some cases, it conflicts with species boundaries differentiated by morphology (Fischer and Binder 2004), reproductive compatibility (i.e., biological species) (Aanen et al. 2001), DNA fingerprinting (Kauserud et al. 2006), or multi-locus phylogenies (O’Donnell et al. 1998; Cruse et al. 2002; Villa et al. 2006). Despite the importance of assessing ITS variation at the community scale, only a few studies have addressed this topic. Thus far, these studies have employed limited DNA sequencing and focused on ITS variation of sexual reproductive structures (sporocarps) rather than hyphae or other somatic structures (Kåren et al. 1997; Horton 2002; Glen et al. 2001; Avis et al. 2006). In this study, we examined intraspecific and intra-sporocarp ITS variation by DNA sequencing of both sporocarps and pooled roots from 68 species of ectomycorrhizal fungi collected at a single site in a *Quercus* woodland. We hypothesized that DNA sequencing would reveal significantly more intraspecific and intra-sporocarp ITS variation than has previously been documented.

Materials and methods

Site characteristics

All research was conducted in the Koch Natural Area at the University of California Sierra Foothill Research and Extension Center (UCSFREC) on the western slope of the Sierra Nevada foothills in Yuba County, California, USA (39°14′ N, 121°18′ W). The terrain consists of low hills 50–650 m above sea level. Overstory vegetation is dominated by three species; *Quercus douglasii* Hook and Arn., *Quercus wislizeni* A. DC., and *Pinus sabiniana* Douglas. The Mediterranean climate is characterized by cool, wet winters and hot, dry summers. Precipitation generally occurs between October and May (annual mean 71 cm, range 23–132 cm) and temperature varies seasonally (mean 17.8°C, range 10–43°C) (UCSFREC online: http://danrrec.ucdavis.edu).

Sampling of sporocarps and ectomycorrhizal roots

Sporocarps of ectomycorrhizal fungi and ectomycorrhizal root tips were collected during 2000–2005 at UCSFREC beneath *Quercus* spp. as part of a study on temporal dynamics in EM fungal communities. Details about the composition and seasonality of the fungi at this site are published elsewhere (Smith et al. 2007). Briefly, sporocarps were collected from a ca 400×400 m plot and identified based on morphological characters with published keys. Taxon identifications were often confirmed, refined, or revised based on BLAST searches (Altschul et al. 1997), phylogenetic analysis of sequence data from ITS and 28s rDNA, and consultation with taxonomic experts. For molecular analysis, a small piece of clean tissue was removed from within each sporocarp with a sterilized

scalpel, placed in CTAB extraction buffer, and frozen until DNA extraction. Ectomycorrhizal roots of *Q. douglasii* were sampled from a total of 94 root cores in a 32×32 m plot of woodland-savanna on four dates in 2003–2004 (5 March 2003, 21 May 2003, 29 February 2004, 3 May 2004). Understory vegetation consists of assorted herbaceous, non-ectomycorrhizal plants. Litter was removed, and round soil cores of ca 900 cm^3 (±8.5 cm deep×11.5 cm diameter) were collected, stored at 4°C, and processed within 15 days of sampling. Soil was sieved and washed by hand with deionized water. After cleaning, root material was viewed under a dissecting microscope and ca 100 healthy, colonized ectomycorrhizal roots per core were randomly selected, pooled, and lyophilized for later molecular analysis.

Molecular techniques

Sporocarp tissue was ground with a micropestle, and DNA was extracted with a modified CTAB method (Gardes and Bruns 1993) or Qiagen Stool Kit (Qiagen, Valencia, CA, USA). We performed PCR with one or more of the following primer combinations: ITS1F and ITS4, ITS1F and ITS4B, ITS1F and LR3, or LROR and LR3 (White et al. 1990; Gardes and Bruns 1993; Hopple and Vilgayls 1994). PCR conditions were optimized for each primer combination, but the general reaction protocol was as follows: initial denaturation of 94°C for 5 min followed by 25 cycles of 1 min (94°C); 1 min (55°C); 2 min (72°C) followed by a final extension of 72°C for 7 min. PCR products were visualized on 1.5% agarose gels with SYBR Green I (Molecular Probes, Eugene, OR, USA). When multiple bands occurred on a gel, PCR products were gel purified with BioRad Freeze 'n' Squeeze Tubes (BioRad, Hercules, CA, USA) and reamplified with appropriate primers. PCR products were cleaned before sequencing with Microcon-PCR Spin Tubes or Montage PCR_{96} Filter Plates (Millipore, Billerica, Mass, USA). DNA sequencing was performed as needed with the same primers as above with the ABI Big Dye Terminator Sequencing Kit (v3.1) and read with an ABI13730xl capillary sequencer (Applied Biosystems, Foster City, CA, USA) at the College of Agricultural and Environmental Sciences Genomics Facility, University of California, Davis. ITS sequences were manually examined and edited with Sequencher v.4.1 (Gene Codes, Ann Arbor, MI, USA).

For ectomycorrhizal roots, DNA extraction with the modified CTAB method was followed by purification with a MO-BIO Soil DNA kit (MO-BIO Laboratories, Solana Beach, CA, USA). We performed PCR on all mixed-template reactions with 1–4 µl of DNA template and high resolution Taq polymerase (Invitrogen, Carlsbad, CA, USA). The reaction protocol was as follows: initial denaturation of 94°C for 10 min followed by 20 cycles of 1 min (94°C); 1 min (55°C); 4 min (72°C) followed by a final extension of 72°C for 7 min. Fresh PCR products were visualized on 1.5% agarose gels with SYBR Green I. Four successful replicate reactions were pooled and then cloned with a TOPO-TA kit (Invitrogen, Carlsbad, CA, USA). At least 46 successful clones per reaction were grown overnight in Luria-Bertani (LB) media amended with 100 µg/ml of ampicillin. Cloned fragments were then re-amplified in a PCR reaction with ±0.5 µl of the bacterial suspension as template following the same PCR protocol as above except that standard, recombinant Taq polymerase (Invitrogen, Carlsbad, CA, USA) was used instead of high resolution Taq polymerase. Amplicons were digested with the restriction enzymes *Alu*I and *Hinf*I then electrophoresed through a 1.5% agarose gel and stained with SYBR Green I. At least 46 clones for each primer combination were visually inspected and scored to determine the number of different restriction fragment length polymorphism (RFLP) types. One to four representative clones of each type were sequenced with ITS1F following the same protocol as for sporocarps.

Quantification of ITS variation

To calculate intraspecific ITS variation, we created species-specific alignments using Sequencher v.4.1, ClustalX (Chenna et al. 2003) and MacClade (Maddison and Maddison 2000). We used all high-quality sporocarp and root clone sequences and quantified variable base pairs (bp) and insertion–deletions (indels). For sporocarps, we examined only those species where two or more high-quality ITS sequences were available (n=31 spp.). For one exception, *Melanogaster* cf. *tuberiformis*, only a single sequence was available. For clones from ectomycorrhizal roots, ITS variation found in a single root core may have been due to PCR or DNA sequencing error and was thus not quantified. A conservative approach was used whereby an ITS type was only considered variable at a particular base pair position or indel if the same variation (either variable bp or indel) occurred in two or more separate soil cores. Based on this criterion, ITS variation from taxa on roots was only analyzed for ITS types that occurred separately in four or more soil cores (n=37 species). Ambiguous base pairs were excluded from all analyses. Representative rDNA sequences of all taxa have been deposited in GenBank (Table S1).

Results

We examined intraspecific variation within 380 ITS sequences (ca 16,800 bp) from 68 of the 161 taxa encountered on ectomycorrhizal roots or as sporocarps with *Q. douglasii*. The remaining 91 taxa were either too rare, or sequences were not of sufficient quality for unambiguous comparisons. Of the 68 species that were considered, we found ITS

variation in 27 (ca 40% of the species examined), and we identified three species complexes (Table 1). For the purposes of this analysis, ITS sequences were considered to belong to different species if they differed in 3% or more of the base pairs across the ITS1/5.8s/ITS2 region, with the exception of the *Cenococcum geophilum* and *Cortinarius flexipes* species groups (Smith et al. 2007). This 97% similarity criterion is similar to that of several previous EM studies (e.g., Izzo et al. 2005; Tedersoo et al. 2003). For *C. geophilum*, sequences of both ITS and a group I intron located in the small subunit (18s) of the rDNA were amplified by the ITS1f/LR3 primer pair. Both regions were used to unambiguously place *Cenococcum* clones into one of the three main lineages described previously by Douhan and Rizzo (2005). For the *C. flexipes* species group, ITS haplotypes were designated as in Smith et al. (2007).

Intraspecific variation was present on several different levels; we detected intraspecific ITS variation between: (1) different samples of the same species from pooled roots (nine occurrences), (2) different samples of the same species from sporocarps (four occurrences), and (3) different samples of the same species found as both pooled roots and as sporocarps (ten occurrences). ITS variation within species ranged between 0.16 and 2.85%, with the number of sequences ranging 1–43 (mean=9.8 sequences) and the length of examined alignments ranging 420–640 base pairs (mean=520 bp). Of the 28 species that exhibited ITS variation, 7 were Ascomycota, and 20 were Basidiomycota.

We also detected putative ITS variation within individual sporocarps (five occurrences, all Basidiomycota). For three species, *Clavulina* cf. *cristata*, *Hygrophorus eburneus*, and *Hygrophorus cf. roseiburneus*, ITS variation was present as 1–2 polymorphic base pair positions with double peaks present (i.e., heterozygous positions) within two or more sporocarps (Table 1). For the other two species, *Russula* cf. *crassotunicata* and *M.* cf. *tuberiformis*, chromatogram patterns were excellent but became abruptly unreadable in the middle of the sequence. The most parsimonious explanation for such a phenomenon is the presence of two heterologous ITS copies that differ by an indel of 1 bp or more. A frame shift of this type obscures the chromatogram pattern only in the downstream direction from the indel site; a pattern we detected in chromatograms generated by both the ITS1f and ITS4 primers.

For the three species complexes (*Genea harknessii* group, *C. geophilum* group, and *C. flexipes* group) ITS variation ranged between 5.7 and 12.5% or more. Scoring ITS variation for these taxa was problematic due to large and/or frequent indels, so variation is presented as conservative estimates. For the species complexes, the number of sequences analyzed ranged 18–29 (mean=23.3), and the length of examined alignments ranged 300–505 bp. The two unalignable sequence regions obtained from *C. geophilum* (ITS and SSU Intron) were analyzed separately (Douhan and Rizzo 2005).

Discussion

Kåren et al. (1997) were the first to study intraspecific ITS variation among ectomycorrhizal fungi using RFLP. They assessed ITS variation in 44 taxa across Scandinavia and detected polymorphism in roughly 16%. On a much smaller spatial scale, Horton (2002) found polymorphism in approximately 14% of the 44 species examined, and Glen et al. (2001) found polymorphism in approximately 29% of the 42 species examined. We detected ITS variation in 28 of 68 taxa (ca 40%), a much greater proportion than in any previous studies. In this study, ITS variation was generally low, ranging between 0.16 and 2.85%, but the spatial scale and number of sporocarps examined here was small compared to the other three studies that have examined ITS variation of ectomycorrhizal fungi at a community scale. The fact that more species showed variation when assessed by ITS sequencing rather than by RFLP analysis is not surprising, as RFLP can only detect variation within a small fraction of nucleotides, while DNA sequencing allows visualization of all nucleotides across a gene region (Kåren et al. 1997). Consequently, the three studies mentioned above could not accurately distinguish between actual ITS hetereogeneity and cryptic species, except for the few polymorphic species where DNA sequencing was used (e.g. *Cortinarius traganus* and *Cortinarius armillatus*—Kåren et al. 1997; *Tricholoma flavorvirens* group—Horton 2002). In contrast, we detected three species complexes where clusters of related ITS sequences were variable in approximately 5.7 to 12.5% of nucleotides. Two of these groups, *C. geophilum* and *Genea harknesii*, were resolved into several well-supported lineages that appear to represent cryptic species (Douhan and Rizzo 2005; Smith et al. 2006). Haplotypes of the *C. flexipes* group, however, could not be easily resolved and may represent a cluster of closely related species undergoing reticulate evolution (e.g., Frøslev et al. 2005). This is not surprising, as phylogenetic resolution using ITS has proven difficult within *Cortinarius*, and this genus is among the most species rich in the Basidiomycota (Frøslev et al. 2005).

In our study, variation in ITS appeared to be nonrandom and was greater among sporocarps than clones of ectomycorrhizal roots. This was expected for several reasons. First, as ectomycorrhizal roots were cloned before DNA sequencing, intraspecific ITS variants may have been present, but have not been sequenced due to the randomness of PCR amplification, cloning, and selection of individual DNA fragments (O'Donnell et al. 1998). Second, sporocarps were sampled from a larger plot than

Table 1 Intraspecific ITS sequence variation in ectomycorrhizal roots and sporocarps associated with *Quercus douglasii*

Species	Source of variation/Source	Number (*n*)	bp Examined	Variable bp	Indels	Indel length	polymorphic bp	Variation (%)
Amanita lanei	Between sporocarps	2	550	6	6	1 bp	–	2.22%
Inocybe cf. *armeniaca*		2	485	1	1	9 bp	–	2.06%
Boletus zellerii		2	250	1	1	2 bp	–	1.20%
Hygrophorus cf. *gliocyclus*		2	540	2	–	–	–	0.37%
Clavulina cf. *cristata*[a]	Within sporocarps	3	600	–	–	–	2 bp	0.33%
Russula cf. *crassotunicata*[b]		2	490	–	1?	?	–	0.20%?
Melanogaster cf. *tuberiformis*[b]		1	500	–	1?	?	–	0.20%?
Hygrophorus cf. *roseiburneus*[a]		2	610	–	–	–	1 bp	0.16%
Thelephoraceae1	Between roots	4	525	5	–	–	–	0.95%
Tomentella cf. *nitellina*		14	575	4	–	–	–	0.84%
Tuber cf. *whetstonense*		11	640	4	–	–	–	0.63%
*Thelephoraceae*9		18	570	3	–	–	–	0.53%
Inocybe sp. 1		9	620	–	1	1 bp	–	0.16%
Inocybe cf. *fraudans*		6	430	–	1	1 bp	–	0.23%
Inocybe sp. 2 (cf. *sindonia*)		16	460	1	–	–	–	0.22%
*Sebacinales*2		16	570	1	–	–	–	0.18%
Inocybe sp. 3 (cf. *lanatodisca*)		10	580	–	1	1 bp	–	0.17%
Tuber cf. *candidum*	Between roots and sporocarps	43	421	10	1	2 bp	–	2.85%
Genabea cerebriformis		7	610	14	2	1 bp	–	2.62%
Genea arenaria		6	500	6	–	–	–	1.20%
*Pyronemataceae*4 (src840)		9	585	6	–	–	–	1.03%
Peziza infossa		13	560	–	2	1 bp, 2 bp	–	0.54%
Tomentella sp. (src857)		32	600	3	–	–	–	0.50%
Thelephora cf. *anthocephala*		9	540	1	–	–	–	0.19%
Marcelleina sp. (src731)		10	530	1	–	–	–	0.19%
Tomentella sp. (src824)		31	605	1	–	–	–	0.17%
Hygrophorus eburneus[c]	Between roots and sporocarps, within sporocarps	6	550	1 bp	–	–	2 bp	0.55%
Species complexes								
Genea harknessii group	Between roots and sporocarps	24	415	47	5	1–27	–	12.5%+
Cortinarius flexipes group[d]	Between roots and sporocarps	22	450	16	13	1–6 bp	–	6.4%+
Cenoccocum geophilum group[e] (ITS)	Between roots and sclerotia	18	300	15	2	1 bp	–	5.67%+
Cenoccocum geophilum group[e] (SSU intron)	Between roots and sclerotia	29	505	34	1	2 bp	–	7.13%+

[a] Sporocarp w/ heterologous ITS copies—some bp consistently polymorphic (double peaks)
[b] Sporocarp w/ heterologous ITS copies—apparently different in length (causing frame shift)
[c] *Hygrophorus eburneus* had sporocarps with heterologous ITS copies (some bp consistently polymorphic) and showed variation between roots & sporocarps
[d] Includes seven ITS haplotypes
[e] The ITS1f/LR3 primer pair amplifies both the ITS and SSU intron. Both regions can be used to place *Cenoccocum*; isolates into one of the three main lineages designated by Douhan and Rizzo (2005)

were roots, and thus, there was a greater probability of sampling a higher number of fungal individuals (genets) in our sporocarp sampling.

A recent study by Avis et al. (2006) used TRFLP, cloning, and DNA sequencing to detect intraspecific and intra-sporocarp ITS diversity in ectomycorrhizal basidiomycetes. In particular, they focused on species that had been previously studied morphologically and were thus less likely to consist of multiple cryptic species. Their results suggest that some variation detected with RFLP or TRFLP may be an artifact of incomplete digestion by restriction enzymes. However, they also found that real, low-level ITS diversity could be found within individual sporocarps, and thus within species. Similar to the findings of Avis et al. (2006), we detected ITS variation within individual sporocarps of some Basidiomycota. Although ITS variation within individual dikaryons and sporocarps of Basidiomycota has been reported in the past (Aanen et al. 2001; Kauserud and Schumacher 2003; Avis et al. 2006), the discovery of putative ITS variation in sporocarps of 5 out of 68 species (ca 7%) indicates this phenomenon may be more widespread than previously thought.

In this study, we also detected ITS variation in seven species of ectomycorrhizal ascomycetes. Although this phenomenon has not been widely reported in the past, it may be due to the fact that ectomycorrhizal ascomycetes have generally received less attention than basidiomycetes (Tedersoo et al. 2006). Population and phylogenetic studies of the edible true truffles (genus *Tuber*), however, have detected significant ITS variation in some species, but not in others. For example, Mello et al. (2002) found relatively high ITS variation (ca 6%) in a small number of *Tuber unicatum* specimens sampled from just a few Italian sites. On the contrary, both *Tuber melanosporum* and *Tuber magnatum* had very low ITS variation (ca 1.6 and 0.4%, respectively), even when many specimens were sampled from several European countries (Murat et al. 2004; Mello et al. 2005).

Although the studies of *Tuber* spp. discussed above indicate that the level of ITS heterogeneity is not fixed within a genus, results from this and previous studies suggest that intraspecific ITS variation is more common in some genera or families than others. Among the ectomycorrhizal genera that have been previously studied, *Cortinarius*, *Inocybe*, *Laccaria*, *Lactarius*, and *Tomentella* appear to have elevated rates of intraspecific ITS variation (Kåren et al. 1997; Kõljalg et al. 2000; Glen et al. 2001; Horton 2002 and references therein). Our results corroborate this pattern; we detected an elevated rate of intraspecific ITS variation among *Inocybe* (5 spp.), Thelephoraceae (6 spp.) and *Hygrophorus* (3 spp.). The genus *Hygrophorus* showed the greatest rate of ITS variation; we found polymorphisms in all three of the species we examined. Although the reasons for this phenomenon are not totally clear, it appears that ITS variation may be higher in taxonomic groups that have undergone recent evolutionary radiation and/or that have high species diversity.

Taken overall, our results suggest that that low level intraspecific ITS variation is probably much more common than previously thought and is not an artifact of molecular methods. For example in this study, we only used exact sequences that were found in more than one sample, and recovering the same 'technical error' between two sampling locations is very unlikely. These findings therefore have important implications for future environmental sampling of fungal communities. Although ITS variation was sufficiently low to allow for the judicious use of RFLP or TRFLP to resolve the majority of fungal taxa at the small scale of the community we studied, our results suggest that ITS variation at larger scales (e.g., landscapes) may be significant. Thus, within the context of fungal ecology, sequencing the ITS region from multiple, morphologically similar specimens of local sporocarps seems an efficient way to detect intraspecific ITS variation that could be accounted for in techniques such as RFLP or TRFLP. Therefore, findings based on ITS fingerprinting methods such as RFLP or TRFLP should be corroborated by at least some DNA sequencing, particularly for taxonomic groups where fingerprint variation suggests the presence of cryptic species. As suggested by Dickie and FitzJohn (2007), sequencing clone libraries together with higher throughput TRFLP may allow both techniques to be utilized to their fullest potential.

Acknowledgements We thank E. Smith, M. Morris, K. Huryn, C. Bledsoe, and R. Albright for their assistance in the laboratory and important feedback throughout the process of this research. The staff at the UCSFREC provided logistical support for fieldwork. This research was supported by grants to D. M. Rizzo by the National Science Foundation (DEB-99-81711) and to M. E. Smith by the North American Truffling Society (NATS) and the Mycological Society of America (MSA).

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