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Multiple species of ectomycorrhizal fungi are frequently detected on individual oak root tips in a tropical cloud forest

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Abstract The ecological importance of ectomycorrhizal (EM) fungi in tropical ecosystems is increasingly recognized, but few studies have used molecular methods to examine EM fungal communities in tropical forests. The diversity and composition of the EM community on *Quercus crassifolia* in a tropical montane cloud forest in southern Mexico were characterized using DNA sequencing of single root tips. Individual root tips commonly harbored multiple fungal species that resulted in mixed polymerase chain reaction (PCR) products. By cloning and performing gel extractions on mixed PCR samples, we identified two or more EM fungi on 26% of the root tips. When non-EM fungi were considered, this figure increased to 31% of root tips. A total of 44 EM taxa and nine non-EM taxa were detected on roots from 21 soil cores (104 root tips). Taxa in the families Russulaceae, Cortinariaceae, Inocybaceae, and Thelephoraceae were frequent. This is the first study to characterize the belowground EM community in a tropical montane cloud forest.

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Introduction

Oaks (*Quercus* spp.) are widely distributed ectomycorrhizal (EM) trees with significant ecological and economic value. In Mexico, oaks are important components of a wide variety of habitats, including subtropical evergreen forest, oak-pine forest, chaparral, and cloud forest (Nixon 1993). Mexico harbors the greatest diversity of oaks (*Quercus* spp.) in the Americas, and the montane forests of southern Mexico are a known center of oak diversity (Nixon 1993, 2002). While the high diversity of oak host species might suggest a correspondingly high EM diversity, no studies have examined belowground EM communities in Mexico.

The use of DNA-based techniques to identify EM fungal symbionts on roots has greatly increased our understanding of the diversity and composition of belowground EM communities (Horton and Bruns 2001). Most molecular studies on EM communities, however, have been carried out in temperate regions, despite the fact that many important EM hosts occur in tropical forests (e.g., Pinaceae, Fagaceae, Diptrocarpaceae, Myrtaceae; Taylor and Alexander 2005). Southeast Asian dipterocarp forests, *Nothofagus*-dominated rainforests of Papua New Guinea, and Caesalpiniaceae rainforests in Guyana and Africa are some notable examples of tropical ecosystems dominated by EM tree species (Torti et al. 2001). However, our knowledge of tropical EM communities is limited (Haug et al. 2005; Moyersoen 2006; Riviere et al. 2007; Tedersoo et al. 2007), and almost nothing is known about EM symbionts on the roots of Mexican *Quercus* spp. (Varela and Estrada-Torres 1997).

EM communities exhibit tremendous diversity at various spatial scales. Small temperate forest stands of a single host plant may contain more than 90 species (Smith et al. 2007), and individual trees can host more than 15 species (Saari et al. 2005). At small spatial scales, EM diversity can be high, and even individual root tips can be colonized by more than one fungal symbiont (Wu et al. 1999; Kaldorf et al. 2004; Koide et al. 2005; D. Linder, unpublished data). Maintenance of the high species diversity found in EM communities has been attributed to various factors, including niche partitioning (Dickie et al. 2002), host specificity (Ishida et al. 2007), succession (Visser 1995; Twieg et al. 2007), and species interactions (Bruns 1995; Koide et al. 2005). Due to high belowground diversity of EM fungi at small spatial scales, interactions between EM species may be common, as they compete to colonize roots and acquire plant carbon. Furthermore, competition between EM species may play an important role in structuring EM communities (Wu et al. 1999; Koide et al. 2005; Kennedy and Bruns 2005; Kennedy et al. 2007).

The objectives of this research were to study the diversity of EM fungi in a tropical cloud forest and to identify EM fungi on root tips that produced mixed polymerase chain reaction (PCR) products. During molecular analyses of individual EM root tips from this tropical cloud forest, we found that samples frequently produced multiple PCR products or unusable DNA sequence data. To determine the identities of fungal taxa that co-occurred on EM root tips, we used cloning and gel extractions followed by DNA sequencing. To facilitate the identification of DNA sequences from root tips, we collected fruiting bodies from the site and developed an internal transcribed spacer (ITS)-DNA sequence database.

Materials and methods

Study site

The study was conducted in a montane cloud forest with a *Quercus* overstory that is located in Huizteco Park (Cerro Huizteco), Guerrero, Mexico, approximately 4.5 km north of Taxco (18°36′ N, 99°36′ W). Huizteco Park is managed for recreational use by the municipality of Taxco de Alarcón. People from the surrounding communities also gather edible mushrooms and edible insects during the appropriate seasons.

The forest canopy is dominated by various species of evergreen (*Quercus laurina* Humb. & Bonpl., *Q. castanea* Née) and deciduous (*Q. crassifolia* Humb. & Bonpl., *Q. magnoliifolia* Née, *Q. candicans* Née) oaks that are typically covered with abundant epiphytes (Valencia-Ávalos 1995). Elevation ranges between 2,200 and 2,550 m, and the climate is humid subtropical (Trewartha 1954). Annual mean temperature is 18°C, and total annual precipitation in this region varies between 1,200 and 1,500 mm with the majority of precipitation falling from June to October. Forest soils, classified as Haplic Phaeozem (Food and Agriculture Organization of the United Nations classification) or Hapludolls (US soil taxonomy), are characterized by a surface layer with high humus content and high base saturation in the upper meter of soil.

EM root tip sampling

We collected a total of 21 soil cores (12 cm depth, 6 cm diameter) from transects around two randomly selected, mature *Q. crassifolia* trees (diameter breast height=30, 37 cm) on 10 October and 2 November 2003. Eighteen of the 21 soil cores were collected from 2-m transects (three to six soil cores/transect) in the cardinal directions around tree #1. Three of the 21 soil cores were collected from 2-m transects in north and east directions around tree #2. On the November sampling, an additional ten soil cores contained no EM roots and were discarded. The lack of EM root tips in these November soil cores may be related to oak phenology, as the deciduous *Q. crassifolia* trees had lost some of their leaves by 2 November. Samples were stored at 4°C and processed within 14 days. We washed roots over a 300-μm sieve and randomly selected five individual EM root tips from each core for molecular analyses. EM roots were determined based on presence of fungal sheath, characteristic branching, color, and swelling. In this study, we defined an individual EM root tip as the terminal end of an EM root that appeared to be colonized by a single morphotype. The majority of these terminal root tips were approximately 1–2 mm long, although a few tips were up to 4 mm long. For EM morphotypes with branching roots, we cut terminal tips off before lateral branching. The terminal ends of individual root tips were cleaned vigorously with deionized water and then stored in cetyltrimethylammonium bromide (CTAB) buffer (Gardes and Bruns 1993) for DNA extraction.

Molecular identification and data analysis of EM fungi

Root tips were crushed with a micropestle, and DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) with several modifications. We used 400 μl of Buffer AP1, 200 μl of Buffer AP2 and preformed an extra wash with 500 μl of Buffer AW. The rDNA ITS region was amplified with the primer pair ITS1F/ITS4 (Gardes and Bruns 1993). For a select number of species, we obtained partial 28s rDNA using the primer pair ITS1F/LR3 (Hopple and Vilgalys 1994). PCR was preformed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA,

USA) thermocycler as follows: 94°C for 15 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, then 72°C for 10 min. Amplifications consisted of 50 μl reactions containing 1 U HotStarTaq DNA polymerase (Qiagen), 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 0.38 μM primers, and 2 μl of DNA template. Negative controls were run with all samples.

For DNA samples that did not amplify, we repeated PCR with varying concentrations of template. We successfully amplified 104 out of 105 root tips (99%). PCR products that resulted in a single band as visualized on 1.5% agarose gels were purified with QIAquick PCR purification kit (Qiagen) and sequenced with ITS1F and ITS4. Many samples, however, produced multiple amplicons when viewed on 1.5% agarose gels or resulted in mixed sequences. For these mixed samples, we either: (1) excised individual bands and purified them with QIAquick gel extraction kit (Qiagen) or (2) cloned PCR products using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Gel purification did not consistently produce usable sequence data, so we used cloning for the majority of mixed samples. Twelve plasmids from each sample were amplified with ITS1F and ITS4 following the protocols of Morris et al. (2008). Amplicons from approximately four to five clones were randomly chosen for sequencing, and in one case, we sequenced amplicons from up to seven clones. Sequencing was preformed by the UC Davis College of Biological Sciences DNA sequencing facility on an ABI 3730 DNA Analyzer (Applied Biosystems) using ITS1F and ITS4 or LR3. Sequences were edited using Sequencher v4.2 (Gene Codes, Ann Arbor, MI, USA) and examined by basic local alignment search tool (BLAST) searches against GenBank and compared to a sequence database of locally collected fruiting bodies.

Sequences with <97% sequence similarity were considered to be unique taxa (Izzo et al. 2005; Walker et al. 2005). EM root tip sequences that matched fruiting body sequences are indicated by collection numbers (MHM#). To compare the effectiveness of sampling various numbers of EM root tips per sample, we randomly selected 1, 2, 3, 4, or 5 root tips from each soil core and calculated species accumulation curves using EstimateS 8.0 (Colwell 2006).

Sampling of EM fruiting bodies

We opportunistically collected EM fruiting bodies from the study site during the rainy season in 2003 and 2004 (six sampling dates). Tissue from fruiting bodies was stored in CTAB buffer, and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). We amplified the ITS region using the primer pairs ITS1F/ITS4B for Basidiomycota and ITS1F/LR3 for Ascomycota. PCR and sequencing were preformed as described above except that we used high fidelity Platinum *Taq*DNA polymerase (Invitrogen) and reaction volumes of 30 μl. Voucher specimens of fruiting bodies were deposited at the Mexican National herbarium (MEXU) in Mexico City and the UC Berkeley herbarium (UC). Sequences were deposited in GenBank with accession numbers EU569230-EU569286.

Results

Based on DNA sequencing of 104 root tips from 21 soil cores, we detected multiple species of fungi on almost one third of the root tips (31%). A few of these multiple occurrences were the result of both EM and non-EM fungi (soil contaminants or rhizosphere associated fungi) on a root tip (Table 1). More than one quarter (26%) of the root tips contained two or more species of EM fungi. Dual occurrences involved many different taxa, but five root tips from three different soil cores contained the same two species: Clavulinaceae 1 and *Cortinarius* sp. 1 (Table 1). A total of 21 root tips harbored two EM species, whereas six roots tips harbored three EM taxa.

We identified a total of 44 EM taxa and nine non-EM fungi on oak root tips (Fig. 1, Table 2). High EM diversity was also apparent at a small spatial scale; we sampled five root tips/core and found an average of 3.9 EM taxa/core. The majority of EM species were Basidiomycota (34 taxa), but diverse Ascomycota (ten taxa) were also detected. The families Russulaceae (seven taxa), Cortinariaceae (three taxa), Inocybaceae (four taxa), and Thelephoraceae (eight taxa) were the most frequently detected groups. Only one core was dominated by a single species, *Russula* sp. MHM071 (Fig. 1). There was little overlap in species composition between soil cores despite the fact that some soil cores were just 20 cm apart. *Cortinarius* sp. 1 was the most frequent (24% of soil cores, 5/21) and abundant species (15% of root tips, 16/104) and was predominantly found on roots tips with multiple species. Species accumulation curves for sampling one to five root tips per soil core did not level off after sampling 21 soil cores; however, we observed similar species richness when sampling four or five root tips per soil core (Figure S1).

We obtained DNA sequences from 57 species of epigeous EM fruiting bodies from 12 different genera (*Amanita*, *Boletus*, *Boletellus*, *Clavulina*, *Cortinarius*, *Lactarius*, *Leccinum*, *Peziza*, *Ramaria*, *Russula*, *Strobilomyces*, and *Xerocomus*). Many taxa could not be identified to species (Table S1). DNA sequences from fruiting bodies of eight species matched DNA sequences found on root tips (Table 2).

Table 1 Root tips containing multiple fungal species from a tropical cloud forest in Guerrero, Mexico

Root tip #	Species 1	Species 2	Species 3	Species 4
Roots with more than one EM fungal species				
1	Thelephoraceae 1	Helotiales 2	Sordariomycete 1	Agaricales 1[a]
2	***Cenococcum geophilum***	Ascomycota 3	*Russula* sp. MHM097	Ascomycota 1[a]
3	***Cenococcum geophilum***	*Inocybe* sp. 2	**Helotiales 1**	
4	*Pseudotomentella* sp. 2	Thelephoraceae 6	Pezizales 1	
5	***Cortinarius* sp. 1**	**Clavulinaceae 1**	*Cortinarius* sp. 2	
6	*Pseudotomentella* sp. 1	*Tricholoma* sp. 1	*Craterellus* sp. 1	
7	***Cenococcum geophilum***	*Craterellus* sp. 1	Ascomycota 5[a]	
8	*Inocybe* sp. 4	*Lactarius* sp. MHM308	Ascomycota (cf. *Cercospora*)[a]	
9	***Cortinarius* sp. 1**	Cortinarius sp. MHM200		
10	***Cortinarius* sp. 1**	*Inocybe* sp. 3		
11	***Cortinarius* sp. 1**	*Tricholoma* sp. 3		
12	***Cortinarius* sp. 1**	*Inocybe* sp. 3		
13	***Cortinarius* sp. 1**	*Inocybe* sp. 3		
14	***Cortinarius* sp. 1**	*Russula* sp. 1		
15	***Cortinarius* sp. 1**	*Russula* sp. 1		
16	***Cortinarius* sp. 1**	**Clavulinaceae 1**		
17	***Cortinarius* sp. 1**	**Clavulinaceae 1**		
18	***Cortinarius* sp. 1**	**Clavulinaceae 1**		
19	***Cortinarius* sp. 1**	**Clavulinaceae 1**		
20	***Cenococcum geophilum***	Basidiomycota 1 (Sistotrema clade)		
21	***Cenococcum geophilum***	*Russula* sp. MHM071		
22	Helotiales 2	Thelephoraceae 1		
23	**Helotiales 1**	*Lactarius chrysorrheus* MHM143		
24	**Helotiales 1**	*Lactarius* sp. MHM308		
25	**Helotiales 1**	*Cortinarius* sp. 2		
26	*Pseudotomentella* sp. 1	*Tricholoma* sp. 2		
27	Helotiales 3	Thelephoraceae 5		
Roots with one EM fungus and one or two non-EM species				
28	Thelephoraceae 8	Ascomycota 2[a]	Basidiomycota 3[a]	
29	*Lactarius chrysorrheus* MHM143	Ascomycota 2[a]		
30	Pezizales 1	Ascomycota 6[a]		
31	Basidiomycota 2 (Hymenochaetoid clade)	*Cryptococcus* sp. 1[a]		
32	*Tuber* sp. 1	*Alternaria* sp. 1[a]		

Taxa in bold were frequently detected on root tips containing more than one EM species. A total of 104 tips were analyzed; 26% (27/104) harbored multiple EM species and 5% (5/104) contained one EM species and one or two non-EM species.
[a] Denotes non-EM taxon

Discussion

Using data from cloning and DNA sequencing, we found that multiple fungal species frequently occurred on individual root tips. Although several researchers have used morphotyping to document the presence of multiple species of EM fungi on single root tips (Mamoun and Olivier 1993; Wu et al. 1999, Kaldorf et al. 2004), the occurrence of dual symbionts is rarely reported in studies using molecular methods for fungal identification (Avis et al. 2003; Koide et al. 2005). Molecular methods have the potential to amplify DNA from nontarget sources, as hyphae and spores are abundant in soil and processing EM root tip involves disturbance of delicate structures (Avis et al. 2006). Although we detected multiple EM species on individual root tips, it is not certain that these species were colonizing the root tip, as they may have been the result of adhering spores or hyphae from surrounding soil or leaf litter. However, since previous studies have used morphological criteria to detect dual colonization (Wu et al. 1999, Kaldorf et al. 2004), we suspect that the high frequency of multiple EM taxa on roots indicates that multiple taxa colonize individual root tips. Regardless of whether multiple EM taxa are colonizing or simply present on individual root

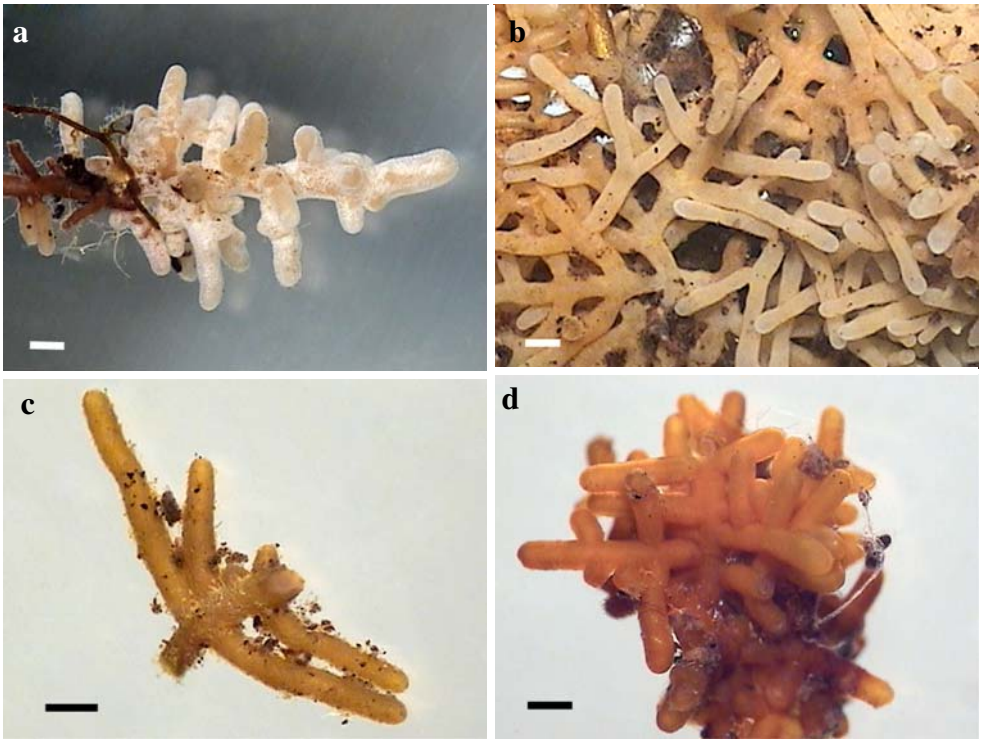

Fig. 1 EM roots from an oak-dominated tropical cloud forest in Guerrero, Mexico. **a** *Boletellus russellii*, **b** Clavulinaceae 1, **c** *Russula* sp. 2, **d** *Russula* sp. MHM071. Scale bar=1 mm

tips, the detection of multiple species at such small spatial scales has important implications for sampling design, molecular analyses, and future studies of fungal–fungal interactions.

The percentage of root tips containing multiple species of EM fungi in this study (26%) was higher than in previous reports. In a *Pinus resinosa* plantation, approximately 10% of EM root tips harbored more than one species of EM fungi (Koide et al. 2005), and 19% of EM root tips from aspen trees harbored multiple fungal taxa (Kaldorf et al. 2004). Root tips containing multiple EM fungi may be overlooked when using molecular methods because these samples result in mixed restriction fragment length polymorphism (RFLP) patterns or DNA sequences. For example, Izzo et al. (2005) found that many RFLP types in a mixed-conifer forest were due to the presence of multiple fungi. In an oak savanna, approximately 22% of samples produced more than one amplicon per mycorrhizal tip (Avis et al. 2003). Parrent et al. (2006) found that many *Pinus* roots contained endophytic and/or pathogenic ascomycetes that resulted in mixed PCR products. One approach to deal with mixed PCR samples is to use basidiomycete-specific primers (Parrent et al. 2006), but this method excludes Ascomycota that are often an important component of EM communities (Tedersoo et al. 2006; Smith et al. 2007). In addition, basidiomycete-specific primers would not work for samples containing multiple Basidiomycota taxa.

In this study, cloning was necessary to identify EM fungi on almost one quarter of the roots. Cloning also provided insights into the most frequent EM species, *Cortinarius* sp. 1, which frequently occurred on root tips with other fungi. Since cloning of individual tips becomes prohibitively expensive as sample size increases, a more efficient approach is to pool root tips from a single soil core for DNA extraction, PCR amplification, and cloning. This approach has been successfully used in a *Quercus*-dominated woodland where individual EM roots are small and difficult to amplify (Smith et al. 2007; Morris et al. 2008). Pooling of EM roots tips has also been used to characterize EM communities on *P. taeda* (Burke et al. 2005, 2006), *Lithocarpus densiflora* (Bergemann and Garbelotto 2006), and *Picea glauca* seedlings (Kernaghan et al. 2003).

The frequent detection of multiple species of fungi on oak root tips from this cloud forest suggests that there may be widespread competition between EM species and that replacement of one EM species by another may be common. Alternatively, it could indicate parasitism or facilitation between fungal species. Although competition among EM fungi has rarely been studied, recent work by Kennedy and Bruns (2005) suggests that competition, including priority effects, may have important effects on EM communities. Although parasitism and facilitation in EM fungi have yet to be studied experimentally, anecdotal and field-based evidence indicates that both interactions may be important. For example, Koide et al. (2005) observed

Table 2 EM and non-EM taxa detected on root tips in an oak-dominated tropical montane cloud forest in Guerrero, Mexico

Species	# cores	# root tips	Phylum[a]	# root tips with:		Accession #	Closest BLAST match[b]
				2 spp.	3 spp.		
EM fungi							
Cortinarius sp. 1	5	16	B	11	1	EU563508	*Cortinarius flexipes* (98% 568 bp)
Lactarius sp. MHM308	5	9	B	2	0	EU569255	*Lactarius alnicola* (95% 618 bp)
Clavulinaceae 1	4	8	B	4	1	EU563504	*Clavulina cinerea* (89% 1,068 bp)
Inocybe sp. 3	4	8	B	3	0	EU563500	*Inocybe* cf. *flocculosa* (90% 1,273 bp)
Lactarius chrysorrheus MHM143	4	9	B	1	0	EU569286	*Lactarius luculentus* (93% 790 bp)
Russula sp. MHM071	4	11	B	1	0	EU569264	*Russula aurata* (87% 712 bp)
Cenococcum geophilum	4	5	A	3	2	EU563491	*Cenococcum geophilum* (98% 971 bp)
Craterellus sp. 1	3	7	B	1	1	EU563479	*Cantharellus tubaeformis* (92% 598 bp)
Thelephoraceae 8	3	3	B	0	0	EU563490	*Tomentella* sp.(93% 794 bp)
Helotiales 1	3	5	A	3	1	EU563498	Uncultured Helotiales (96% 480 bp)
Tuber sp. 1	3	3	A	0	0	EU563484	*Tuber californicum* (90% 755 bp)
Cortinarius sp. 2	2	2	B	1	1	EU563509	*Cortinarius bulliardi* (93% 595 bp)
Inocybe sp. 1	2	2	B	0	0	EU563499	*Inocybe armeniaca* (91% 1,007 bp)
Russula sp. 1	2	2	B	2	0	EU563497	*Russula betularum* (96% 724 bp)
Russula sp. MHM087	2	4	B	0	0	EU569267	*Russula decolorans* (94% 700 bp)
Sebacinales 1	2	3	B	0	0	EU563487	*Sebacina incrustans* (98% 1,167 bp)
Pseudotomentella sp. 1	2	2	B	1	1	EU563503	*Pseudotomentella tristis* (96% 580 bp)
Thelephoraceae 6	2	2	B	0	1	EU563486	*Tomentella* sp. (94% 615 bp)
Basidiomycota 1 (Sistotrema clade)	1	1	B	1	0	EU563507	*Sistotrema alboluteum* (90% 1,141 bp)
Basidiomycota 2 (Hymenochaetoid clade)	1	1	B	0	0	EU563480	*Coltricia* cf. *oblectans* (90% 240 bp)
Xerocomus sp. MHM129	1	1	B	0	0	EU569235	*Xerocomus zelleri* (90% 847 bp)
Boletellus russellii MHM166	1	1	B	0	0	EU569284	*Boletellus mirabilis* (85% 636 bp)
Cortinarius sp. MHM200	1	3	B	1	0	EU569253	*Cortinarius cinnabarinus* (94% 702 bp)
Inocybe sp. 2	1	1	B	0	1	EU563505	*Inocybe rufuloides* (93% 650 bp)
Inocybe sp. 4	1	2	B	1	0	EU563510	*Inocybe* aff. *lanuginose* (86% 1,200 bp)
Russula sp. 2	1	2	B	0	0	EU563492	*Russula integriformis* (93% 690 bp)
Russula sp. MHM097	1	2	B	0	1	EU569272	*Russula persicina* (96% 450 bp)
Sebacinales 2	1	2	B	0	0	EU563483	*Sebacina* sp. (93% 1,167 bp)
Sebacinales 3	1	1	B	0	0	EU563494	*Sebacina* sp. (96% 556 bp)
Thelephoraceae 1	1	2	B	1	1	EU563502	*Tomentella* sp. (90% 805 bp)
Pseudotomentella sp. 2	1	1	B	0	1	EU563488	*Pseudotomentella tristis* (89% 699 bp)
Thelephoraceae 4	1	1	B	0	0	EU563496	*Tomentella* sp. (96% 507 bp)
Thelephoraceae 5	1	1	B	1	0	EU563493	*Tomentella* sp. (94% 627 bp)
Thelephoraceae 7	1	1	B	0	0	EU563485	*Tomentella* sp. (92% 792 bp)
Tricholoma sp. 1	1	1	B	0	1	EU563477	*Tricholoma fulvum* (95% 637 bp)
Tricholoma sp. 2	1	1	B	1	0	EU563482	*Tricholoma ustale* (95% 806 bp)
Tricholoma sp. 3	1	1	B	1	0	EU563478	*Tricholoma muricatum* (96% 664 bp)
Ascomycota 3	1	1	A	0	1	EU563489	Ericoid mycorrhizal sp. (88% 513 bp)
Helotiales 2	1	2	A	1	1	EU563501	*Hyphodiscus hymeniophilus* (93% 1,088 bp)
Helotiales 3	1	1	A	1	0	EU563495	cf. Hymenoscyphus sp. (92% 1,078 bp)
Pezizaceae 1	1	1	A	0	0	EU563481	*Pachyphloeus* sp.(90% 986 bp)
Pezizales 1	1	2	A	0	1	EU563475	*Helvella compressa* (87% 294 bp)
Pyronemataceae 1	1	1	A	0	0	EU563476	*Cheilymenia stercorea* (91% 530 bp)
Sordariomycete 1[c]	1	1	A	0	1	EU563511	Uncultured sordariomycete clone (96% 520 bp)
Non-EM fungi							
Basidiomycota 3	1	1	B	0	0	EU624338	*Hemimycena gracilis* (83% 472 bp)
Agaricales 1	1	1	B	0	1	EU563506	*Hemimycena gracilis* (83% 605 bp)
Alternaria sp. 1	1	1	A	0	0	EU624337	*Alternaria tenuissima* (99% 539 bp)
Ascomycota (cf. *Cercospora*)	1	1	A	1	0	EU624336	*Cercospora sorghi f. maydis* (100% 500 bp)

Table 2 (continued)

Species	# cores	# root tips	Phylum[a]	# root tips with:		Accession #	Closest BLAST match[b]
				2 spp.	3 spp.		
Ascomycota 1	1	1	A	0	1	EU624335	*Cladophialophora chaetospira* (98% 1,131 bp)
Ascomycota 2	2	2	A	0	0	EU624334	*Alternaria japonica* (89% 926 bp)
Ascomycota 5	1	1	A	1	0	EU624333	*Rhinocladiella* sp. (83% 590 bp)
Ascomycota 6[d]	1	2	A	0	0	EU624332	*Geoglossum nigritum* (90% 440 bp)
Cryptococcus sp. 1	1	1	A	1	0	EU624331	*Cryptococcus oeirensis* (99% 609 bp)

Taxa with DNA sequences that matched DNA sequences from fruiting bodies collected at the study site are indicated by MHM#. A total of 21 soil cores were collected, and five root tips per core were subject to DNA sequencing.

[a] Ascomycota (A); Basidiomycota (B)

[b] Closest named BLAST species were used and the percentage similarity, and total number of base pairs aligned is indicated in parentheses.

[c] Taxa within Sordariomycetes have been found on EM root tips of *Vateriopsis seychellarum* (Tedersoo et al. 2007) and *L. densiflorus* (Bergemann and Garbelotto 2006).

[d] The closest named BLAST match for this species was *Geoglossum nigritum* (DQ491490), and some members of the Geoglossaceae are suggested as being EM by Agerer (2006), but this has yet to be confirmed.

positive correlations between several pairs of EM species. Mamoun and Olivier (1993) noted putative facilitation between *Tuber brumale* and *T. melanosporum* under conditions of high soil moisture. *Boletopsis leucomelaena* forms haustoria on an unknown EM fungus on root tips (Agerer 1992), and *Xerocomus parasiticus* is known to parasitize *Scleroderma citrinum* (Raidl 1997). Similarly, members of the Gomphidiaceae regularly parasitize the tuberculate ectomycorrhizas formed by species of *Suillus* and *Rhizopogon* (Agerer 1990; Olsson et al. 2000).

Similar to temperate *Quercus* forests, the EM community in this tropical montane cloud forest was dominated by Russulaceae, Cortinariaceae, Inocybaceae, and Thelephoraceae (Avis et al. 2003, 2008; Walker et al. 2005; Smith et al. 2007). Sebacinales and Pezizales, notable components of temperate *Quercus* EM communities, were represented in this tropical cloud forest as well. One frequent species found in this study, *Lactarius chrysorheus,* was also identified as a frequent symbiont on *Q. rubra* and *Q. prinus* in mixed forests in the southern Appalachians (Walker et al. 2005).

Our study is the first to use DNA sequencing to describe belowground EM communities in a tropical montane cloud forest. Fine-scale sampling showed high diversity at multiple scales from tree (meters) to soil core (centimeters) to root tip (millimeters). There was little overlap in species composition between soil cores, and more than one quarter (26%) of root tips contained more than one species of EM fungi. This study provides insights into field sampling methodology and DNA sequencing strategies for future studies and indicates the need for further studies of fungal–fungal interactions.

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