Hypogeous Ectomycorrhizal Fungal Species on Roots and in Small Mammal Diet in a Mixed-Conifer Forest

Antonio D. Izzo, Marc Meyer, James M. Trappe, Malcolm North, and Thomas D. Bruns

Abstract: The purpose of this study was to estimate the portion of an ectomycorrhizal (ECM) fungi root community with a hypogeous fruiting habit. We used molecular methods (DNA sequence analysis of the internally transcribed spacer [ITS] region of rDNA) to compare three viewpoints: ECM fungi on the roots in a southern Sierra Nevada *Abies*-dominated old-growth forest, fungi in scat samples collected from small mammals in the same forest, and hypogeous sporocarps found throughout the Sierra Nevada. We found that hypogeous taxa accounted for a minimum of 21% of the species and 25–40% of the dry root biomass of all samples. This estimate is two to three times greater than estimates from previous studies. This difference may be due to methodological advantages of this study, but may also be related to conditions in dry forests typical of western North America where prolonged drought may favor this form of fruiting. Although molecular analysis of scat samples did not add to our view of the ECM roots, we readily isolated sequences from *Rhizopogon* species. From these results we inferred that two species, *R. occidentalis* and *R. olivaceotinctus*, are represented primarily in the spore bank and may be dependent on substantial disturbance to become abundant on roots. FOR. SCI. 51(3): 243–254.

Key Words: Mycophagy, community structure, fecal pellet.

ECTOMYCORRHIZAL (ECM) FUNGI with a hypogeous (i.e., belowground) fruiting habit are intricately connected with the food webs and dynamics of a number of forest systems (Johnson 1996, Claridge 2002). These fungi can make up considerable portions of the diet of small mammals and, in some cases, are the primary food source (Maser et al. 1978, Johnson 1996). From a fungal perspective, the mammals act as primary dispersal agents for the spores and may play important roles in maintenance of ECM diversity in the forests because of variations in ranges and dietary preferences exhibited by the mammals (Maser et al. 1978, Pyare and Longland 2002). Hypogeous fruiting is hypothesized to be an advantage in situations where moisture is seasonally or otherwise limited, or there is a physical barrier to sporocarp emergence (Thiers 1984, Trappe 1988). These hypotheses are supported by the high diversity of hypogeous taxa seen in the dry forests of western North America and Australia and by the strong interdependence of small mammals with these fungal taxa (Maser et al. 1978, Johnson 1996). Estimates based on the fruiting record show that fungi forming hypogeous sporocarps are often the dominant fruiters in such systems (Fogel 1981, North et al. 1997). Several studies, however, have shown that sporocarp composition and abundance are not representative of dominant species of ECM root composition (Dahlberg 2001, Horton and Bruns 2001). Thus it remains unclear whether the abundant diversity and fruiting of hypogeous taxa also translate into an increased colonization of the roots by these species.

Among the numerous recent studies of ectomycorrhizal root communities, relatively few hypogeous taxa are typically reported (e.g., Horton and Bruns 1998, Jonsson et al. 1999, Stendell et al. 1999, Taylor and Bruns 1999). This may be because species-level identifications are often not reported, and that hypogeous fungi are not a distinct evolutionary unit that can be identified by phylogenetic analysis. Instead, hypogeous fungi are derived forms that have evolved multiple times (Thiers 1984, Hibbett et al. 1997). Hypogeous taxa are often very closely related to epigeous taxa (Bruns et al. 1989, Miller et al. 2001) and are therefore difficult to separate by ECM morphology. Molecular methods have partially alleviated this problem by matching restriction fragment length polymorphism (RFLP) patterns of sporocarps with those of ECM roots. However, most molecular ECM studies are noticeably deficient in locally collected hypogeous sporocarps for comparison. Identifications are still possible by using sequence databases such as Genbank, but these databases are currently undersampled for most hypogeous fungal taxa.

The overall goal of our study was to make a directed

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effort to assess what portion of the ECM roots in an old-growth forest is composed of hypogeous fungi. We used two approaches to determine whether fungi identified on roots in a previous study (Izzo et al. 2005) were likely to form hypogeous sporocarps. In the first approach, we obtained DNA sequence data from the sporocarps of an array of hypogeous fungi collected across the Sierra Nevada (California, USA) to compare to those obtained previously from the roots. Our second approach took advantage of the fact that hypogeous fungi are an important food source for small mammals in this forest (Meyer 2003). These mammals are therefore likely to be more effective at sampling the true diversity of sporocarps than humans are. We obtained fungal DNA sequence data directly from small mammal scat that had been collected across the same forest (Meyer 2003) and in the same years that the ECM roots were studied. In addition to providing an estimate of how prominent hypogeous fruiters are on the roots, the ability to interconnect these three life history phases of hypogeous taxa allows greater insight into the ecology of ECM fungi and their dispersers in dry forests, where the strength of this coupling may be more pronounced.

Methods

Site Description

This study was conducted at the Teakettle Experimental Forest, Sierra National Forest (SNF), CA (36°58′N; 119°2′W) on the southwestern slope of the Sierra Nevada. This forest has been described in greater detail by North et al. (2002). The elevation at this site is approximately 2,100 m. Of the ectomycorrhizal conifers found throughout the study range, *Abies concolor* (white fir) and *Abies magnifica* (red fir) are dominant, and *Pinus jeffreyi* (Jeffrey pine) and *Pinus lambertiana* (sugar pine) are very common. *Calocedrus decurrens* (incense cedar) is not ectomycorrhizal, but is prominent in the overstory as well. Annual precipitation is 125 cm, most of which comes in the form of snow between Nov. and May. The average monthly rainfall between May and Nov., as measured 1.2 km from the Teakettle Experimental Forest, is less than 2 cm. Air temperatures average 15.5°C in the summer and 0.7°C in the winter.

ECM Root Sampling Overview

The sampling of the ECM root community was performed in a previous study that examined changes in community structure across time and at a range of spatial scales, and has been described in more detail elsewhere (Izzo et al. 2005). In brief, soil samples were collected across nine plots located in stands dominated by *Abies* sp. These plots were distributed across one of the 4-ha compartments (UN3) established as part of the ongoing studies in this forest (North et al. 2002). Four small (2.8 cm^2) cores were taken per plot across a 20-cm mini-transect. Each core sampled through the O horizon and 20 cm into the mineral soil. This sampling was repeated each year from 1999 to 2002, with subsequent transects being parallel 5 cm away from the previous year. The 1999 sampling was performed in late July following the typical drought period experienced in this region. In contrast, the 2001 and 2002 samplings were conducted in late May to early June following the last snowmelt. Samples from 2000 were not included in the study. The composition of fungal taxa on the roots was characterized by a combination of morphotyping under a dissecting scope and DNA analysis of the internally transcribed spacer (ITS) region of the rDNA gene region (Izzo et al. in press). The dry weight biomass of the roots was used as the abundance measure within each core. BLAST searches (Altschul et al. 1997) of the Genbank database were used to estimate the taxonomic affinities of the fungi, and resulted in the identification of eight species that likely formed hypogeous sporocarps.

Collection and Molecular Analysis of Small Mammal Scat

To increase the DNA database for local hypogeous fungi, we recovered fungal ITS sequences from rodent scat samples. Scat samples were obtained from a separate study in which they had been collected from *Glaucomys sabrinus* (northern flying squirrel) and *Tamias speciosus* (lodgepole chipmunk) in the Teakettle Experimental Forest across three seasons and their spore content microscopically characterized to genus (Meyer 2003). A subset (roughly 10%) of the samples from that study was selected for molecular analysis. Samples were chosen to maximize the number of unique recoverable sequences. This was done by choosing samples that contained different genera as determined by spore morphology, by sampling greater numbers of those that contained the most common genera (e.g., *Rhizopogon*), and by selecting samples from different years (14 from 1999, 17 from 2000, 27 from 2001), seasons (33 in spring, 25 in fall), mammal (28 from *G. sabrinus* and 30 from *T. speciosus*), and locations (across 15 compartments). Whenever it was possible, samples that contained predominantly one type were selected over those that contained complex mixtures to facilitate direct sequence recovery. Scat samples from Meyer (2003) had previously been resuspended in 1,000 μl dH_2O for microscopic analysis and then stored frozen at −80°C. Approximately 100 μl of each resuspended scat sample was crushed with a 35-mm glass bead in a mini bead-beater (Biospec Products, Bartlesville, OK) and added to 900 μl CTAB buffer (2% CTAB, 0.1 M Tris pH 8.0, 1.4 M NaCl, 0.02 M EDTA). Following a 60-min incubation at 65°C, samples were vortexed with 600 μl chloroform/isoamyl alcohol (24:1) and centrifuged (13,000 *g* for 5 min). The resulting upper layer was further cleaned with Qiagen DNeasy genomic isolation kits (Qiagen Inc., Valencia, CA). Samples were resuspended in 100 μl of the AE buffer supplied by the manufacturer and stored at −20°C. Fungal ITS DNA was amplified from the scat using the ITS1F, ITS4, and ITS4B primers (White et al. 1990, Gardes and Bruns 1993) with varying PCR conditions (96° denature, 51–53°C annealing temp, 72°C extension for 35 cycles or touchdown PCR by lowering the annealing from 67°C to

51°C in 1°C increments). Sequencing reactions were performed using BigDye chemistry at manufacturer's recommended conditions (Applied Biosystems, Foster City, CA). Sequenced DNA fragment separation was performed on an ABI3100 Genetic Analyser and was analyzed with Sequence Analysis 3.4.1 software (Applied Biosystems) to obtain the final DNA sequence.

Collection and Molecular Analysis of Hypogeous Fruiting Bodies

To further increase the DNA database of hypogeous fungal samples likely to be found at Teakettle Experimental Forest, we took advantage of hypogeous sporocarps collected in an intensive sampling effort (Table 1) (North 2002) and herbarium specimens collected primarily in the Sierra National Forest. The North study (2002) involved a total inventory of the hypogeous sporocarps from a 1-ha plot at one time point in mixed conifer forest approximately 7 km from Teakettle Experimental Forest. To increase the chances of detecting maximum species richness, we biased molecular sampling toward genera that were unique to or underrepresented in Genbank, and toward genera whose taxonomy is problematic and therefore might be expected to reveal greater DNA sequence diversity. Nonmycorrhizal fungal samples that are known to be part of the small mammal diet were included to allow us to identify them in the scat samples as well. Molecular analysis of the sporocarps was performed in the same manner as with the scat samples except that approximately 3 mm^3 of tissue was used for nucleic acid isolation.

Sequence Analysis and Comparison

The ITS1 region from all ECM root taxa sequences were compared against Genbank sequences, sequences derived from the sporocarps, and sequences obtained from the small mammal scat using both web-based and standalone BLAST search analysis tools. The standalone BLAST programs (National Center for Biotechnology Information) allow for BLAST searches against unpublished sequences maintained in a lab database. Root samples were considered to be from hypogeous taxa if the best match was a strong match to a hypogeous sporocarp. When sequences matched equally to a hypogeous and epigeous sporocarp, the sequence was considered to be unknown relative to its likely fruiting habit. However, if one of the samples was collected locally, then it was considered to be the better match.

Calculations of Relative Frequency, Abundance, and Species Number of Hypogeous Taxa

Relative frequency of the hypogeous taxa was calculated by dividing the total number of root sequence types that were identified as hypogeous by the total number of identified mycorrhizal sequence types. Relative abundance was calculated by dividing the total amount of hypogeous taxa root biomass by total root biomass of ECM roots that successfully amplified. It was assumed that taxa that did not amplify would not be biased toward or against being hypogeous taxa. Inasmuch as ECM proliferation and individual fungal taxa are known to have patchy distributions, we used bootstrapping techniques to refine our estimates. In the bootstrapping analysis, plots were summed and randomly chosen with substitution to obtain a more general estimate of the hypogeous contribution to the root community. To estimate the number of *Rhizopogon* species present in the small mammal diet, a species accumulation curve was constructed using EstimateS v5.0 (Colwell, R. 1997. EstimateS: Statistical estimation of species richness and shared species from samples. Version 5. User's Guide and application published at http://viceroy.eeb.ucon.edu/estimates). For this analysis, scat samples from which *Rhizopogon* sequences had been obtained were treated as the sample unit ($n = 18$).

Results

PCR and Direct Sequencing from Small Mammal Scat Samples

We were able to amplify fungal DNA from 55 of the 58 scat samples. A total of 15 unique sequence types was identified (Table 2). The fungal-specific primer pairs ITS1F and ITS4 yielded usable DNA sequences from 28 samples. However, DNA sequence comparison revealed that only two sequences obtained with this primer pair were clearly hypogeous taxa (*Geopora* and *Rhizopogon*), and that the rest were an ascomycete of unclear taxonomic affinity assumed to be a coprophilic or gut fungus. Use of the basidiomycete-specific primer pair ITS1F and ITS4B was successful with 43 samples, 28 of which yielded more ectomycorrhizal sequences for comparison and one *Cryptococcus* sp. sequence as well. In total, three of the scat sequences were matches to sequences obtained from the ECM roots: two *Rhizopogon* sp. and one *Geopora*.

Sequencing of Sporocarp Samples

Single pass sequence data for the ITS1 region were obtained from 73 sporocarp samples representing 29 genera. Of these genera, 14 were hypogeous taxa previously not represented by ITS sequence in Genbank at the time of this analysis (Table 3).

ECM Root Community and Occurrence of Hypogeous Taxa

PCR amplification of the ITS region was successful on 65% of the root samples collectively representing approximately 80% of the biomass for each year (Izzo et al. in press). By initial BLAST searches alone, hypogeous species made up 8% of the total species and 26% of the root biomass. There were three instances where an ECM root sequence equally matched an epigeous and hypogeous species within the Russulaceae (Table 4). The hypogeous matches for two of these—AY702793 and AY702797—matched local collections of *Gymnomyces*, and so these were considered hypogeous,

Table 1. Fruiting bodies used in sequence analysis

Species	Collection location	Voucher	Accession no.
Alpova trappei	Sierra Nevada, CA	SNF105	AY558738
Alpova sp.	Teakettle forest, CA		AY558739
Arcangeliella crassa	Sierra Nevada, CA	SNF203	AY558740
Balsamia cf. magnata	Sierra Nevada, CA	SNF222	AY558741
Balsamia nigrens	Ross Crossing, CA†	JMT19926	AY558742
Barssia oregonensis	Oregon	JMT27997	AY558743
*Bryoria fremontii**	Teakettle forest, CA		AY558744
*Endoptychum depressum**	Sierra Nevada, CA	SNF294	AY558745
Endoptychum sp.*	Sierra Nevada, CA	SNF190	AY558746
Gastroboletus sp.	Sierra Nevada, CA	SNF181	AY558747
Gastroboletus sp.	Sierra Nevada, CA	SNF309	AY558748
Gastroboletus vividus	Sierra Nevada, CA	SNF167	AY558749
Gautieria caudata	Ross Crossing, CA†	JMT19937	AY558750
Gautieria sp.	Teakettle forest, CA		AY558751
Gautieria sp.	Teakettle forest, CA		AY558752
Genebea cerebriformis	Ross Crossing, CA†	JMT19067	AY558753
Geopora cooperi	Sierra Nevada, CA	SNF96	AY558754
Geopora sp.	Pt. Reyes, CA		AY558755
Geopora sp.	Teakettle forest, CA	LG1193	AY558756
Gymnomyces alveolatus	Sierra Nevada, CA	SNF35	AY558757
Gymnomyces fallax	Ross Crossing, CA†	JMT19916	AY558758
Gymnomyces sp.	Sierra Nevada, CA	SNF269	AY558759
Gymnomyces sp. nov.	Ross Crossing, CA†	JMT19941	AY558760
Gymnomyces abietis	Sierra Nevada, CA	SNF168	AY558761
Hydnoplicata sp. nov.	Ross Crossing, CA†	JMT19945	AY558762
Hydnotrya cerebriformis	Ross Crossing, CA†		AY558763
Hydnotrya cerebriformis	Sierra Nevada, CA	SNF86	AY558764
Hydnotrya cerebriformis	Ross Crossing, CA†	JMT19066	AY558765
Hydnotrya cerebriformis	Sierra Nevada, CA	SNF97	AY558766
Hydnotrya cf variiformis	Sierra Nevada, CA	SNF234	AY558767
Hydnotrya sp.	Sierra Nevada, CA	SNF160	AY558768
Hydnotrya sp.	Sierra Nevada, CA	SNF82	AY558769
Hydnotrya variiformis	Teakettle forest, CA		AY558770
Hydnotryopsis gautierioides sp. nov.	Ross Crossing, CA†	JMT19933	AY558771
Hydnotryopsis setchellii	Sierra Nevada, CA	SNF289	AY558772
Hydnotryopsis setchellii	Ross Crossing, CA†	JMT19932	AY558773
Hymenogaster gilkeyae	Ross Crossing, CA†	JMT19948	AY558774
Hymenogaster subalpinus	Oregon	JMT27992	AY558775
Hysterangium crassirhachis	Ross Crossing, CA†		AY558776
Hysterangium fallax Castellano & Trappe, nom. prov.	Sierra Nevada, CA	SNF233	AY558777
Hysterangium sp.	Ross Crossing, CA†	JMT19073	AY558778
Leucogaster microsporus nom prov.	Ross Crossing, CA†	JMT19949	AY558779
Leucogaster rubescens	Sierra Nevada, CA	SNF171	AY558780
Leucogaster rubescens	Ross Crossing, CA†		AY558781
Leucogaster sp.	Teakettle forest, CA		AY558782
Leucophleps magnata	Ross Crossing, CA†		AY558783
Leucophleps sp.	Teakettle forest, CA		AY558784
Leucophleps spinispora	Sierra Nevada, CA	SNF226	AY558785
Leucophleps spinispora	Sierra Nevada, CA	SNF91	AY558786
Macowanites sp.	Santa Cruz Islands, CA	LG1052	AY558787
Martellia sp.	Ross Crossing, CA†		AY558788
Melanogaster sp.	Sierra Nevada, CA	SNF162	AY558789
Melanogaster tuberiformis	Teakettle forest, CA		AY558790
Mycolevis siccigleba	Sierra Nevada, CA	SNF206	AY558791
Mycolevis sp.	Teakettle forest, CA		AY558792
*Nivatogastrium nubigenum**	Sierra Nevada, CA	SNF338	AY558793
Pyrenogaster atrogleba	Sierra Nevada, CA	SNF83	AY558794
Pyrenogaster atrogleba	Sierra Nevada, CA	SNF152	AY558795
Radiigera fuscogleba	Sierra Nevada, CA	SNF305	AY558796
Radiigera sp.	Sierra Nevada, CA	SNF297	AY558797
Radiigera sp.	Ross Crossing, CA†		AY558798
Rhizopogon pedicellus Smith	Ross Crossing, CA†	JMT19931	AY558799
Rhizopogon rubescens	Ross Crossing, CA†		AY558800
Rhizopogon sp. (#1)	Teakettle forest, CA		AY558801

Continued

Table 1. (continued).

Species	Collection location	Voucher	Accession no.
Rhizopogon sp. (#2)	Teakettle forest, CA		AY558802
Rhizopogon sp. nov.	Ross Crossing, CA†	JMT19920	AY558803
Rhizopogon subcaerulescens	Teakettle forest, CA		AY558804
Thaxterogaster pingue	Sierra Nevada, CA	SNF137	AY558805
Thaxterogaster sp. nov.	Sierra Nevada, CA	SNF121	AY558806
Tuber californicum	Ross Crossing, CA†	JMT19070	AY558807
Tuber gardneri	Ross Crossing, CA†	JMT19947	AY558808
Tuber maculatum	Sierra Nevada, CA	SNF54	AY558809
Zelleromyces sp. nov.	Ross Crossing, CA†	JMT19934	AY558810

* Not mycorrhizal but potentially part of mammal scat content.
† Sierra National Forest collections from North (2002).

whereas AY702792 was only matched to Genbank and therefore was considered unknown. Based on sequence matches either to fruiting bodies or sequences available on Genbank, 21% of the species representing 39% of the total ECM root biomass were hypogeous taxa. *Rhizopogon* had the largest number of species in the North (2002) study and was also abundant on ECM roots (Table 3). *Melanogaster* and *Tuber* were also speciose in the North study but were hardly detected on the ECM roots. One occurrence of AY702790, a *Rhizopogon* species, within a single plot in 2001, accounted for 16% of the total root biomass in the 3 years. Bootstrapping of plot values provided a more conservative estimate that hypogeous taxa make up 25% of the overall biomass. The relative abundance and relative biomass of hypogeous species were similar across years and seasons, only varying 9% and 15%, respectively (Table 5). Hypogeous relative abundance varied across the nine plots (range 8–43%, mean 17% ± 12% S.D.).

Discussion

Using molecular techniques, we have found that hypogeous fungi make up a much larger portion of both the ECM root community species and the ECM root biomass than has been reported in any other mature forest to date. Compared to previous studies, we can attribute a three to four times greater percentage of species, and a two to three times greater percentage of root biomass to the ECM fungi that makes hypogeous sporocarps (Table 6). To some extent, their prominence on the roots is not surprising because, in the coniferous forests of the western United States, hypogeous fungi produce large quantities of sporocarps and are clearly important in the small mammal diet and, therefore, the carbon flow in these forests (i.e., Maser et al. 1978, North et al. 1997). However, sporocarp-based estimates generally do not account for ECM taxa that are asexual or

Table 2. Results of BLAST analysis of fungal ITS1 sequences obtained from scat samples of *Glaucomys sabrinus* and *Tamias speciosus*

bp	Best match (accession number) {frequency in scat}	% Similarity/bp overlap	*E*-value[2]	Accession no.
247	*Alpova trappei* (AF074920) {1}	97%/275	1.00*E*–135	AY558727
230	*Cryptococcus nyarrowii* (AF400697) {1}[1]	99%/230	1.00*E*–118	AY558726
234	*Endoptychum* sp. SNF190 {1}	97%/234	1.00*E*–118	AY558723
220	*Geopora cooperi* SNF96 {1}[3]	100%/184	2.00*E*–98	AY558737
173	*Melanogaster tuber* {2}	90%/159	3.00*E*–22	AY558728
197	*Ramaria apiculata* (AJ408385) {1}	92%/69	6.00*E*–18	AY558725
273	*Rhizopogon arctostaphyli* isolate JPT5705 holotype (AF377167) {2}	100%/223	1.00*E*–121	AY558729
224	*Rhizopogon subcaerulescens*[3]	99%/224	1.00*E*–123	AY558730
223	*Rhizopogon vulgaris* (AF062931), *Rhizopogon* sp. TK1616 {2}	100%/223	1.00*E*–121	AY558731
223	*Rhizopogon rubescens* (AF158018) {6}	99%/223	1.00*E*–120	AY558732
195	*Rhizopogon subsalmonius* strain BCC-MPM 1,653 (AJ515424){3}[1]	97%/128	6.00*E*–58	AY558733
294	*Rhizopogon* sp. (#1) {1}[3]	99%/294	1.00*E*–160	AY558734
240	*Pholiota spumosa* (AF345654) {4}	89%/226	2.00*E*–55	AY558735
220	*Pholiota spumosa* (AF345654) {1}	87%/79, 89%/57	2.00*E*–12	AY558736
163	unidentified ascomycota sp. RH 10-1 (AJ301722) {26}	98%/163	6.00*E*–79	AY558724

Fifteen unique sequences were obtained from a total of 58 scat samples. The sequences matching *Cryptococcus* and the unidentified ascomycetous fungus are assumed to be nonmycorrhizal occupants of the mammal gut.

[1] Only the ITS2 region was used for identification.
[2] The number of different alignents with scores equivalent to or better than observed that are expected to occur in BLAST search by chance (Altschul et al., 1997).
[3] Sequence matched those found on ECM roots in Teakettle Experimental Forest.

Table 3. Composition of hypogeous genera found or included in this study

	ECM root taxa		Scat taxa		Sporocarps	ITS sequences	
Genus	No. sp.	Rel biomass All roots	Sequences No. sp.	Spore type Frequency[2]	No. sp.[3] in 1 ha	Genbank[4]	Added this study
Alpova	0	0.00	1	0.00	1	2	2
Arcangeliella	1	0.03	0	0.00	1	2	1
Balsamia	0	0.00	0	0.00	2	0	2
Barssia	0	0.00	0	0.00	0	0	1
Choiromyces	1	0.00	0	0.00	0	7	0
Endogone	0	0.00	0	0.01	3	3	0
Gastroboletus	0	0.00	0	0.00	0	0	3
Gautieria	3	0.04	0	0.19	6	53	3
Genebea	1	0.00	0	0.07	1	0	1
Genea	0	0.00	0	0.00	1	0	0
Geopora	1	0.00	1	0.00	1	3	3
Gymnomyces	3	0.03	0	0.00	3–4	49	5
Hydnoplicata	0	0.00	0	0.00	1	0	1
Hydnotrya	1	0.01	0	0.03	1	0	8
Hydnotryopsis	0	0.00	0	0.00	2	0	3
Hymenogaster	0	0.00	0	0.00	4–5	13	2
Hysterangium	0	0.00	1	0.03	4	0	3
Leucogaster	3	0.05	0	0.06	2	0	4
Leucophleps	2	0.03	0	0.04	1	0	4
Macowanites	0	0.00	0	0.00	1	5	1
Martellia	1	0.00	0	0.00	6	2	1
Melanogaster	0	0.00	1	0.12	1	28	2
Mycolevis	0	0.00	0	0.00	1	0	2
Nivatogastrium	0	0.00	1	0.00	0	0	1
Pachyphloeus	0	0.00	0	0.01	0	0	0
Pyrenogaster	0	0.00	0	0.00	0	0	2
Radiigera	0	0.00	0	0.06	1	0	3
Rhizopogon	3	0.19	6	0.38	10–11	285	6
Sclerogaster	0	0.00	0	0.01	0	0	0
Thaxterogaster	0	0.00	0	0.00	0	13	2
Trappea	0	0.00	0	0.07	1	0	0
Tuber	1	0.01	0	0.03	8	283	3
Zelleromyces	0	0.00	0	0.00	1	6	1
Sum of hypogeous	21	0.39					
All epigeous[1]	20	0.16					
All nonfruiting	2	0.13					
All resupinate	17	0.15					
All unknown habit	43	0.17	3				
Total	100	1.00	14		64–67		

[1] Includes *Wilcoxina* sp. (3 sequence types with 0.08 relative abundance).
[2] Relative frequency of genus relative to other named taxa in scat samples analyzed for this study.
[3] Minimum number of species named by sporocarp morphology collected in North (2002) study.
[4] Based on total number ITS sequences within a genus in a general Genbank search at time of submission. Some species may be represented more than once.

that produce inconspicuous sporocarps such as the hypogeous or resupinate fungi. Therefore, it has not been clear until now that the hypogeous fungi are strong components of the ECM community as a whole.

Our estimates are higher than those reported in other root-based studies because of unique aspects of our methodological approaches, of the forest type in our study, or some combination of both. Both morphological and molecular methodologies of past studies have been limited in the ability to distinguish many root fungi as being either hypogeous or epigeous, whereas, in this study, sequence analysis coupled with the growing sequence database of hypogeous sporocarps allowed us a more refined estimate. For example, the identification of root taxa resulting from the addition of previously unsequenced genera allowed us to increase our estimates of both species richness and root biomass by almost 10% (Table 3). These molecular database limitations, however, cannot account for the absence of taxa such as *Rhizopogon* that were already well represented in the databases, and accounted for the most root biomass in our study. If *Rhizopogon* species were prominent in other studies as well, then they would likely have been identified, especially given the molecular biases toward the boletoid taxa inherent with the original design of the primers common to these studies (Gardes and Bruns 1993). The long, dry summer conditions in this forest type may therefore favor the hypogeous fruiting habit. Although we did not quantify epigeous sporocarps, our observations suggest that spring fruiting is sparse outside of a narrow window, and that fruiting bodies are quickly exposed to the hot dry air

Table 4. BLAST results of ITS1 of ECM mature root sequence types matching hypogeous taxa

	bp	Best match (accession number if not from this study)	% Similarity/bp overlap	*E* value
ECM sequence type				
AY702740	206	*Genebea ceribriformis* JMT19067	95%/167	2.0*E*–72
AY702762	206	*Leucogaster* sp. from Teakettle Forest	92%/190	1.0*E*–72
AY702763	201	*Leucophleps* sp. from Teakettle Forest	96%/178	1.0*E*–83
AY702764	177	*Leucogaster rubescens* from Ross Crossing	97%/150	5.0*E*–60
AY702765	194	*Leucophleps spinispora* SNF91	98%/183	2.0*E*–92
AY702767	171	*Leucogaster rubescens* SNF171	91%/135	1.0*E*–45
AY702769	212	*Choiromyces alveolatus* (AF501258)	91%/212	8.0*E*–70
AY702775	650	*Gautieria* sp. Dinkey2230CA (AF377085)	99%/652	0
AY702776	656	*Gautieria monticola* isolate SNF346CA (AF377101)	99%/656	0
AY702777	196	*Gautieria monticola* isolate ORHF20 (AF377094)	98%/189	2.0*E*–94
AY702782	230	*Arcangeliella crassa* SNF203	98%/230	1.0*E*–118
AY702783	284	*Hydnotrya variiformis* from Teakettle Forest	91%/284	1.0*E*–123
AY702785	207	*Geopora cooperi* SNF96	100%/207	1.0*E*–108
AY706753	224	*Rhizopogon subcaerulescens/salebrosus*	100%/224	1.0*E*–118
AY702790	293	*Rhizopogon subsalmonius* (AJ419212)	80%/280[2]	3.0*E*–31
AY702791	296	*Rhizopogon* sp. (#1) from Teakettle Forest	99%/296	1.0*E*–155
AY702793[1]	190	*Gymnomyces abietis* SNF168	95%/168	3.0*E*–73
AY702794	188	*Gymnomyces abietis* (AY239348)	99%/187	7.0*E*–98
AY702796	217	*Martellia* sp. from Ross Crossing	99%/217	1.0*E*–113
AY702797[1]	216	*Gymnomyces fallax* JMT19916	99%/216	1.0*E*–111
AY702816	220	*Tuber maculatum* SNF54	83%/179	7.0*E*–34
ECM sequence types that are potentially hypogeous but not included in the analysis				
AY702784	184	Motifs similar to *Hydnotryopsis* sp., no close Genbank match	n/a	n/a
AY702792	216	*Macowanites ammophilus* (AF230890) *Russula pectinata* (AY061706)	98%/216	1.0*E*–110

[1] Taxa that matched Genbank epigeous taxa and a Sierra Nevada hypogeous fruiting body equally.
[2] Results of discontinuous BLAST search.

Table 5. Annual ECM root species composition by fruiting habit

	1999	2001	2002	All years
Relative number of species				
Total species	26	72	62	100
Relative portion				
Hypogeous	0.19	0.22	0.27	0.21
Epigeous	0.23	0.15	0.13	0.17
Inconspicuous epigeous	0.04	0.04	0.02	0.03
Nonfruiting	0.04	0.03	0.03	0.02
Resupinate	0.12	0.14	0.16	0.17
Unknown	0.38	0.58	0.39	0.43
Relative biomass				
Total biomass (mg)	84.8	479.5	359.8	924.1
Relative portion of root biomass				
Hypogeous	0.41	0.43	0.33	0.39
Epigeous	0.18	0.04	0.12	0.08
Inconspicuous epigeous	0.11	0.07	0.08	0.08
Nonfruiting	0.16	0.12	0.13	0.13
Resupinate	0.06	0.16	0.15	0.15
Unknown	0.08	0.18	0.19	0.17

Total biomass indicates dry weight biomass of ECM roots that were analyzed by molecular means. The fruiting habit designation is the best estimate based on sequence BLAST analysis.

that would limit their effectiveness in dispersal of spores. Emerging sporocarps in this forest also face physical barriers. Because of fire suppression and forest age, the duff layer and woody debris across this forest are often quite thick. As a result, epigeous sporocarps that did not emerge through this barrier are commonly observed. The ability of hypogeous fungi to continue to be well dispersed by small mammals, therefore, may allow them to maintain themselves across space and time better than epigeous fungi in these conditions. However, in the increasing time period following fire, some hypogeous taxa are actually less likely to be detected in forests as the time passed since the last fire increases (Claridge et al. 2000), and there is no obvious increase in hypogeous sporocarp relative contribution to the

Table 6. Estimates of hypogeous taxa on roots from other comparable studies

Host type	Total sp.	Hypogeous sp.	Estimated % sp.	Estimated % Biomass	% unknown	Freq. Mass
Pinus muricata (Taylor and Bruns 1999)	20	1	5%	<1%	25%	2%
Pseudotsuga menziesii/Pinus muricata (Horton and Bruns 1998)	16	1	6%	5–10%	25%	15%
Picea abies/Pinus sylvestris (Jonsson et al. 1999)	43	1	2%	0.5%		>50%
Pinus ponderosa (Stendell et al. 1999)	53	2	4%	15%	64%	50%
This study	100	21	21%	39%	17%	43%

The listed studies were chosen based on (1) their potential to have identified the fungi as being a hypogeous taxon, and (2) having made an estimate of abundance of the fungal species on the roots.

overall fruiting community (Smith et al. 2002). Still, the Smith et al. study was conducted in mesic forests, where fire suppression may have less effect on forest floor conditions.

A higher abundance of hypogeous taxa in drier forests could have important implications for fungal consumers and ECM dispersal. There is less seasonal variability in fruiting and abundance of hypogeous than epigeous sporocarps (North et al. 1997, Smith et al. 2002). Several small mammals that are opportunistic mycophagists may supplement more of their diet with fungal sporocarps in these forests if hypogeous sporocarps are consistently available. Our study suggests that it would be interesting to compare the relative abundances of hypogeous fungi on ECM roots in both mesic and xeric forest conditions, where seasonality of fungal sporocarps differs. The higher abundance of hypogeous taxa also implies the importance of mycophagists in xeric forests as ECM dispersers. Effective ECM dispersal and inoculation may be important for tree seedling establishment in forest openings (Perry et al. 1982) where ectomycorrhizae increase drought tolerance (Parke et al. 1983).

Our methods of sampling the roots and identifying hypogeous species on them had limitations, but the basic conclusions are not expected to change. Our study sample was biased toward two prominent host species in the forest, *A. concolor* and *A. magnifica*; thus, we do not know whether our results are unique to *Abies* sp. or if they apply to the forest as a whole. Very few studies have sampled ectomycorrhizae of *Abies* in mature forest settings. Studies of sporocarps in sampled *Abies* forests (Matsuda and Hijii 1998, Salerni et al. 2000) reveal few hypogeous taxa, but in these cases they did not intentionally seek them out. In the previous studies of *Abies* ECM root communities, few taxa were identified to species and there did not appear to be many hypogeous sporocarps used for comparison (Hagerman et al. 1999, Kernaghan 2001). *Rhizopogon ellenae,* in particular, was found in high abundance on *Abies magnifica* roots elsewhere in the Sierra Nevada, but this report was biased by localized root stimulation by the mycoheterotroph *Sarcodes sanguinea* (Bidartondo et al. 2000). The other ectomycorrhizal overstory hosts in our forest are both from the genus *Pinus*; therefore, comparable analysis of the *Pinus* ECM community would be needed to confirm that our measurements on *Abies* roots reflects that of the entire forest. Again, this is not expected to change our overall conclusions, because *Pinus* species are known to host *Rhizopogon* as well, and because fungi capable of colonizing many hosts in a mixed-species forest are often the most common fungi present (Horton and Bruns 1998, Cullings et al. 2000, Kennedy et al. 2003).

Although our estimates of both the number and abundance of species have inaccuracies associated with them, neither source of error affects the basic conclusion that hypogeous fungi are a dominant class of fungi on the roots in this system. The inaccuracies associated with species numbers are due to our ITS-based definition of species, our undersampling of the root community, and the number of unidentified root-associated taxa. We used a 4% ITS difference as our cutoff for recognizing unique species. This value was chosen based on (1) preliminary analyses of other ectomycorrhizal studies that suggested the closest sister species in the community would fall no closer than this range, (2) error rates of our single-pass sequencing that were estimated to be 0.5–1%, and (3) observed rates of 1.5% change across the entire ITS within a species across a 7-km survey (Horton 2002). It is likely that this 4% cutoff will incorrectly group some species, but we would not expect a systematic bias toward or away from hypogeous taxa. Similarly, we know that species richness was undersampled, as it almost always is (Taylor 2002), but the relative species numbers between the 2 years of early season sampling were very similar (Table 5) and support a consistent trend. Because species whose fruiting habit could not be identified made up only a small percentage of the root biomass (Table 3), estimates of hypogeous contribution to this measure seem stable. These unknown species, however, composed roughly a quarter of the total species observed, making this measure more tentative.

Abundance of species as measured by colonized root biomass has a high variance because many species are clustered (Taylor 2002). For example, a single occurrence of one of the *Rhizopogon* species (AY702790) accounted for 31% of the total hypogeous contribution in 2001. This is not expected to affect the biomass estimates drastically, however, because "spikes" in local abundance occurred at each plot in different years and therefore would be expected to be a consistent trend across the forest as well. In support

of this interpretation, biomass estimates showed little variation between years (Table 5) and a more conservative bootstrap estimate of hypogeous ECM biomass (25%) was still higher than has been previously reported.

We were successful in our direct molecular analysis of the small mammal fungal diet, although this approach did not fulfill our original purpose of matching sequences to the root community, and clearly had some limitations. Using multiple primer pair combinations we obtained 57 sequences (Table 2) to compare to the ECM root community. Unfortunately, only three sequences matched those found on the ECM root community, and these were either already well represented in Genbank (*Rhizopogon*) or already found from sporocarps. However, the method did work in that sequences of additional hypogeous taxa were recovered. *Endoptychum*, *Geopora*, and *Melanogaster* species were all successfully amplified from scat and identified by matches to the sporocarp-derived sequences.

The problems and biases that arose from our direct-sequencing approach were primarily the result of complexities associated with the molecular analysis of multiple-taxa samples. Based on visual spore abundance, *Rhizopogon*, *Gautieria*, and *Melanogaster* together made up roughly 70% of the typed spores (40% of all spores) in the scat samples we used in this study (Table 3). However, although *Rhizopogon* and *Melanogaster* sequences were readily obtained, *Gautieria* was not. In at least half of the samples where it was present, *Gautieria* was the clearly dominant taxon and should have been amplified at some dilution. Our PCR methods may have biased against its detection for two reasons. First, many *Gautieria* samples detected in the Sierra Nevada forests have very long ITS segments that might amplify less efficiently than shorter sequences of other taxa present in the same samples. Second, the sequences used to design the basidiomycete-specific primer ITS4B were based heavily on taxa in the Boletales (Gardes and Bruns 1993), and therefore may bias the primer's specificity toward these groups (e.g., *Melanogaster* and *Rhizopogon*). It also proved to be difficult to obtain direct sequence of ascomycetes because of the presence of a presumably coprophilic ascomycete species in all of the scat samples that amplified preferentially in over 70% of the samples. More detailed or directed access to the mycorrhizal species should be possible by cloning methods or by use of taxon-specific primers. Lastly, as with any complex community sample, care must be taken because the potential for chimeric sequences remains an issue (Wang and Wang 1997), and therefore sequences that did not match other confirmed sequences must remain suspect until a true match has been demonstrated. Despite the aforementioned limitations, we have demonstrated that sequences of fungi vectored by mammals can be obtained from their scat. This has the potential to refine microscopic spore analysis by providing near-species-level identifications.

Rhizopogon is currently the best example of this potential, because sequences of this genus were readily obtained from the scat samples, and the large ITS database for *Rhizopogon* enables identification of all sequences to at least a subgeneric level. The addition of small mammal mycophagy adds a unique view of the ecology of *Rhizopogon* to three other views of this genus in the Sierra National Forest (Table 7). Studies of different life phases of ECM fungi each have their own particular bias and limitations, but when they are taken together we can infer more about the fruiting and dispersal frequency of some of the *Rhizopogon* species. ECM root studies are the most limited in sampling intensities that can be attained, and, expectedly, they yielded the lowest estimates of *Rhizopogon* species richness. The 1-ha sporocarp survey of North (2002) reported 10–12 species of *Rhizopogon*, but the sampling was limited to a single time point. The spore bank study of Kjøller and Bruns (2003) identified species that have the potential to occupy roots, but this does not guarantee that they actually do so in the present forest setting (Taylor and Bruns 1999). The scat study covers the greatest spatial (72 ha) and temporal (3-year) scales, and, unlike the mycorrhizal studies and bioassay study, is not directly biased by host tree. However, the scat sample is likely biased by fruiting abundance and possibly by rodent taste preferences. Species accumulation curves (Figure 1) suggest that 6 *Rhizopogon* species are being commonly eaten and dispersed even though we know there are potentially up to 18 (Table 7). Despite these biases, two important observations come from comparing these studies. First, two species, *R. arctostaphyli* and *R. salebrosus*, stand out for being detected by practically every method. These species appear to be very numerous or responsive as spores, are active on the roots, fruit abundantly, and are frequently dispersed by the mammals. Second, two species, *R. olivaceotinctus* and *R. occidentalis*, have been detected in the spore bank in two separate Sierra National Forest studies (Kjøller and Bruns 2003, Izzo, unpublished data) but appear absent from the sporocarp records as documented by multiple years of collecting by humans and by small mammals, as documented in our study. These species may be more reliant on disturbances such as diggings, windthrow, or fire for maintaining their

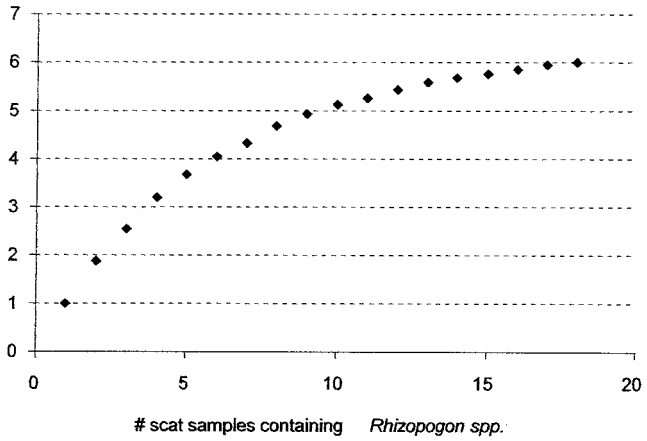

Figure 1. Species accumulation curve for *Rhizopogon* in scat samples. Only scat samples where *Rhizopogon* sequences were obtained were used in this analysis ($n = 18$). The projected peak of the curve would occur at 8.6 species observed.

Table 7. The occurrence of *Rhizopogon* species in the Sierra National Forest as viewed by different sampling approaches

Species group of *Rhizopogon*	Small mammal scat (this study)	ECM roots *Abies* sp. biased (Izzo et al. 2005)	Sporocarps (North 2002)	Spore bank SNF *Pinus* bioassay (Kjoller and Bruns 2003)	ECM roots monotropoid, SNF (Bidartondo and Bruns 2002)	Sporocarps collections SNF	ECM roots SNF *Pinus* (Stendell et al. 1999)
Section *Amylopogon* (Mixed-conifer)							
Rh. arctostaphyli	⊕		⊕[1]	⊕	⊕	⊕	
Rh. salebrosus/ subcaerulescens	⊕	⊕	⊕[2]	⊕	⊕	⊕	⊕
Rh. subgelatinosus			+				
Rh. ellenae					⊕	⊕	
Rh. sp. nov. (North 2002)			+				
Section *Roseoli* (*Pinus* host)							
Rh. vulgaris	⊕		+	⊕		+	
Rh. roseolus	⊕		+				
Rh. variabilisporus			+				
Rh. hysteriangiodes						+	
Rh. burlinghamii						+	
Sections *Rhizopogon/Versicolores* (*Pinus/Abies* hosts)							
Rh. sp. (#1)	⊕	⊕				⊕	
Rh. sp. (scat accession AY558733)	⊕						
Rh. sp. (root accession AY702790)		⊕					
Rh. ellipsosporus			⊕				
Rh. evadens			+				
Rh. brunnescens			+				
Rh. occidentalis				⊕			
Rh. olivaceotinctus				⊕			

Sections and host specificity are as per Grubisha et al. (2002).
+, Species detected.
⊕, ITS sequence from this sample used for comparison.
[1] Collection sample originally identified as *Rh. rubescens*.
[2] Sequence identical to *Rh. pedicellus*.

presence. There is already suggestive evidence that *R. olivaceotinctus* behaves in this way. In the California coastal *Pinus muricata* forests, this species is absent from the mature forest but detected both on postfire seedlings and in spore bank bioassays (Gardes and Bruns 1996, Baar et al. 1999, Taylor and Bruns 1999, Grogan et al. 2000). Suppression policies have kept fire out of Teakettle Forest and much of the Sierra National Forest, which could explain the absence of *R. olivaceotinctus* on roots, as sporocarps, or in small mammal diet. The potential need for specific disturbances to maintain some ECM species has important implications both for forest management and conservation. However, more studies will be needed to confirm these observations and test for the factors that may be involved.

In this study we have attempted to use molecular techniques to connect three different aspects of ECM ecology in a mixed-conifer old-growth forest: ectomycorrhizal root community structure, small mammal diet (and subsequent spore dispersal), and the hypogeous fruiting habit. Although spatial scale differences among the three sampling schemes limit our inferences, two general conclusions can be reached. The first is that fungal species exhibiting the hypogeous fruiting habit seem to make up a much larger portion of both total species and biomass of the ECM root community than has been previously observed. The second conclusion is that species-level DNA information can be readily obtained from scat samples; this will allow more detailed analysis of mammal dietary preferences and diversity, while also adding a new dimension to our understanding of ECM fungal ecology.

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