High diversity of fungi recovered from the roots of mature tanoak (*Lithocarpus densiflorus*) in northern California

S.E. Bergemann and M. Garbelotto

Abstract: We collected mature tanoak (*Lithocarpus densiflorus* (Hook. & Arn.) Rehder) roots from five stands to characterize the relative abundance and taxonomic richness of root-associated fungi. Fungi were identified using polymerase chain reaction (PCR), cloning, and sequencing of internal transcribed spacer (ITS) and 28S rDNA. A total of 382 cloned PCR inserts were successfully sequenced and then classified into 119 taxa. Of these taxa, 82 were basidiomycetes, 33 were ascomycetes, and 4 were zygomycetes. Thirty-one of the ascomycete sequences were identified as *Cenococcum geophilum* Fr. with overall richness of 22 ITS types. Other ascomycetes that form mycorrhizal associations were identified including *Wilcoxina* and *Tuber* as well as endophytes such as *Lachnum*, *Cadophora*, *Phialophora*, and *Phialocephela*. The most abundant mycorrhizal groups were Russulaceae (*Lactarius*, *Macowanites*, *Russula*) and species in the Thelephorales (*Bankera*, *Boletopsis*, *Hydnellum*, *Tomentella*). Our study demonstrates that tanoak supports a high diversity of ectomycorrhizal fungi with comparable species richness to that observed in *Quercus* root communities.

Key words: Cenoccocum geophilum, community, dark septate endophytes, ectomycorrhiza, species richness.

Résumé : Les auteurs ont prélevé des racines de *Lithocarpus densiflorus* (Hook. & Arn.) Rehder) dans cinq peuplements, afin de caractériser l'abondance relative et la richesse taxonomique des champignons associés à ses racines. On a identifié les champignons à l'aide du PCR, par clonage et séquençage de l'ITS et du 28S rADN. On a séquencé avec succès 382 segments clonés par PCR avant de les classifier en 119 taxons. De ces taxons 82 appartiennent aux basidiomycètes, 33 aux ascomycètes et 4 aux zygomycètes. On a identifié 31 des séquences ascomycètes au *Cenococcum geophilum* Fr. avec une richesse d'ensemble constituée de 22 types ITS. On a identifié d'autres ascomycètes qui forment des associations mycorhiziennes, incluant les genres *Wilcoxina* et *Tuber* ainsi que des endophytes, soient des *Lachnum*, *Cadophora*, *Phialophora* et *Phialocephala*. Les groupes mycorhiziens les plus abondants sont les Russulaceae (*Lactarius*, *Macowanites*, *Russula*) et des espèces de Théléphorales (*Bankera*, *Boletopsis*, *Hydnellum*, *Tomentella*) Cette étude démontre que le *Lithocarpus densiflorus* supporte une grande diversité de champignons ectomycorhiziens et une richesse en espèces comparable à celles observées chez les racines de communautés de *Quercus*.

Mots clés : Cenococcum geophilum, communauté, endophytes septés sombres, ectomycorhize, richesse en espèces.

[Traduit par la Rédaction]

Introduction

In recent years, there has been considerable interest in assessing the structure and diversity of fungal communities prompted mostly by the increased popularity and utility of polymerase chain reaction (PCR)-based molecular methods (Horton and Bruns 2001; Anderson and Cairney 2004). Assessments of ectomycorrhizal (EM) diversity based on root tips has challenged our perception of EM community dominance based on sporocarp surveys by demonstrating that the majority of EM fungi on colonized roots are not the producers of sporocarps above ground (Gardes and Bruns 1996; Horton and Bruns 2001). For example, *Tomentella* species are often dominant components of root cores but form very inconspicuous, resupinate sporocarps that are usually overlooked in sporocarp surveys (Gardes and Bruns 1996; Erland et al. 1999; Kõljalg et al. 2000). Molecular profiling of EM root communities has led to newly described EM partnerships (Selosse et al. 2002; Villarreal-Ruiz et al. 2004) and has unequivocally illustrated the high diversity of EM fungi (Gardes and Bruns 1996; Horton and Bruns 2001; Erland and Taylor 2002).

One of the pivotal tasks when examining community structure is to determine the degree to which community assemblages are predictable in terms of common taxonomic assemblages. Thus far, EM community structure has been primarily assessed for members of the Pinaceae family, and most of these studies have found that assemblages are quite similar in terms of overall species richness and dominance by particular mycorrhizal groups (Horton and Bruns 2001, for a review). In contrast, there are fewer reports about the composition of the EM fungi associated with the family Fagaceae (oak family) under natural conditions (Smith and Read 1997). Richard et al. (2004, 2005) demonstrated that the EM community assemblages of old-growth *Quercus ilex*

Received 12 December 2005. Published on the NRC Research Press Web site at http://canjbot.nrc.ca on 13 October 2006.

S.E. Bergemann[1] and M. Garbelotto. University of California, Berkeley Environmental Science, Policy and Management Ecosystem Sciences Division 137 Mulford Hall, MC#3114 Berkeley, CA 94720, USA.

[1]Corresponding author (e-mail: sbergemann@nature.berkeley.edu).

 doi:10.1139/B06-097

L. are diverse in terms of sporocarp diversity and EM root tip composition. In eastern North America, high estimates of EM diversity of seedlings were also found in mixed *Quercus* forests with similar dominance of EM taxa to *Q. ilex* (*Cenococcum geophilum* Fr., species of Cortinariaceae, Russulaceae, and Thelephorales) (Walker et al. 2005). In contrast, in western North America, lower EM richness was observed on tanoak (*Lithocarpus densiflorus* (Hook. & Arn.) Rehder)) seedlings and *Quercus garryana* Hook. (Kennedy et al. 2003; Valentine et al. 2004).

Lithocarpus densiflorus constitutes a substantial component of forests in coastal regions of California and southern Oregon (Niemiec et al. 1995). These forests are often highly productive in terms of sporocarp richness and have been targeted in compliance with the Pacific Northwest Forest Plan to establish baseline research for the evaluation of forest sustainability and functioning of ecosystems (McFarland and Largent 2001). In northern California, most of the Bureau of Land Management "sensitive" (considered as "sensitive" by rarity or being endemic to old-growth forests) fungi are more predominant in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco – tanoak forests (J.M. McFarland, personal communication, 2005). In addition to the high sporocarp richness of tanoak forests, the susceptibility of tanoak to an aggressive pathogen, *Phytophthora ramorum* (often referred to as "sudden oak death", or "SOD"), make the characterizing of tanoak communities important, if we are to evaluate the resilience of the EM community to disease introduction and spread across the range of *L. densiflorus* (Garbelotto and Rizzo 2005).

This study was initiated to provide the first assessment of the EM root community of mature tanoak stands. Although the EM composition of tanoak root tips has been assessed on seedlings (Kennedy et al. 2003), there has not yet been any examination of fungi associated with roots of mature tanoak stands. In this paper, we characterize *L. densiflorus* root communities by amplifying rDNA genes using fungal-specific primers, cloning, and sequencing these loci to determine the taxonomic composition of fungi using phylogenetic inference and relative abundance in a *L. densiflorus* forest.

Methods and materials

The study was conducted on a privately owned experimental forest (40°00′30″N, 123°57′00″W) in southern Humboldt county, California, USA, in January of 2003. The forest was originally dominated by Douglas-fir (*Pseudotsuga menziesii*) and tanoak (*L. densiflorus*). In 1951, all merchantable Douglas-fir was harvested after a stand-replacing wildfire. Rapid regeneration of tanoak from sprouts resulted in widespread canopy dominance that excluded the establishment of Douglas-fir. We focused our root-tip sampling in areas where tanoak constituted the dominant canopy cover. There are other EM hosts that make up a minor component of the vascular plant flora including madrone (*Arbutus menziesii*) and huckleberry (*Vaccinium ovatum*). Although no attempt was made to estimate the proportion of ericaceous roots, it was assumed that the potential inclusion of a small percentage of ericoid root tips would not likely influence our results, since the most frequent EM colonizers of tanoak and ericoid hosts are often generalists that form associations with multiple hosts (Vrålstad et al. 2002; Kennedy et al. 2003; Villarreal-Ruiz et al. 2004; Richard et al. 2005).

We established 20 m × 60 m plots in tanoak stands, which were further subdivided into three 20 m × 20 m subplots. Within each subplot, we placed two, 6 m perpendicular transects by random selection of two intersecting points along 15 m × 15 m grids positioned in the center of each plot. Within each subplot, three soil cores were sampled at 2 m intervals along each transect using PVC pipe with an internal diameter of 2.5 cm from all 15 subplots (90 cores). The cores were transported from the study site to the University of California–Berkeley on ice. Each core was divided into 10 sections of equal volume, and two subsections (each 16 cm^3) were randomly chosen from each core (n = 180 total) and excised from each core with an electric saw (Sawzall, Milwaukee Tool Company, Winnenden, Germany). Soil and roots were freeze-dried for approximately 36 h, after which all fine root mass from each section was separated from soil using a forceps, weighed and stored at –80 °C.

Characterization of tanoak root communities

DNA extraction and polymerase chain reaction amplification

After fine roots were separated from each core section, roots were pulverized prior to extraction by suspension of root tissue in liquid nitrogen for 30 s and beating with 2.5 mm glass beads using a FastPrep Instrument (Qbiogene Inc., Carlsbad, California). DNA from the fine roots was extracted using the UltraClean Soil DNA kit (Mo Bio Laboratories, Carlsbad, California) following the manufacturer's instructions. After each section was extracted, equal volumes from each subsection (n = 12) were pooled for each subplot and diluted (1:20) prior to PCR amplification.

The internal transcribed spacer (ITS1), 5.8S, ITS2, and a portion of the 28S (variable domains D1, D2) rDNA region were amplified with primers ITS-1F and TW13 (White et al. 1990; Gardes and Bruns 1993). PCR reactions were carried out in a 25 μL total volume containing 1× PCR buffer (200 mmol/L Tris–HCl, pH 8.4, 500 mmol/L KCl) (Invitrogen Corporation, Carlsbad, California), 2 mmol/L $MgCl_2$, 2 mmol/L deoxynucleoside triphosphate (dNTPs), 0.5 mg/mL bovine serum albumin (BSA), 0.5 μmol/L forward and reverse primers, 0.05 U of *Taq* polymerase (Invitrogen Corporation) and approximately 10–100 ng of template DNA. Thermocycling was conducted as follows: 94 °C for 1 min, 25 cycles of 93 °C for 45 s, 58 °C for 50 s, 72 °C for 45 s with a 1 s increment every cycle, and 72 °C for 10 min.

Cloning and screening of multiple PCR fragments from tanoak root pools

Two to four microlitres of PCR fragments amplified from pooled DNA sections from each subplot were cloned using the TOPO TA cloning kit version K following the manufacturer's (Invitrogen Corporation) protocol. Briefly, 1–2 μL of the cloning reaction was transformed with TOP 10 *Escherichia coli* competent cells. Cultures were plated on four Luria–Bertani broth (LB) (EMD Chemicals, Darmstadt, Germany) plates containing 50 μg/mL of kanamycin and incubated overnight at 37 °C. Each colony was isolated and incubated at 37 °C overnight in 96-well culture

Table 1. Cloned PCR inserts from tanoak roots, ITS and 28S GenBank accessions, putative identity based on alignment and phylogenetic analyses, the ecological status, and the number of plots from which the taxon was sequenced.

Clone identification and taxonomic rank assigned GenBank accessions	GenBank ITS accession No.	GenBank 28S accession No.	Sequence identity	Ecological status*	No. of plots detected (n = 15)
Ascomycota					
Ascomycota					
N19	DQ273286	DQ273444	Unknown	?	2
F22	DQ273287	DQ273445	Unknown	?	1
F6	DQ273288	DQ273446	Unknown	?	1
Cenococcum geophilum					12
P62	DQ273289	DQ273447	*Cenococcum geophilum*	M	
A27	DQ273290	DQ273448	*Cenococcum geophilum*	M	
F39	DQ273291	DQ273449	*Cenococcum geophilum*	M	
A4	DQ273292	DQ273450	*Cenococcum geophilum*	M	
N78	DQ273293	DQ273451	*Cenococcum geophilum*	M	
P14	DQ273294	—	*Cenococcum geophilum*	M	
K57	DQ273295	—	*Cenococcum geophilum*	M	
K58	DQ273296	—	*Cenococcum geophilum*	M	
F67	DQ273297	—	*Cenococcum geophilum*	M	
G14	DQ273298	—	*Cenococcum geophilum*	M	
X58	DQ273299	—	*Cenococcum geophilum*	M	
K7	DQ273300	—	*Cenococcum geophilum*	M	
Y36	DQ273301	—	*Cenococcum geophilum*	M	
N69	DQ273302	—	*Cenococcum geophilum*	M	
F20	DQ273303	—	*Cenococcum geophilum*	M	
B6	DQ273304	—	*Cenococcum geophilum*	M	
R7	DQ273305	—	*Cenococcum geophilum*	M	
K2	DQ273306	—	*Cenococcum geophilum*	M	
R54	DQ273307	—	*Cenococcum geophilum*	M	
T60	DQ273308	—	*Cenococcum geophilum*	M	
T33	DQ273309	—	*Cenococcum geophilum*	M	
D69	DQ273310	—	*Cenococcum geophilum*	M	
K29	DQ273311	—	*Cenococcum geophilum*	M	
X11	DQ273312	—	*Cenococcum geophilum*	M	
N72	DQ273313	—	*Cenococcum geophilum*	M	
F39	DQ273314	—	*Cenococcum geophilum*	M	
A31	DQ273315	—	*Cenococcum geophilum*	M	
B19	DQ273316	—	*Cenococcum geophilum*	M	
T45	DQ273317	—	*Cenococcum geophilum*	M	
T49	DQ273318	—	*Cenococcum geophilum*	M	
F14	DQ273319	—	*Cenococcum geophilum*	M	
Q17	DQ273320	—	*Cenococcum geophilum*	M	
Geoglossaceae					
Y43	DQ273321	DQ273452	*Geoglossum nigritum*	S	1
Helotiales					
F13	DQ273322	DQ273453	*Cadophora*	EN†	1
K19	DQ273323	DQ273454	*Cadophora*	EN†	1
C34	DQ273324	DQ273455	*Cadophora*	EN†	2
F11	DQ273325	DQ273456	*Phialocephela*	EN†	2
S15	DQ273430	DQ273457	*Chalara*	P	1
Hyaloscyphaceae					
D12	DQ273326	DQ273458	*Lachnum*	EN	2
F3	DQ273327	DQ273459	*Lachnum*	EN	7
Pezizomycotina					
Y7	DQ273328	DQ273460	Unknown	?	1
L17	DQ273329	DQ273461	Unknown	?	1
G60	DQ273330	DQ273462	Unknown	?	4
N2	DQ273331	DQ273463	Unknown	?	1

Table 1 (*continued*).

Clone identification and taxonomic rank assigned GenBank accessions	GenBank ITS accession No.	GenBank 28S accession No.	Sequence identity	Ecological status*	No. of plots detected (n = 15)
P2	DQ273332	DQ273464	Unknown	?	1
A8	DQ273333	DQ273465	Unknown	?	4
N8	DQ273334	DQ273466	Unknown	?	1
X35	DQ273335	DQ273467	Unknown	?	1
L10	DQ273336	DQ273468	Unknown	?	1
D44	DQ273338	DQ273470	*Phialophora*	EN†	4
T11	DQ273337	DQ273469	*Phialophora*	EN†	1
D21	DQ273339	DQ273471	*Phialophora*	EN†	1
C14	DQ273340	DQ273472	*Capronia*	P†	1
Pyrenomycetaceae					
C45	DQ273342	DQ273474	*Wilcoxina*	M	1
Sarcosomataceae					
S7	DQ273341	DQ273473	*Galiella*	S	2
Sordariomycetes					
D11	DQ273343	DQ273475	Unknown	?	1
F41	DQ273344	DQ273476	Unknown	?	1
D38	DQ273345	DQ273477	Unknown	?	1
D49	DQ273346	DQ273478	Unknown	?	1
D35	DQ273347	DQ273479	*Neonectria*	P	3
Tuberaceae					
D68	DQ273348	DQ273480	*Tuber*	M	1
Basidiomycota					
Amanitaceae					
O16	DQ273349	DQ273481	*Amanita*	M	1
K11	DQ273350	DQ273482	*Amanita*	M	1
Atheliaceae					
P19	DQ273351	DQ273483	*Piloderma*	M	2
A1	DQ273352	DQ273484	*Byssocorticium*	M	1
X34	DQ273353	DQ273485	*Byssocorticium*	M	4
R1	DQ273354	DQ273486	*Piloderma*	M	4
C13	DQ273355	DQ273487	*Amphinema*	M	1
N3	DQ273356	DQ273488	*Piloderma*	M	1
T2	DQ273357	DQ273489	Unknown	M	1
Basidiomycota					
X32	DQ273358	DQ273496	Unknown	?	1
G20	DQ273359	DQ273490	Unknown	?	5
D34	DQ273360	DQ273491	Unknown	?	1
C11	DQ273361	DQ273492	Unknown	?	1
S33	DQ273362	DQ273493	Unknown	?	2
Boletaceae					
F12	DQ273363	DQ273495	*Boletus*	M	3
B8	DQ273364	DQ273497	*Melanogaster*	M	3
A34	DQ273365	DQ273498	*Boletus*	M	1
G5	DQ273366	DQ273499	*Alpova*	M	1
C15	DQ273367	DQ273500	Unknown	M	1
W13	DQ273368	DQ273501	Unknown	M	3
Cantharellales					
C52	DQ273369	DQ273502	*Clavulina*	M	1
Q3	DQ273370	DQ273503	*Clavulina*	M	1
P4	DQ273371	DQ273504	Unknown	M	2
Ceratobasidiaceae					
X20	DQ273372	DQ273505	*Ceratobasidium*	EN	1
P40	DQ273373	DQ273506	*Ceratobasidium*	EN	1
Cortinariaceae					
Y1	DQ273374	DQ273507	*Cortinarius*	M	2

Table 1 (*continued*).

Clone identification and taxonomic rank assigned GenBank accessions	GenBank ITS accession No.	GenBank 28S accession No.	Sequence identity	Ecological status*	No. of plots detected (n = 15)
N43	DQ273375	DQ273508	*Cortinarius*	M	1
O25	DQ273376	DQ273509	*Cortinarius*	M	2
S4	DQ273377	DQ273510	*Cortinarius*	M	1
K4	DQ273378	DQ273511	*Inocybe*	M	1
L6	DQ273379	DQ273512	*Inocybe*	M	1
C25	DQ273380	DQ273513	*Inocybe*	M	2
A6	DQ273381	DQ273514	*Inocybe*	M	1
F59	DQ273382	DQ273515	*Inocybe*	M	1
Entolomataceae					
D16	DQ273383	DQ273516	*Entoloma*	M	1
Gomphaceae					
F21	DQ273384	DQ273517	Unknown	M	3
G3	DQ273385	DQ273518	Unknown	M	2
O4	DQ273386	DQ273519	*Ramaria*	M	4
C8	DQ273387	DQ273520	Unknown	M	2
D43	DQ273388	DQ273521	Unknown	M	1
Hygrophoraceae					
A61	DQ273389	DQ273522	*Hygrophorus*	M	1
Russulaceae					
D20	DQ273390	DQ273523	*Lactarius*	M	6
A16	DQ273391	DQ273524	*Lactarius*	M	1
A22	DQ273392	DQ273525	*Lactarius*	M	1
X59	DQ273393	DQ273526	*Lactarius*	M	1
Y42	DQ273394	DQ273527	*Lactarius*	M	1
Q52	DQ273395	DQ273528	*Russula*	M	2
K10	DQ273396	DQ273529	*Russula*	M	1
P50	DQ273397	DQ273530	*Russula*	M	1
Q1	DQ273398	DQ273531	*Russula*	M	2
B11	DQ273399	DQ273532	*Macowanites*	M	5
F4	DQ273400	DQ273533	*Russula*	M	1
N17	DQ273401	DQ273534	*Russula*	M	3
Sebacinaceae					
X9	DQ273402	DQ273535	*Sebacina*	M	1
Y2	DQ273403	DQ273536	*Sebacina*	M	1
O17	DQ273404	DQ273537	*Sebacina*	M	1
N11	DQ273405	DQ273538	*Sebacina*	EN†	1
Thelephorales					
D1	DQ273406	DQ273539	*Hydnellum*	M	1
A73	DQ273407	DQ273540	*Tomentella*	M	1
A20	DQ273408	DQ273541	*Tomentella*	M	1
A14	DQ273409	DQ273542	Unknown	M	1
C70	DQ273410	DQ273543	*Tomentella*	M	1
A21	DQ273411	DQ273544	*Tomentella*	M	1
T1	DQ273412	DQ273545	Unknown	M	1
W54	DQ273413	DQ273546	*Tomentella*	M	1
D3	DQ273414	DQ273547	Unknown	M	1
F45	DQ273415	DQ273548	Unknown	M	1
G1	DQ273416	DQ273549	Unknown	M	1
L11	DQ273417	DQ273550	Unknown	M	1
A17	DQ273418	DQ273551	*Tomentella*	M	1
X33	DQ273419	DQ273552	*Boletopsis*	M	2
F57	DQ273420	DQ273553	*Tomentella*	M	1
R69	DQ273421	DQ273554	*Bankera*	M	1
P5	DQ273422	DQ273494	Unknown	M	1
Tricholomataceae					
X19	DQ273423	DQ273555	*Lyophyllum*	M	1

Table 1 (*concluded*).

Clone identification and taxonomic rank assigned GenBank accessions	GenBank ITS accession No.	GenBank 28S accession No.	Sequence identity	Ecological status*	No. of plots detected (n = 15)
G58	DQ273424	DQ273556	*Tricholoma*	M	1
R24	DQ273425	DQ273557	*Tricholoma*	M	3
F16	DQ273426	DQ273558	*Tricholoma*	M	2
S1	DQ273427	DQ273559	*Tricholoma*	M	2
W74	DQ273428	DQ273560	*Tricholoma*	M	2
T14	DQ273429	—	*Armillaria gallica*	P	3
S37	DQ273432	—	*Mycena murina*	S	1
Zygomycota					
F58	DQ273431	—	*Mortierella*	EN	2
D74	DQ273433	DQ273563	*Umbelopsis*	EN	1
N77	DQ273434	DQ273562	*Umbelopsis*	EN	1
K21	DQ273435	DQ273561	*Umbelopsis*	EN	1

Note: All ITS GenBank accession numbers for *Cenococcum geophilum* are shown because of ITS divergence detected from bulked root pools (details in Fig. 5).

* M, mycorrhizal; P, root pathogen; EN, endophyte; S, saprobe; ?, unknown.

†In some cases, the putative ecological status is the subject of debate (see text for details).

plates containing 150 µL of LB broth with the same concentration of kanamycin.

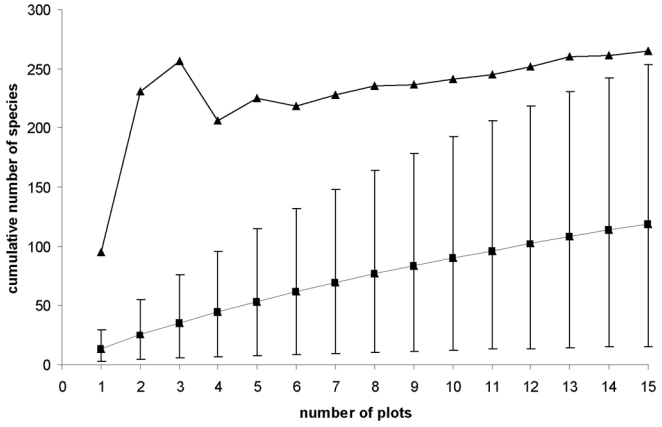
To screen the library for PCR cloned inserts that exhibited sequence variation, real-time PCR was used to generate melt temperature profiles (MTPs) of the ITS2 region by denaturation of PCR fragments at high temperatures followed by rapid cooling of the PCR amplicon to disassociate the fluorophores from the double-stranded DNA (Ririe et al. 1997). Initial screening of two clone libraries created from PCR amplification of pooled DNA from one subplot were used to estimate the number of colonies that were necessary to achieve saturation of MTPs. MTPs were analyzed from plasmid colonies using the Bio-Rad iCycler iQ Real Time PCR System (Hercules, California) with real-time thermal capabilities. PCR amplification of the ITS2 region using primers ITS3 and ITS4 (White et al. 1990) was chosen, since it often exhibits ample length and sequence variation and because the small size of the amplicon is optimal for analysis of product differentiation of MTPs (Ririe et al. 1997). Cloned PCR inserts were amplified in a 25 μL reaction containing: 1× PCR buffer, 3 mmol/L $MgCl_2$, 2 mmol/L each dNTP, 0.5 µmol/L of primers ITS3 and ITS4, 0.05 U *Taq* polymerase, and 10^{-5}× solution of SYBR green fluorophore (Sigma-Aldrich, St. Louis, Missouri), 10^{-5}× fluorescein (Bio-Rad), and 1–2 μL of plasmid broth. PCR cycling conditions were as follows: 94 °C for 10 min, 30 cycles of 94 °C for 35 s, 58 °C for 55 s, 72 °C for 50 s with an increase of 5 s increment every cycle, and 72 °C for 10 min. After PCR amplification, MTPS of 120 fragments were determined by: 110 cycles at 62 °C for 10 s, adding 0.3 °C at each curve. If cloning and transformation efficiency resulted in fewer than 80 clones, PCR amplifications from each subplot generated after combining equal volumes of DNA extracted from the roots of 12 core sections, cloning, and plasmid transformation were repeated. After amplification and sequencing (see below), 50 μL of 80% glycerol was added to plasmid broth and stored at –80 °C.

We were able to differentiate ITS2 sequences by real-time PCR differentiation using MTPs within a clone library (data not shown). In some instances, MTPs of identical ITS sequences differed by 0.3–0.6 °C as a result of variable peak heights, which likely reflected differences in amplification efficiency (data not shown). In several of the samples with no visible MTPs, primer mismatches were found between the ITS3 priming site near the 3′ end for a *Ramaria* sp. (C8) and an unidentified Basidiomycota species (X29) (data not shown). In addition to false negatives due to mismatch in forward priming sequences, MTPs with several sequences with long ITS2 sequence lengths (>500 bp) failed to produce a profile including *Armillaria gallica* (T14), *Boletus* sp. (A34), *Entoloma* sp. (D16), and Basidiomycota species (G20) (Table 1). Despite the occurrence of overlapping profiles with similar sequences and PCR bias, the sensitivity of our analyses suggests that this method is sufficient for rapid determination of ITS sequence types within a clone library.

Sequencing and editing

To select ITS fragments for sequencing, the following criteria were used to maximize the sequence variation retrieved from plots and to verify the reliability of melt temperature technique at differentiating ITS2 profiles (see above): (*i*) all cloned inserts with MTPs that differed by 0.3 °C within a single plot; (*ii*) several cloned PCR inserts from each library with overlapping MTPs within each plot; (*iii*) several cloned inserts from each library with no peak and were potentially a result of false negatives. PCR amplifications were completed in a 25 µL reaction containing 1× PCR buffer, 3 mmol/L $MgCl_2$, 2 mmol/L each dNTP, 0.5 µmol/L M13, T7 primers, 0.03 U *Taq*, 1–2 µL of plasmid broth. Cycling conditions were as follows: 95 °C for 10 min, 35 cycles of 95 °C for 45 s, 57.5 °C for 50 s, 72 °C for 2 min with a 1 s increment every cycle, 72 °C for 7 min. ITS fragments were sequenced in both directions with primers ITS-1F and ITS4 (White et al. 1990; Gardes and Bruns 1993) using the Big Dye 3.1 Terminator reaction kit (ABI, Foster City, California). Sequencing for 28S regions for taxa with unique ITS profiles was completed with primers CTB6 and TW13 (White et al. 1990; O'Donnell 1993). Cycle sequencing was

Fig. 1. Species richness accumulation curves plotted as a function of the number of plots sampled (n = 15). Mean estimates of observed species (■, S_{obs}) and estimated total richness (▲, Chao2). Bars indicate 95% lower and upper bound confidence intervals.



performed using the following parameters: 25 cycles of 96 °C 10 s, 50 °C for 5 s, 60 °C 4 min. Products were precipitated with 1 μL of 25 mmol/L EDTA, 1 μL 3 mol/L NaOAc, and 25 μL of 95% EtOH and washed in 70% EtOH. Sequence reactions were loaded on ABI 3100 automated sequencer. Fragments were edited and aligned using Sequencher 4.2 (Gene Codes, Ann Arbor, Michigan).

Phylogenetic placement of sequence unknowns

Given the large number of ITS fragments sequenced, an initial BLAST search with GenBank accessions was conducted to group sequences into smaller alignment blocks. ITS sequences with similarities >90% were aligned using Sequencher 4.2 (Gene Codes) to identify duplicate sequence fragments that exhibited overall sequence similarity of ≥98%. Once duplicate fragments were identified, sequences were aligned with GenBank accessions. To provide a more highly conserved region for the practical purposes of alignment and phylogenetic tree building, a portion of the 28S that includes two variable domains (D1, D2) was manually aligned with GenBank accessions. DNA sequences were aligned using ClustalX version 1.8 (Thompson et al. 1997) with manual editing using Se-Al (Rambaut 1996).

Phylogenetic analyses of the sequence data were performed with maximum-parsimony methods, as implemented in PAUP 4.0b (Swofford 2003), and with Bayesian inference, using MrBayes version 2.01 (Huelsenbeck and Ronquist 2001). For MP analyses, an initial heuristic search of 10–100 random taxon addition replicate searches was conducted with TBR branch-swapping, MAXTREES set to 10 000, unordered and equally weighted nucleotides, and retention of two shortest trees. The shortest trees were used as starting trees in a second heuristic search, with TBR branch swapping and MAXTREES at 5000 to find the most parsimonious trees. Bootstrap support for clades was estimated from 100 replicate heuristic searches with simple taxon addition sequence, retention of one tree per replicate, TBR branch swapping and MAXTREES set to 5000. Bayesian analyses were conduced with each alignment with six incrementally heated simultaneous Monte Carlo Markov chains (MCMC) run over one to two million generations using the general time-reversible model of DNA substitution, additionally assuming a percentage of invariable alignment sites with gamma-distributed substitution rates and random starting trees. All samples taken prior to burn-in were discarded, and the remaining samples were used to determine posterior probability distributions. From these distributions, a 50% majority rule consensus tree was computed.

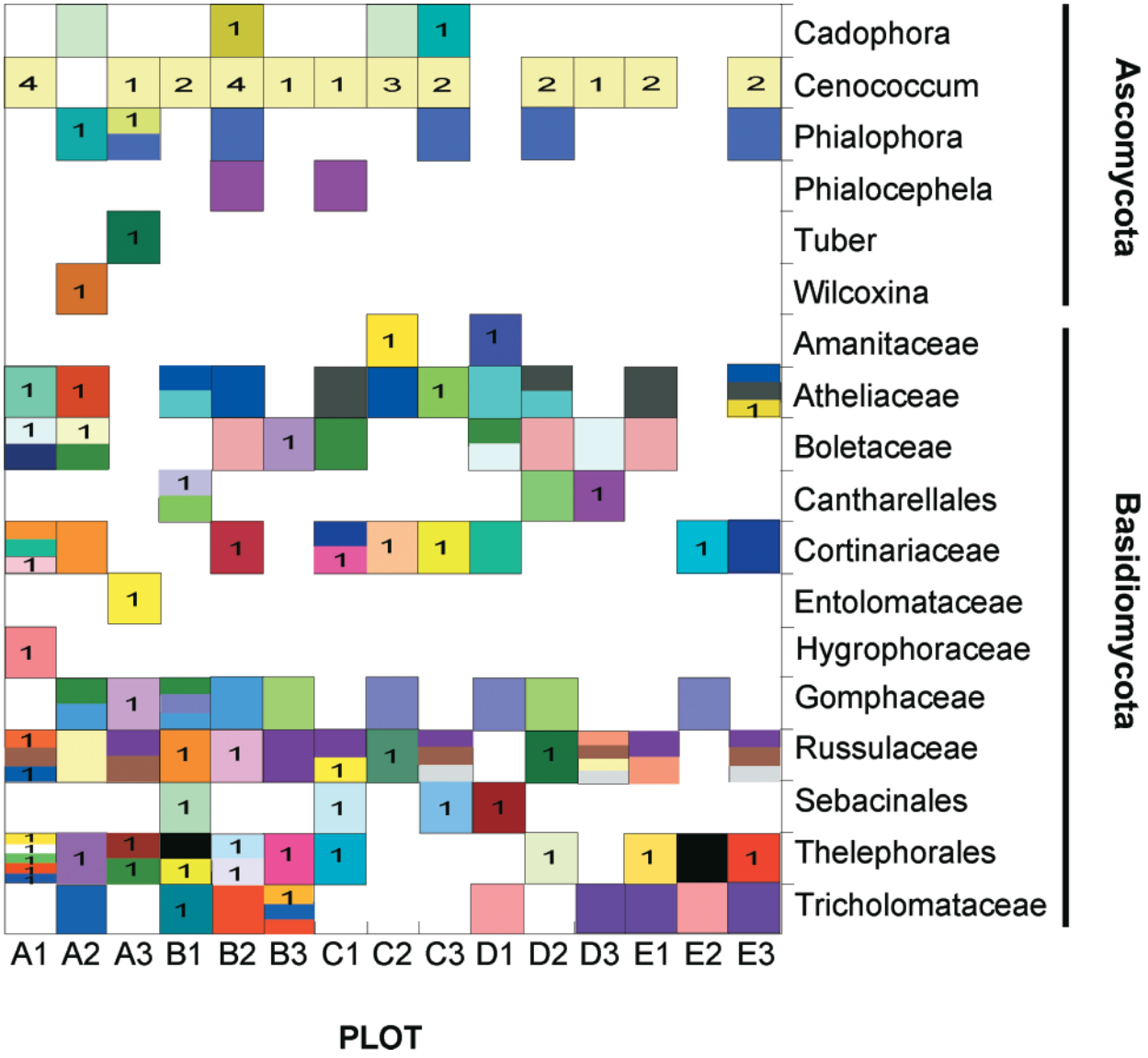
DNA sequences from cloned PCR products were analyzed together with sequences available in GenBank (http://www.ncbi.nlm.nih.gov/) (Table 1). We analyzed three 28S data sets: (*i*) 68 sequences covering ascomycetes known to form ectomycorrhizae with both ericoid and EM hosts, several taxa that comprise endophytes, epiphytes, or pathogens, unknown ascomycete sequences identified by nearest BLAST searches, and root-associated tanoak unknowns; (*ii*) 138 sequences of basidiomycete taxa including EM and endophytic fungi in nearest blast searches, a small subset of sporocarps collected within the plots, and our basidiomycete unknowns; (*iii*) 58 sequences of taxa belonging to the Sebacinales (Weiss et al. 2004) including environmental sequences, sequences of *Sebacina* fruitbodies and closely related taxa to differentiate EM taxa from those that are not likely involved in obligate tanoak root mutualisms. For the ascomycetes, the topology of the tree was rooted with *Schizosaccharomyces japonicus* (GenBank No. U94943). The basidiomycete tree was rooted with *Ustilago maydis* (GenBank No. L20287) (Taylor and Bruns 1999). The *Sebacinales* tree was rooted with *Geastrum saccatum* (GenBank No. AF287859) (Weiss et al. 2004). Several of the generic relationships between cloned inserts were unresolved using the 28S; therefore, we further analyzed the most representative groups including the Atheliaceae and Thelephorales (*Tomentella* and *Thelephora*) and sequences belonging to what appear to be *Cenococcum geophilum* sensu lato. Because of the variation observed in ITS sequences of *C. geophilum*, comparisons were made between our tanoak unknowns and isolates from *Quercus douglasii* and *Q. garryana* (Douhan and Rizzo 2005), *Q. ilex* (Richard et al. 2005), accessions from mixed coniferous forests in California (Izzo et al. 2006; A.D. Izzo, D.T. Nguyen, and T.D. Bruns, unpublished results), and *C. geophilum* sequences from Oregon (Horton et al. 2005; Horton and Ashkannejhad 2006) (see details in Fig. 5). For ITS data sets, we made no attempt to root the trees with outgroup comparison but used midpoint rooting as a suitable alternative.

Estimates of species richness and abundance

We calculated both the mean estimate of observed species (S_{obs}) and the nonparametric incidence-based estimator (Chao2) to estimate the true species richness with 100 randomizations and clusters of 10 individuals (m = 10) using EstimateS (Colwell 2005). S_{obs} estimates the mean number of taxa, which is equivalent to the species richness accumulation curve. The Chao2 estimator takes into account the effect of rare species on total richness to provide a better estimate of "true" species richness for small sample sizes (Colwell and Coddington 1994). We plotted both S_{obs} with 95% upper and lower confidence intervals and classic Chao2 estimator for comparison.

For simplicity, we considered *C. geophilum* as a single taxon and ranked the fungi in terms of overall species rich-

Fig. 2. Distribution of mycorrhizal fungi sequenced from tanoak roots across the plots sampled. Letters correspond with the five stands (A, B, C, D, E) and numbers correspond to the three plots (1, 2, 3) within each stand. Within each group of Ascomycota and Basidiomycota, each species is represented by a unique color. Species sequenced from a single plot are indicated by the number one (1). For *Cenococcum*, each plot was coded by the number of ITS sequence types recovered (1–4).



ness of taxonomic subphyla (Ascomycota, Basidiomycota, Zygomycota) and overall richness observed for each ecological status (mycorrhizal, endophyte, saprobe, unknown). We plotted the species composition across plots for dominant groups in the Ascomycota and Basidiomycota to determine whether their dominance was a function of the overall richness of species within a group or whether the distribution of species contributed to overall dominance in the tanoak root community.

Results

A total of 382 cloned PCR ITS inserts were successfully amplified and sequenced from 15 plots. Of these, 71% were basidiomycetes, 26% were ascomycetes, and 3% were zygomycetes (Table 1). Sixty-four percent of fungi were mycorrhizal, 11% were endophytes, 3% were saprobes, 3% were root pathogens, and 18% are of unknown ecological status (Table 1). The number of species sequenced from 15 plots ranged from 8 to 20 species. The number of observed species (S_{obs} = 119) was significantly lower than the estimate of total species richness (Chao2 = 265) indicating that the diversity of tanoak root-associated fungi is much higher than the mean observed richness (Fig. 1).

Four sequences were identified as *Mortierella* (1 sp.) and *Umbelopsis* (3 spp.) within the subphylum Zygomycota (Table 1). Of the remaining taxa, 267 basidiomycete and 111 ascomycete sequences were recovered from root pools from 15 plots (Table 1; Figs. 3, 4). The most common taxon recovered from tanoak root pools was *C. geophilum*, which was present in 80% of the plots sampled (Table 1: Fig. 2). Phylogenetic analyses supported the separation of 22 *C. geophilum* ITS types from tanoak into four clades (Fig. 5). Of the remaining sequences, 32 ascomycete spe-

Fig. 3. Ascomycetes. Maximum parsimony phylogram produced from the alignment of nuclear DNA sequences from the D1 and D2 region of the large ribosomal subunit is shown. The topology was rooted with *Schizosaccharomyces japonicus*. GenBank accessions are provided with corresponding accession numbers. Bootstrap values equal to or greater than 80 are shown above the branches. Sequences from cloned PCR inserts from tanoak roots are indicated by a clone library (A–Y) and numeric values (1–80).

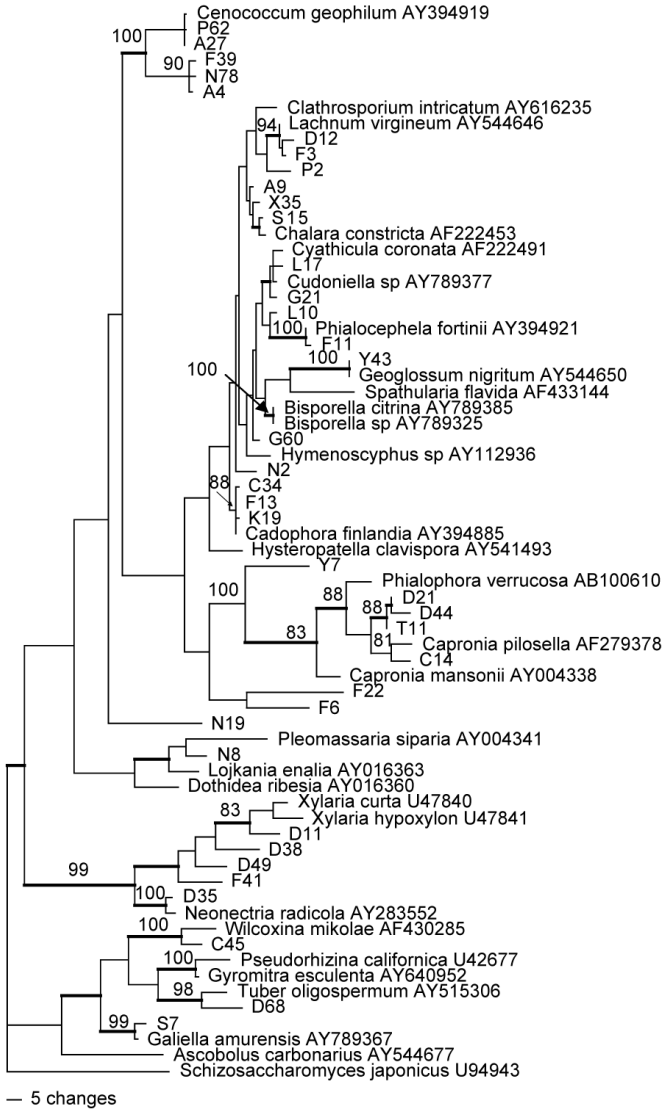
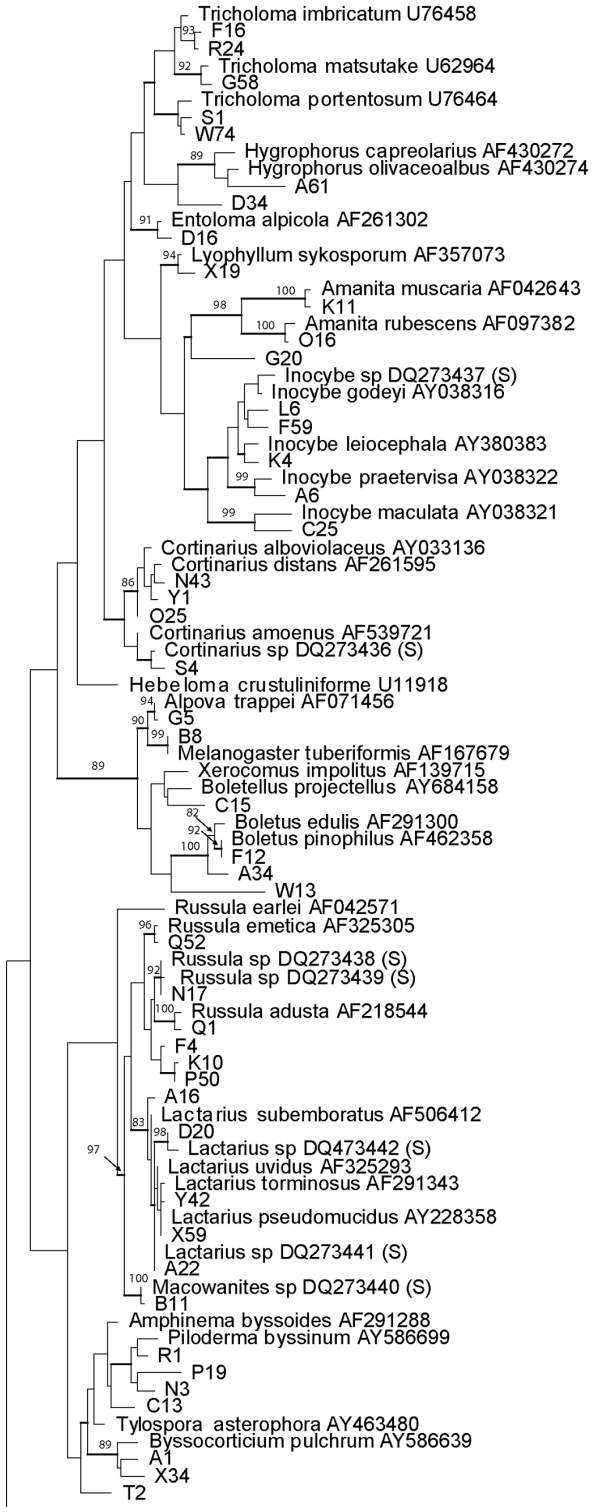


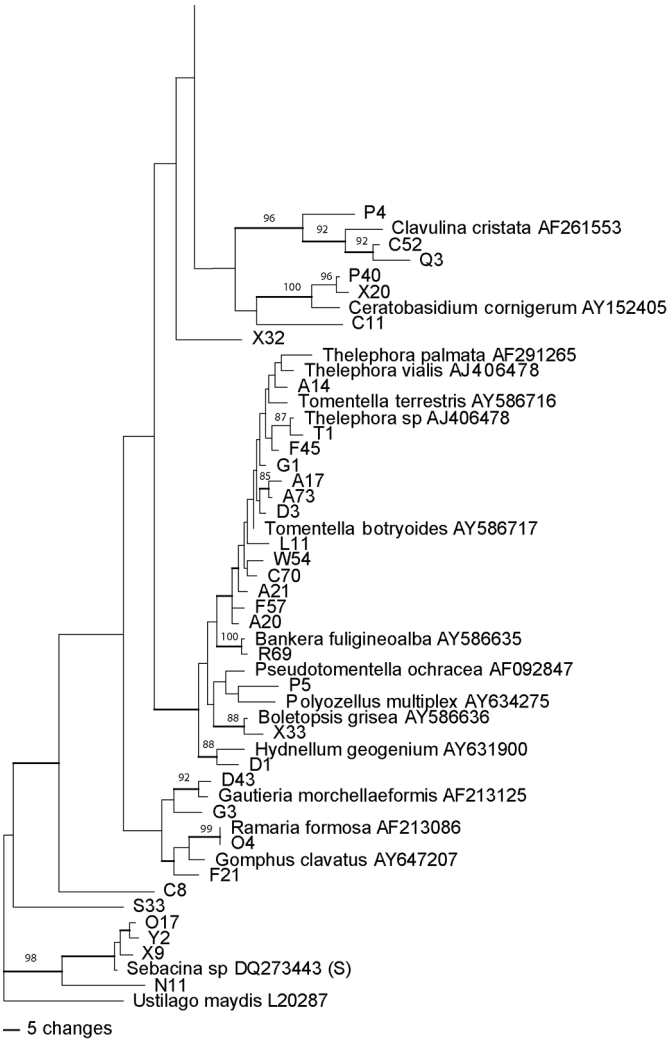
Fig. 4. Basidiomycetes. Maximum parsimony phylogram produced from the alignment of nuclear DNA sequences from the D1 and D2 region of the large ribosomal subunit is shown. The topology was rooted with *Ustilago maydis*. Accessions from sporocarps collected from plots and nearby vicinities are labeled with the letter S. GenBank accessions are provided with corresponding accession numbers. Bootstrap values equal to or greater than 80 are shown above the branches. Sequences from cloned PCR inserts from tanoak roots are indicated by a clone library (A–Y) and numeric values (1–80).



cies were identified (Table 1; Fig. 3). Ascomycetes previously reported as mycorrhizal symbionts include *Tuber* (D68) and *Wilcoxina* (C45) (Table 1; Fig. 3). Others such as *Cadophora* (F13, K19, C34), *Capronia* (C14), *Chalara* (S15), *Lachnum* (D12, F3), *Galiella* (S7), *Geoglossum nigritum* (Y43), *Neonectria* (D35), *Phialophora* (C14, D44, T11, D21), and *Phialocephela* (C14) have been classified as endophytes, saprobes or pathogens in previous studies that have evaluated their ecological status (Kile and Walker 1987; Nitare 1988; Cao et al. 1992; Mantiri et al. 2001; Dimitrova 2002; Addy et al. 2005) (Table 1; Fig. 3).

A total of 267 basidiomycete sequences were classified into 83 taxa (Table 1; Fig. 4). In the Atheliaceae, three species shared phylogenetic similarities with *Piloderma* (P19,

Fig. 4 (*concluded*).



R1, N3), two sequences were identified as *Byssocorticium* species (A1, X34), one was identified as *Amphinema* (C13), and one is unknown (T2) (Fig. 6). ITS alignment of *Thelephora* and *Tomentella* (A17, A20, A21, A73, C70, F57, W54) demonstrated that the majority of Thelephorales sequences are likely *Tomentella* spp. (Fig. 7). Four sequences matched most closely non-EM basidiomycetes including *Armillaria gallica* (Q65), a well-known root pathogen supported by ITS similarity to GenBank accession AY253570 (99%), a PCR clone (S37) with 99% sequence similarity to *Mycena* aff. *murina* (AF335444), a litter saprobe, and *Ceratobasidium* spp. (X20, P40), which we classified as endophytes, because they are commonly reported as a necrotrophic or ectotrophic with conifers (Bidartondo et al. 2004) (Table 1; Fig. 4). Analyses of the 28S Sebacinales rDNA alignment supported the separation of three sequences that are likely EM (O17, X9, Y2) from one (N11) that shared phylogenetic similarities with non-EM hosts (Fig. 8). Six other sequences could not be resolved to the family or generic rank (Table 1; Fig. 4). Nearest blast searches for S33 exhibited 28S sequence similarities with *Sporobolomyces oryzicola* (AJ510199); however, sequence similarity to support close affinities were inconclusive (68%).

In terms of species richness, the most diverse groups included the Thelephorales and Russulaceae (Fig. 2). When each taxonomic group was ranked in terms of abundance, Russulaceae spp. (*Lactarius*, *Macowanites*, *Russula*) and *Cenococcum* were the dominant fungi identified from 87% and 80% of the plots, respectively. The least diverse fungal groups sequenced only from a single plot included the Entolomataceae, Hygrophoraceae, *Tuber*, and *Wilcoxina* (Fig. 2). Overall dominance by Atheliaceae and Russulaceae groups was a function of both multiple occurrences of taxa and species richness (9 taxa and 7 taxa, respectively) (Fig. 2). In contrast, nearly all of the Thelephorales (94%) and Cortinariaceae (67%) species were restricted to one plot (Fig. 2).

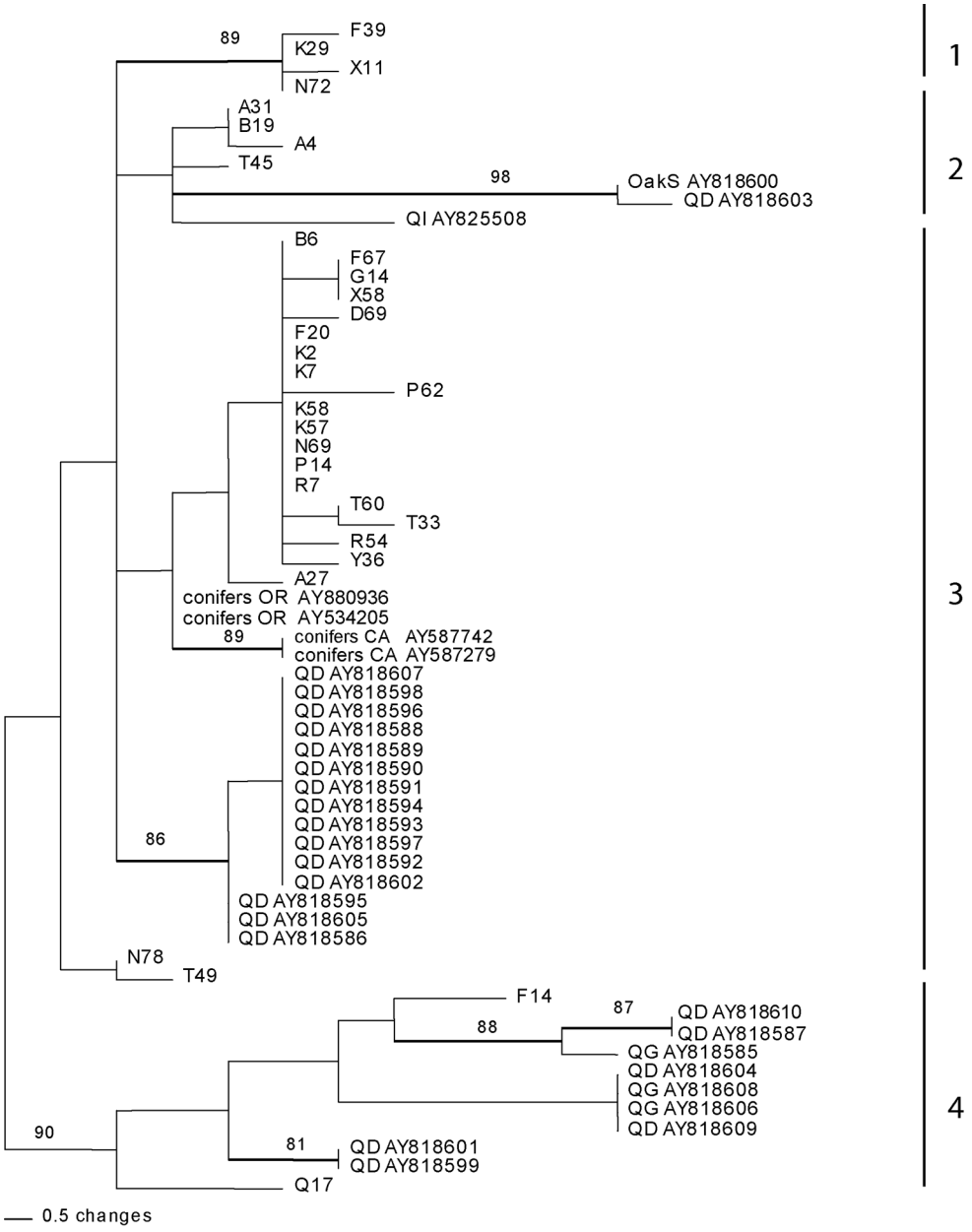
Discussion

Our finding of 119 taxa across a broad range of EM families supports the conclusion that tanoak supports a diverse and complex root community. The total diversity in the stands is likely higher than stated, given that the observed number of species (S_{obs} = 119) never reached an asymptote, and the total estimated species richness (Chao2 = 265) was higher than the observed values. Regardless of our success of sampling our root community, it was possible to identify 83 sequences at the generic rank. Seventy-six percent of all basidiomycetes were identified to the generic rank, while only 52% of ascomycete sequences were assigned to the same rank, and this disparity between the groups is likely to reflect under-representation of ascomycete lineages in GenBank (Horton and Bruns 2001).

Our estimates of mycorrhizal species richness (83 species) were much higher than those reported using morphotype richness of tanoak seedlings (10 morphotypes) (Massicotte et al. 1999) and sequence diversity of tanoak seedlings (29 ITS sequence types) (Kennedy et al. 2003). There are several factors that may contribute to the difference between the higher estimates of species richness observed in our tanoak root community and other studies on tanoak seedlings. Johnson et al. (2005) suggested that the variance among EM species richness associated with a single host varies with host age, inoculum levels from outside sources, host composition, and edaphic composition. In our study site, Douglas-fir was once the predominant EM associate and is known to support high levels of EM fungi on root communities (Sakakibara et al. 2002; Horton et al. 2005). Because multiple-host fungi are the predominant colonizers of Douglas-fir and tanoak, many of the EM fungi found on tanoak are likely shared with Douglas-fir (Massicotte et al. 1999; Kennedy et al. 2003). This may explain why *Amphinema, Byssocorticium*, and *Piloderma* species are abundant on tanoak roots but are absent from other EM communities of *Quercus* (Richard et al. 2005; Walker et al. 2005).

The high estimates of species richness from tanoak roots and the large percentage of rare taxa suggest that the EM community of tanoaks may be underestimated with our sampling effort. Our results suggest that the EM community of tanoak is similar to those seen in studies of EM diversity in the Pinaceae family, in that much of the species richness and dominant ITS types are Russulaceae and Thelephorales species (Gardes and Bruns 1996; Horton and Bruns 2001).

Fig. 5. Phylogenetic relationships within *Cenococcum geophilum*. Maximum parsimony phylogram produced from the alignment of ITS1, 5.8S, and ITS2 demonstrates that 22 tanoak sequences are divided into four clades. Sequences from *Quercus douglasii* (QD), *Quercus garryana* (QG), Oak Savanna from Maryland (OakS), and corresponding accession numbers are shown (AF819585–AF818609) (Douhan and Rizzo 2005). Sequences also include *Quercus ilex* (QI) (AY825508) (Richard et al. 2005) and mixed coniferous forests in California (conifers CA) (AY587742, AY587A279) (Izzo et al. 2006; A.D. Izzo, D.T. Nguyen, and T.D. Bruns, unpublished results) and Oregon (conifers OR) (AY534205, AY880936) (Horton et al. 2005, 2006). GenBank accessions are provided with corresponding accession numbers. Bootstrap values equal to or greater than 80 are shown above the branches. Sequences from cloned PCR inserts from tanoak roots are indicated by a clone library (A–Y) and numeric values (1–80).



Dominance by the Russulaceae in the community was driven by both the relative abundance of taxa sampled across multiple plots and the overall richness of EM types. In contrast, the overall dominance of the Thelephorales group was driven by the observed richness of species (18 species), of which 94% were sampled from one plot. As is true of many EM communities, *Tomentella* species comprise the majority of Thelephorales spp. present in root communities (Gardes and Bruns 1996; Kõljalg et al. 2000; Horton and Bruns 2001).

Most of the basidiomycetes amplified share taxonomic affinities with fungi known to form EM associations (Gardes and Bruns 1996; Horton and Bruns 2001). Basidiomycetes identified from tanoak roots span a broad range of EM lineages including Amanitaceae, Atheliaceae, Boletaceae, Cantharellales, Cortinariaceae, Entolomataceae, Gomphaceae,

Fig. 6. Atheliaceae: Maximum parsimony phylogram produced from the alignment of the ITS1, 5.8S, and ITS2. GenBank accessions are provided with corresponding accession numbers. Bootstrap values equal to or greater than 80 are shown above the branches. Sequences from cloned PCR inserts from tanoak roots are indicated by a clone library (A–Y) and numeric values (1–80).

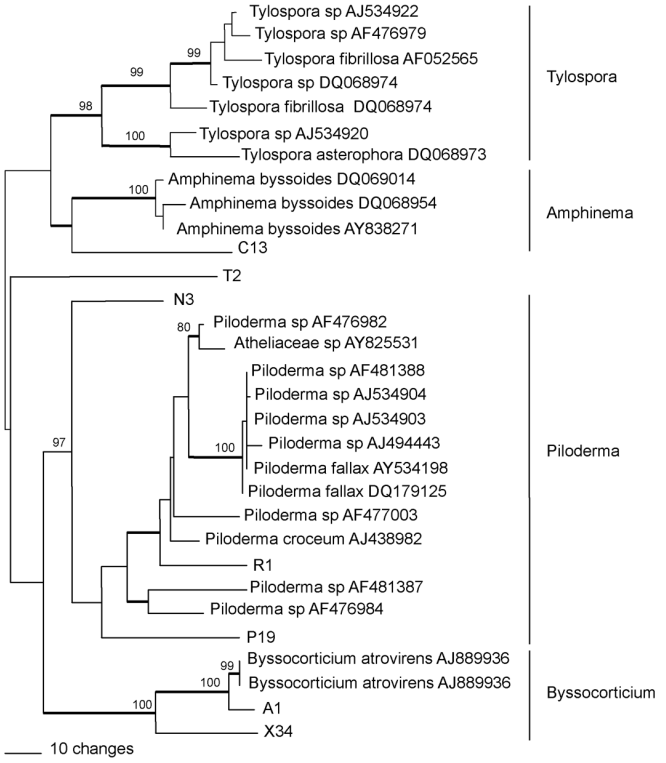
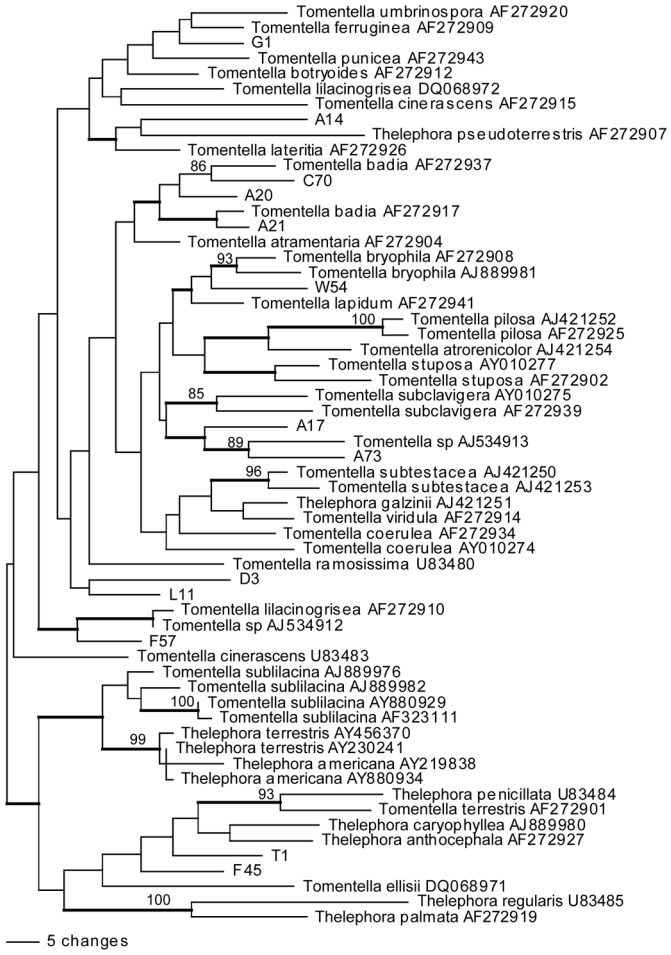


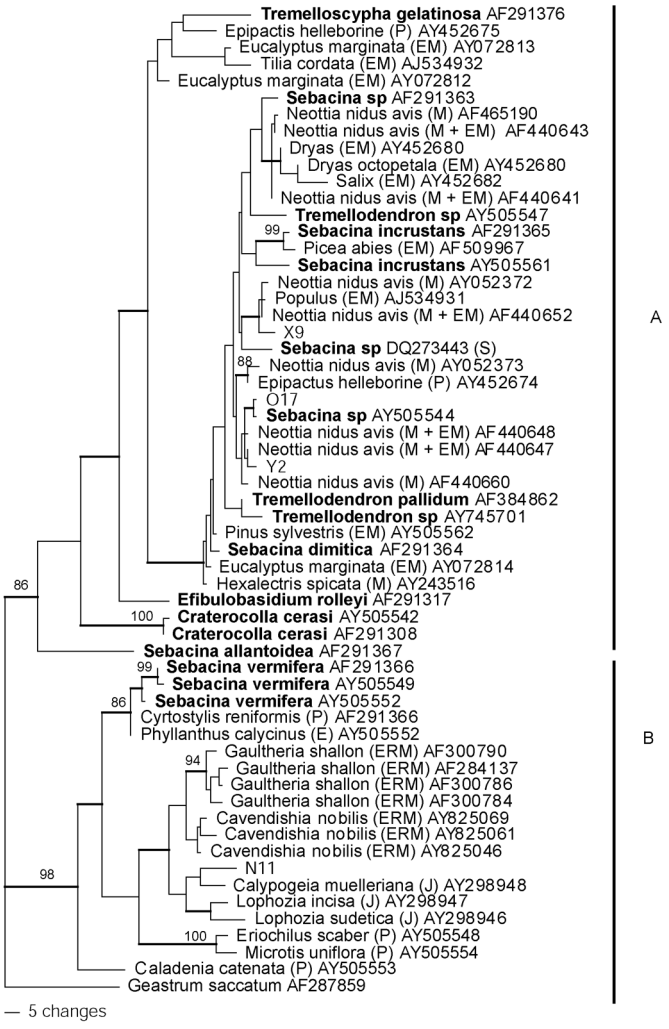
Fig. 7. *Thelephora* and *Tomentella*. Maximum parsimony phylogram produced from the alignment of the ITS1, 5.8S, ITS2 is shown. GenBank accessions are provided with corresponding accession numbers. Bootstrap values equal to or greater than 80 are shown above the branches. Sequences from cloned PCR inserts from tanoak roots are indicated by a clone library (A–Y) and numeric values (1–80).



Hygrophoraceae, Russulaceae, Sebacinales, Thelephorales, and Tricholomataceae (*Lyophyllum*, *Tricholoma*). In terms of representation of EM lineages, this study closely parallels the taxa recovered from oak seedlings in eastern North America (Walker et al. 2005) with the exception of the high richness of the Atheliaceae spp. observed in this study. Several basidiomycete sequences classified in this study including *Cortinarius*, *Inocybe*, *Russula*, *Sebacina*, and *Tomentella* were also common on tanoak seedlings (Kennedy et al. 2003) and *Quercus* seedlings (Richard et al. 2005; Walker et al. 2005). Until its recent isolation from eucalypt roots and other hosts, *Sebacina* was not widely recognized as an EM genus (Glen et al. 2002; Selosse et al. 2002). Weiss et al. (2004) demonstrated that two clades are supported: (*i*) one clade includes *Sebacina* spp. that form EM associations and (or) are likely involved in tripartite relationships between EM hosts and mycoheterotrophic orchids (e.g, *Neottia*, *Hexalectris*) or partially mycoheterotrophic orchids (e.g., *Epipactis*) and; (*ii*) *Sebacina* spp. that are not associated with EM hosts but colonize ericaceous plants (e.g, *Gaultheria*, *Cavendishia*), photosynthetic orchids (e.g., *Caladenia*, *Cyrtostylis*, *Eriochilus*, *Microtis*), euphorbs (e.g, *Phyllanthus*), and leafy liverworts (e.g., *Calypogeia*, *Lophozia*). In the studied tanoak community, both clades are present.

The diversity of the ascomycetes recovered from tanoak roots was markedly high compared with root communities of *Quercus* (Richard et al. 2005; Walker et al. 2005). The disparity between our estimates of diversity and studies on *Quercus* could be the result of several factors. First, the techniques in this study used PCR amplifications from bulked root pools rather than morphotyping methods. In several instances, ascomycete taxa that form E-strain mycorrhizas such as *Wilcoxina* spp. with undefined mantles could be overlooked when morphotyping root tips (Yu et al. 2001). The fungi that form the so-called dark septate endophytes or DSEs are often combined into a single category using morphological criteria (Addy et al. 2005), but are easily differentiated by ITS divergence and phylogenetic analyses. The cloning technique employed using PCR amplicons from fine root mass is likely to amplify root endophytes (e.g., Sordariomycetes) in addition to EM fungi, because the primers are fungal-specific; therefore, abundant endophytic hyphae are co-amplified with root-associated mutualists. In tanoak roots, we have identified 33 ascomycete taxa, but only two species (*Tuber* sp., *Wilcoxina* sp.) shared phyloge-

Fig. 8. Sebacinales. Maximum parsimony phylogram produced from the alignment of nuclear DNA sequences from the D1 and D2 region of the large ribosomal subunit. The topology was rooted with *Geastrum saccatum*. Bold taxa indicate sequences from teleomorph stages. The remaining sequence types are listed according to the host from which the sequence was obtained. Two clades (A, B) are supported, which is similar to the results from Weiss et al. (2004). Clade A contains species that were found on EM roots (EM), or nonphotosynthetic orchids or "mycoheterotrophs" (M), or photosynthetic orchids or "partial mycoheteotrophs" (P). Clade B includes ericoid mycorrhizal types (ERM) and specialized associations with photosynthetic orchids (P), Jungermanniales (J), and Euphorbiaceae (E) hosts. Accessions from sporocarps collected from plots and nearby vicinities are labeled with the letter S. GenBank accessions are provided with corresponding accession numbers. Branches with posterior probabilities equal to or above 0.95 based on Bayesian inference are shown in bold; bootstrap values equal to or greater than 80 are shown above the branches. Sequences from cloned PCR inserts from tanoak roots are indicated by a clone library (A–Y) and numeric values (1–80).



netic affinities with documented examples of mutualistic fungi.

The dark septate endophytes (DSEs) are a poorly defined group of fungi that share similar morphology (Jumpponen 2001). Several taxa that form DSEs including *Cadophora*, *Phialocephela*, and *Phialophora* are now recognized as several lineages supported by considerable phylogenetic divergence (Jumpponen and Trappe 1998; Jumpponen 2001). Although their mutalistic status is often debated (see Addy et al. 2005), evidence exists that DSEs can enhance nutrient uptake and stimulate growth under certain conditions (Jumpponen 2001). Several studies have demonstrated that DSEs are prevalent in root communities. For example, Allen et al. (2003) demonstrated that *Cadophora* was common in *Gautheria shallon* roots and closely related to *Hymenoscyphus ericae*, a well-known ericaceous symbiont. Both Vrålstad et al. (2002) and Villarreal-Ruiz et al. (2004) demonstrated that *Cadophora* genotypes occurred on both coniferous and ericaceous hosts. In our study, we identified three sequences with phylogenetic affinities to *Phialophora*, one species of *Phialocephela* with close sequence similarities to *Phialocephela fortinii* and three *Cadophora* species. We are unaware of any other studies of oak root communities that have documented the diversity of several species commonly referred to as DSEs. It would be interesting to compare the diversity of the tanoak root EM community with that of *Arbutus* and *Vaccinium* to determine whether identical genotypes are recovered from ectomycorrhizal and ericoid mycorrhizal hosts in the same ecosystem.

In addition to those in ericaceous hosts, our analyses of EM fungi from tanoak communities have demonstrated that *C. geophilum*, *Tuber*, and *Lachum* species are found on tanoak. Both *C. geophilum* and *Tuber* were sampled on tanoak seedlings (Massicotte et al. 1999; Kennedy et al. 2003) and were common on *Quercus* roots (Valentine et al. 2004; Douhan and Rizzo 2005; Walker et al. 2005). Walker et al. (2005) reported *Lachnum* as an abundant component of *Quercus* seedlings, and Dimitrova (2002) reported *Lachnum* as a prevalent endophytic component of conifer roots; however, its ecological role in tanoak remains unclear. It is not surprising that *C. geophilium* was the dominant colonizer of tanoak roots considering its broad host range (Trappe 1964) and ubiquitous detection in EM community studies (Horton and Bruns 2001). However, its dominance on *Quercus* (Valentine et al. 2004; Richard et al. 2005; Walker et al. 2005) and our tanoak study suggest that *C. geophilum* may be more prevalent on trees in the Fagaceae. Whether *C. geophilum* is a single "species" is the subject of debate (Shinohara et al. 1999; Douhan and Rizzo 2005). Shinohara et al. (1999) found evidence of significant phylogenetic divergence among isolates of *C. geophilum* across continents, and Jany et al. (2002) found evidence of divergence across a 300 km transect in France in *Fagus sylvatica* L. forests. In contrast, Douhan and Rizzo (2005) reported similar diversity from isolates sampled from three *Q. douglasii* trees. Our findings suggest that several "phylotypes" of *C. geophilum* are found in tanoak stands and that levels are similar sequence diversity to that observed in *Q. douglasii* (Douhan and Rizzo 2005).

In summary, our study supports the idea that tanoak stands sustain a diverse root community and supports a broad assemblage of EM taxa. Future research may incorporate analyzing changes in the EM community of tanoak caused by the expansion and intensification of "SOD", an

exotic disease caused by an introduced pathogen, *P. ramorum*. These effects are likely to be more significant in areas where no other EM hosts are present such as tanoak–redwood forests or in areas where tanoak is the dominant EM host. It may be important to examine the overall loss of diversity across regions largely impacted by "SOD" and whether nutrient cycling or resource allocation may be significantly affected by declining EM richness and abundance.

Acknowledgements

We thank Peter Kennedy and Kabir Peay for comments on earlier drafts of this manuscript, Tim Metz for allowing us the use of his property and his assistance throughout the project, and Nick Kordesch and Will VanSant for laboratory assistance and support. Several people provided additional field support including Katy Hayden, Rachel Linzer, Matt Meshriy, and Steven Swain. Funding was provided by the Gordon and Betty Moore Foundation.

References

Addy, H.D., Piercy, M.M., and Currah, R.S. 2005. Microfungal endophytes in roots. Can. J. Bot. **83**: 1–13. doi:10.1139/b04-171.

Allen, T.R., Millar, T., Berch, S.M., and Berbee, M.L. 2003. Culturing and DNA extraction find different fungi from the same ericoid mycorrhizal roots. New Phytol. **160**: 255–272. doi:10.1046/j.1469-8137.2003.00885.x.

Anderson, I.C., and Cairney, J.W.G. 2004. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ. Microbiol. **6**: 769–779. doi:10.1111/j.1462-2920.2004.00675.x. PMID:15250879.

Bidartondo, M.I., Burghardt, B., Gebauer, G., Bruns, T.D., and Read, D.J. 2004. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc. R. Soc. Lond. B. Biol. Sci. **271**: 1799–1806.

Cao, J.Z., Fan, L., and Liu, B. 1992. Notes on the genus *Galiella* in China. Mycologia, **84**: 261–263.

Colwell, R.K. 2005. *EstimateS*: Statistical estimation of species richness and shared species from samples ver. 7.5. Available from http://purl.ococ.org/estimates.

Colwell, R.K., and Coddington, J.A. 1994. Estimating terrestrial biodiversity through extrapolation. Philos. Trans. R. Soc. Lond. B, Biol. Sci. **345**: 101–118.

Dimitrova, E. 2002. Discomycetous fungi of the Leotiales found on the Betulaceae in Bulgaria. Turk. J. Bot. **26**: 253–258.

Douhan, G.W., and Rizzo, D.M. 2005. Phylogenetic divergence in a local population of the ectomycorrhizal fungus *Cenococcum geophilum.* New Phytol. **166**: 263–271. doi:10.1111/j.1469-8137.2004.01305.x. PMID:15760369.

Erland, S., and Taylor, A.F.S. 2002. Diversity of ecto-mycorrhizal fungal communities in relation to the abiotic environment. *In* Ecological studies. Vol. 157. *Edited by* M.G.A. van der Heijden and I. Sanders. Springer-Verlag, Berlin. pp. 163–200.

Erland, S., Jonsson, T., Mahmood, S., and Finlay, R.D. 1999. Below-ground ecto-mycorrhizal community structure in two *Picea abies* forests in southern Sweden. Scand. J. For. Res. **14**: 209–217.

Garbelotto, M., and Rizzo, D.M. 2005. A California-based chronological review (1995–2004) of research on *Phytophthora ramorum*, the causal agent of sudden oak death. Phytopathol. Mediterr. **44**:127–143.

Gardes, M., and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes — application to the identification of mycorrhizae and rusts. Mol. Ecol. **2**: 113–118. PMID:8180733.

Gardes, M., and Bruns, T.D. 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Can. J. Bot. **74**: 1572–1583.

Glen, M., Tommerup, I.C., Bougher, N.L., and O'Bren, P.A. 2002. Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? Mycorrhiza, **12**: 243–247. PMID:12375135.

Horton, T.R., and Ashkannejhad, S.M. 2006. Ectomycorrhizal ecology under primary succession on coastal sand dunes: interactions involving *Pinus contorta*, suilloid fungi and deer. New Phytol. **169**: 345–354. PMID:16411937.

Horton, T.R., and Bruns, T.D. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol. Ecol. **10**: 1855–1871. doi:10.1046/j.0962-1083.2001.01333.x. PMID:11555231.

Horton, T.R., Molina, R., and Hood, K. 2005. Douglas-fir ectomycorhizae in 40- and 400-year-old stands: mycobiont availability to late successional western hemlock. Mycorrhiza, **15**: 393–403. doi:10.1007/s00572-004-0339-9. PMID:16021480.

Huelsenbeck, J.P., and Ronquist, F.R. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics, **17**: 754–755. doi:10.1093/bioinformatics/17.8.754. PMID:11524383.

Izzo, A.D., Canright, M., and Bruns, T.D. 2006. The effects of heat treatments on ectomycorrhizal resistant propagules and their ability to colonize bioassay seedlings. Mycol. Res. **110**: 196–202. doi:10.1016/j.mycres.2005.08.010. PMID:16387485.

Jany, J.-L., Garbaye, J., and Martin, F. 2002. *Cenococcum geophilum* populations show a high degree of genetic diversity in beech forests. New Phytol. **154**: 651–659. doi:10.1046/j.1469-8137.2002.00408.x.

Johnson, D., Ijdo, M., Genney, D.R., Anderson, I.C., and Alexander, I.J. 2005. How do plants regulate the function, community structure, and diversity of fungi? J. Exp. Bot. **56**: 1751–1760. doi:10.1093/jxb/eri192. PMID:15928014.

Jumpponen, A. 2001. Dark septate endophytes — are they mycorrhizal? Mycorrhiza, **11**: 207–211. doi:10.1007/s005720100112.

Jumpponen, A., and Trappe, J.M. 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytol. **140**: 295–310. doi:10.1046/j.1469-8137.1998.00265.x.

Kennedy, P.G., Izzo, A.D., and Bruns, T.D. 2003. There is high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. J. Ecol. **91**: 1071–1080. doi:10.1046/j.1365-2745.2003.00829.x.

Kile, G.A., and Walker, J. 1987. *Chalara australis* sp. nov. (Hyphomycetes), a vascular pathogen of *Nothofagus cunninghamii* (Fagaceae) in Australia and its relationship to other *Chalara* species. Aust. J. Bot. **35**: 1–32. doi:10.1071/BT9870001.

Kõljalg, U., Dahlberg, A., Taylor, A.F.S., Larsson, E., Hallenberg, N., Stenlid, J., Larsson, K.-H., Fransson, P.M., Kåren, O., and Jonsson, L. 2000. Diversity and abundance of resupinate thelephoroid fungi as ectomycorrhizal symbionts in Swedish boreal forests. Mol. Ecol. **9**: 1985–1996. doi:10.1046/j.1365-294X.2000.01105.x. PMID:11123611.

Mantiri, F.K., Samuels, G.J., Rahe, J.E., and Honda, B.M. 2001. Phylogenetic relationships in *Neonectria* species having *Cylindrocarpon* anamorphs inferred from mitochondrial ribosomal DNA sequences. Can. J. Bot. **79**: 334–340. doi:10.1139/cjb-79-3-334.

Massicotte, H.B., Molina, R., Tackaberry, L.E., Smith, J.E., and Amaranthus, M.P. 1999. Diversity and host specificity of ectomycorrhizal fungi retrieved from three adjacent forest sites by

five host species. Can. J. Bot. **77**: 1053–1076. doi:10.1139/cjb-77-8-1053.

McFarland, J., and Largent, D.L. 2001. Headwaters Forest Reserve nonvascular plant survey. Bureau of Land Management, Arcata, Calif.

Niemiec, S.S., Ahrens, G.R., Willits, S., and Hibbs, D.E. 1995. Hardwoods of the Pacific Northwest. Res. Contrib. 8. Oregon State University, Corvallis, Oreg.

Nitare, J. 1988. Earth-tongues, a declining group of macrofungi in seminatural grasslands. Sven. Bot. Tidskr. **82**: 341–368.

O'Donnell, K. 1993. *Fusarium* and its near relatives. *In* The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. *Edited by* D.R. Reynolds and J.W. Taylor. CAB International, Wallingford, UK. pp. 25–233.

Rambaut, A. 1996. Se-Al: Sequence alignment editor. Available from http://evolve.zoo.ox.ac.uk/.

Richard, F., Moreau, P.-A., Selosse, M.-A., and Gardes, M. 2004. Diversity and fruiting patterns of ectomycorrhizal and saprobic fungi in an old-growth Mediterranean forest dominated by *Quercus ilex* L. Can. J. Bot. **82**: 1711–1729. doi:10.1139/b04-128.

Richard, F., Millot, S., Gardes, M., and Selosse, M.-A. 2005. Diversity and specificity of ectomycorrhizal fungi retrieved from an old-growth Mediterranean forest dominated by *Quercus ilex.* New Phytol. **166**: 1011–1023. doi:10.1111/j.1469-8137.2005.01382.x. PMID:15869659.

Ririe, K.M., Rasmussen, P., and Wittwer, C.T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. **245**: 154–160. doi:10.1006/abio.1996.9916. PMID:9056205.

Sakakibara, M., Jones, M.D., Gillespie, M., Hagerman, S.M., Forrest, M.E., Simard, S.W., and Durall, D.M. 2002. A comparison of ectomycorrhiza identification based on morphotyping and PCR-RFLP analysis. Mycol. Res. **106**: 868–878. doi:10.1017/S0953756202006263.

Selosse, M.-A., Bauer, R., and Moyersoen, B. 2002. Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol. **155**: 183–195. doi:10.1046/j.1469-8137.2002.00442.x.

Shinohara, M.L., LoBuglio, K.F., and Rogers, S.O. 1999. Comparison of ribosomal DNA ITS regions among geographic isolates of *Cenococcum geophilum.* Curr. Genet. **35**: 527–535. doi:10.1007/s002940050449. PMID:10369960.

Smith, S.E., and Read, D.J. 1997. Mycorrhizal symbiosis. 2nd ed. Academic Press, London.

Swofford, D.L. 2003. PAUP*: Phylogenetic analysis using parsimony (* and other methods), version 4.0b 10. Sinauer Associates, Sunderland, Mass.

Taylor, D.L., and Bruns, T.D. 1999. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol. Ecol. **8**: 1837–1850. doi:10.1046/j.1365-294x.1999.00773.x. PMID:10620228.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24**: 4876–4882.

Trappe, J.M. 1964. Mycorrhizal host and distribution of *Cenococcum graniforme.* Lloydia, **27**: 100–106.

Valentine, L.L., Fiedler, T.L., Hart, A.N., Petersen, C.A., Berninghausen, H.K., and Southworth, D. 2004. Diversity of ectomycorrhizas associated with *Quercus garryana* in southern Oregon. Can. J. Bot. **82**: 125–135.

Villarreal-Ruiz, L., Anderson, I.C., and Alexander, I.J. 2004. Interaction between an isolate from the *Hymenoscyphus ericae* aggregate and roots of *Pinus* and *Vaccinium.* New Phytol. **164**: 183–192. doi:10.1111/j.1469-8137.2004.01167.x.

Vrålstad, T., Myhre, E., and Schumacher, T. 2002. Molecular diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the Helotiales in burnt and metal polluted habitats. New Phytol. **155**: 131–148.

Walker, J.F., Miller, O.K., Jr., and Horton, J.L. 2005. Hyperdiversity of ectomycorrhizal fungal assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. Mol. Ecol. **14**: 829–838. doi:10.1111/j.1365-294X.2005.02455.x. PMID:15723674.

Weiss, M., Selosse, M.-A., Rexer, K.-H., Urban, A., and Oberwinkler, F. 2004. *Sebacinales*, a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol. Res. **108**: 1003–1010. doi:10.1017/S0953756204000772. PMID:15506013.

White, T.J., Bruns, T.D., Lee, S.B., and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA gene for phylogenetics. *In* PCR protocols: a guide to methods and applications. *Edited by* M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White. Academic Press, New York. pp. 315–322.

Yu, T.E., Egger, K.N., and Peterson, R.L. 2001. Ectendomycorrhizal associations — characteristics and functions. Mycorrhiza, **11**: 167–177. doi:10.1007/s005720100110.