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Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities

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Abstract

We have investigated colonization strategies by comparing the abundance and frequency of ectomycorrhizal fungal species on roots in a mature *Pinus muricata* forest with those present as resistant propagules colonizing potted seedlings grown in the same soil samples. Thirty-seven fungal species were distinguished by internal transcribed spacer (ITS) restriction fragment length polymorphisms (RFLPs); most were identified to species level by sporocarp RFLP matches or to genus/family level by using sequence databases for the mitochondrial and nuclear large-subunit rRNA genes. The below-ground fungal community found in the mature forest contrasted markedly with the resistant propagule community, as only four species were found in both communities. The dominant species in the mature forest were members of the Russulaceae, Thelephorales and Amanitaceae. In contrast, the resistant propagule community was dominated by *Rhizopogon* species and by species of the Ascomycota. Only one species, *Tomentella sublilacina* (Thelephorales), was common in both communities. The spatial distribution of mycorrhizae on mature roots and propagules in the soil differed among the dominant species. For example, *T. sublilacina* mycorrhizae exhibited a unique bias toward the organic horizons, *Russula brevipes* mycorrhizae were denser and more clumped than those of other species and *Cenococcum* propagules were localized, whereas *R. subcaerulescens* propagules were evenly distributed. We suggest that species differences in resource preferences and colonization strategies, such as those documented here, contribute to the maintenance of species richness in the ectomycorrhizal community.

Keywords: bioassay, colonization, fire, life-history, mycorrhizal ecology, ribosomal large subunit sequence databases

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Introduction

Ectomycorrhizal (EM) fungal communities are species rich. Monoculture forest stands of 0.1 hectare (ha) or less typically support tens of fungal species that intermix on root tips only mm apart (Bruns 1995). The processes that maintain such diversity, within what is generally thought of as a single functional guild, remain largely unknown, but by extrapolating from studies of plant and invertebrate marine communities, one might expect that differences in reproductive strategies (Hirschfield & Tinkle 1975), resource utilization (Grime *et al*. 1997) and recovery from disturbance (Sousa 1979) would play crucial roles. Each of these three attributes is interrelated with the epidemiology of host colonization in EM fungi owing to the obligate nature of the symbiosis (Smith & Read 1997).

In this study, we focused on two aspects of the structure of ectomycorrhizal (EM) fungal communities associated with *Pinus muricata* at the Point Reyes National seashore. First, we examined the composition of the resident EM fungi comprising a mature forest community (MFC) and compared it with the composition of the fungi comprising a resistant propagule community (RPC). Second, we tested for depth stratification of fungal species in each of these communities. These aspects of the fungal community relate to disturbance and resource partitioning, and

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can thus provide insight into both the autecology of individual species and the maintenance of species richness.

In general, the species of EM fungi that colonize seedlings in disturbed sites or potted soil are different from those that colonize seedlings in undisturbed sites. This pattern has been demonstrated repeatedly in diverse EM settings (Fleming 1983; Fox 1983; Fleming *et al*. 1985; Last *et al*. 1987; See & Alexander 1996; Simard *et al*. 1997). The EM species that respond to soil disturbance appear to do so via resistant propagules, while those that dominate undisturbed habitats appear to do so through vegetative expansion from existing mycelium rather than propagules (Fox 1983; Deacon & Fleming 1992).

Although this pattern is clear, and generally accepted, its ecological significance and associated terminology have been controversial. Initially the pattern was described in terms of succession. The fungi that colonized seedlings effectively by spore were referred to as 'early-stage' species because in plantation settings they were the first to appear. Another group, the so called 'late-stage' fungi, dominated the roots of older trees (Fleming 1983; Fox 1983; Deacon & Fleming 1992). The appropriateness of the early/late terminology has been questioned because variation in colonization patterns appear to be more continuous than dichotomous (Keizer & Arnolds 1994; Visser 1995) and because patterns for specific fungi vary across habitats (Newton 1992). The term multistage has been applied to species that arrive early and persist in the mature forest, and other more elaborate temporally based classifications have also been proposed (Molina & Trappe 1982; Keizer & Arnolds 1994; Visser 1995). In response, Dighton & Mason (1984) and Last *et al*. (1987) have de-emphasized temporal aspects, instead relating the early/late dichotomy to r vs. K selection theory and to Grimes's (1977) ruderal/stress-tolerant/competitive (R/S/C) model of plant life-history strategies. According to this view, early-stage fungi can be equated to ruderals, while late-stage fungi are K-selected and S or C strategists (Dighton & Mason 1984; Last *et al*. 1987). This categorization has the advantages of forging a connection to general ecological theories and of being process orientated (i.e. colonization and competition) rather than purely descriptive. On the other hand, the R/S/C model of life-history strategies, as applied to plants, has been controversial, with particular concern over the assumption that density-dependent selection is rare in disturbed or stressful environments and ubiquitous in undisturbed, productive habitats (Boyce 1984; Grubb 1985).

The Point Reyes *P. muricata* forests are primarily even-aged pine monocultures that regenerate following periodic crown fires. *P. muricata* is a serotinous coned species that is normally killed by fire but re-seeds prolifically afterwards (Vogl *et al*. 1977). The historical fire-return interval in the Point Reyes pine forest is ≈ 40 years (Sugnet 1985). Thus, these are forests that reach maturity at an early age and experience predictable disturbance. Both factors could contribute to the persistence of EM fungi employing r-selected or ruderal strategies via resistant propagules.

Previous work on EM fungi in the *P. muricata* forests of Point Reyes focused on the community structure of two mature (32–38 year old) stands. The dominant EM fungi of the MFC at these two sites were members of the Russulaceae and Thelephoraceae (Gardes & Bruns 1996a; Horton & Bruns 1998), but nothing was known about EM-resistant propagules in these forests. However, we expected that a RPC would exist and that it might play a key role in community dynamics because of the importance of fire in this ecosystem.

There is some evidence of differing preferences for soil depths or soil components (i.e. humus, wood, charcoal, mineral soil) among EM fungi (Harvey *et al*. 1978, 1997; Reddell & Malajczuk 1984; Stendell *et al*. 1999). Similarly, EM fungi vary widely in their enzymatic nutrient mobilization capacities (Smith & Read 1997). The possibility that ecological specialization in the form of resource partitioning contributes to species coexistence therefore deserves further study.

Materials and methods

Study site

The study site was a mature (34–38-year-old) natural stand of *Pinus muricata* forest on the south side of Limantour road, ≈ 0.2 km west of the Bayview trail, Point Reyes National Seashore, Marin County, CA, USA (38 04′ 10″N by 122 50′ 24″W). It is 0.5 km from one (Gardes & Bruns 1996a), and 0.9 km from another (Horton & Bruns 1998), previously described site. The site is a small (< 1000 m^2) island of pine surrounded by a coastal scrub community dominated by *Baccharis pilularis*, *Rhamnus californica*, *Toxicodendron diversiloba*, *Rubus* species, *Lupinus* species and *Ceanothus* species. Some of these same shrubs, particularly *Toxicodendron* and *Rubus*, were also common understory components in the forest; none are EM. No pine seedlings were present.

Sampling scheme

Five plots, separated by a minimum of 5 m each, were located along a rough transect within the central portion of this pine island. Within each plot, three soil cores (10 cm diameter × 40 cm depth) were taken; these were equally spaced along the outside of a 1-m circle. This compartmental sampling scheme was employed to achieve the aim of estimating overall diversity within the stand, as well as assessing spatial patterns at several scales. Each core was divided roughly into three portions, which were

bagged separately as 'top', 'middle' and 'bottom.' The top layer always included all the organic horizons, and often the uppermost portion of mineral soil. Samples were kept at 4 °C until further processing. Owing to the time required to process cores, plots 1 and 2 were cored in February, plot 3 in March, and plots 4 and 5 in May of 1994. In this Mediterranean climate, EM activity peaks in late winter/early spring, corresponding with our sampling times. Previous work at Point Reyes provided no evidence for seasonal trends in fungal community composition over a broader sampling period than the one employed here (M. Gardes and T. D. Bruns, unpublished). We therefore treated all these samples together when describing the fungal communities.

In situ *colonization by the MFC*

Ectomycorrhizae and roots were removed by sieving the 15 soil cores. For the bottom two layers, sieving was carried out without washing, but the top layer, which was predominantly organic material, was washed gently with a small volume of water because it was found to be impractical to remove EM root tips from dry organic material. The EM tips recovered from these cores were sorted by morphology and lyophilized as described previously (Gardes & Bruns 1996a). Each layer of each core was treated separately. Thus, only ectomycorrhizae with the same morphology, and from the same layer of the same core, were combined in a given morphotype sample. Following lyophilizing, each morphotype sample was weighed, providing a quantitative measure of active mycorrhizal biomass.

Seedling bioassays for the RPC

P. muricata seeds, collected near the study site, were surface sterilized in 30% hydrogen peroxide for 30 min, then imbibed overnight. The seedlings were germinated and grown in sterile sand/vermiculite or sand/peat for 2 to 3 weeks before transplanting to individual prewatered pots.

The soil and detritus recovered from the cores, after root removal from a given layer of the three cores comprising a plot, was pooled and then air-dried in the paper bags for 4–6 weeks. The drying period resulted in final water content means of 0.76% in the mineral layers and 7.9% in the organic layers. In order to estimate relative spore densities, 'inoculum' soil was combined with sterilized soil in a dilution series. Extra soil was collected from the site in the corresponding three layers and then steamed (for 2 h on four consecutive days) for use in controls and dilutions. Dried inoculum soil from each of the 15 plot/layer combinations was added directly to Cone-tainer® pots (RLC-4 Pine Cell, 2.5 × 16 cm; Stuewe and Sons) in the undiluted treatment, or mixed with steamed soil from the appropriate layer to produce 1:10 and 1:100 dilutions. Five replicate pots were planted for each treatment (five plots × three layers × three dilutions × five replicates = 225 seedlings). As a control, seedlings were also planted into tubes containing steamed soil only (five for each layer; total 15).

Seedlings killed by damping-off or thrips were replaced for up to 11 months after the initial planting. Some samples were split 1:1 with sand owing to poor drainage, which increased the total to 242 seedlings. Pots were maintained under fluorescent lights inside the laboratory. Fertilizers were not used in these experiments. Safer soap® and a pyrethrin insecticide were sprayed on the seedlings four to six times, as required, to combat the thrips. Harvest was conducted from 15 to 20 months after planting. Live mycorrhizal root tips were morphotyped, lyophilized and weighed, as described above. In this part of the study, a morphotype sample comprised all the root tips with the same EM morphology from a single seedling.

Molecular identification of EM fungi

DNA extraction and polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat, and subsequent restriction digests, were performed as described previously (Gardes & Bruns 1993, 1996a,b). In most cases, the two extracts from a single morphotype sample had identical restriction fragment length polymorphism (RFLP) patterns. In cases where the two RFLPs were not identical, from two to 10 additional root tips were extracted and analysed, and the total dry weight of the morphotype sample was divided, proportionally, among the resulting RFLP types. For subsequent fungal identification, morphotype samples with identical ITS RFLP patterns for both *Alu*I and *Hin*fI were considered to belong to the same taxon. We performed directed searches for matches between each unique ITS type and identified sporocarps via side-by-side ITS RFLP comparisons (Gardes & Bruns 1996b).

Owing to the fact that certain easily recognizable morphotypes were encountered repeatedly on the bioassay seedlings, we performed ITS RFLP analysis on a random subset of 20 samples from each of these consistent morphotypes to test the accuracy of the morphotyping. When all 20 samples were identical, the remaining samples assigned by morphology to that type were not subject to molecular analysis. On the other hand, when multiple RFLPs were found among individual tips within a single recognizable morphotype, every amplifiable sample was subject to ITS RFLP analysis. Unamplifiable samples from mature roots or seedlings were usually dead, and were excluded from subsequent analyses.

In cases where RFLP matches between an ITS type and a sporocarp were not quickly found, a portion of the fungal mitochondrial ribosomal large subunit was amplified from the unknown mycorrhizae, sequenced and added to

the database, as described previously (Bruns *et al*. 1998). Because this region could not be amplified from several unknowns, either the ITS (using the primers ITS1F, ITS2, ITS3 and ITS4) or the nuclear large subunit (28S) were sequenced for these taxa. We chose the 28S because it is sufficiently conserved to align across a diverse group of fungi for which hundreds of sequences are available, and fungal-specific amplification of the region from

Table 1 Sequence sources and accession numbers

Taxon	GenBank no.	Taxon	GenBank no.
Nuclear large subunit, 5′ end:		*Eocronartium muscicola*	L20280
Balsamia magnata	U42683	*Ganoderma tsugae*	X78778
Barssia oregonensis	U42684	*Hebeloma crustuliniforme*	U11918
Byssoascus striatosporus	U17912	*Helicobasidium mompa*	L20281
Cazia flexiascus	U42694	*Hygrocybe citrinopallida*	U66435
Choiromyces venosus	U42688	*Lactarius corrugis*	U11919
Ctenomyces serratus	U17913	*Leucocoprinus cepaestipes*	U85306
Dingleya verrucosa	U42686	*Marasmius delectans*	U11922
Elsinoe veneta	U43484	*Multiclavula corynoides*	U66440
Epichloe clarkii	U68413	*Multiclavula vernalis*	U66439
Gymnoascella citrina	U17915	*Phylloporus rhodoxanthus*	U11925
Helvella lacunosa	U42681	*Pleurotus cornucopiae*	U04146
Hemicarpenteles paradoxus	U29819	*Russula marei*	U11926
Hydnotrya cerebriformis	U42676	*Sirobasidium magnum*	AFO42242
Labyrinthomyces varius	U42689	*Tilletia caries*	L20285
Leucangium carthusianum	U42674	*Tremella griffa*	AFO42271
Lojkania striatispora	U43468	*Tricholoma matsutakae*	U62964
Melanomma radicans	U43479	*Ustilago hordei*	L20286
Morchella esculenta	U42669	*Ustilago maydis*	L20287
Nectria ventricosa	L36613	*Zygodesmus eriophori*	L20288
Oosporidium margaritiferum	U40090	28S unknowns	
Ophiostoma piliferum	U47837	RPC-5 (*Phialophora*-like)	AF156922
Peziza quelepidotia	U42693	MFC-16 (*Cantharelloid*-2)	AF156923
Phialophora sp.	U17158	RPC-19 (*Tricharina* sp.)	AF156921
Plectosphaerella cucumerina	L36639	RPC-9 (*Tuber californicum*)	AF156927
Pyrenophora trichostoma	U43477	RPC-8 (*Tuber* sp.-3)	AF156924
Reddellomyces donkii	U42687	RPC-18 (*Tuber* sp.-4)	AF156925
Rhizina undulata	U42691	RPC-10 (*Wilcoxina* sp.)	AF156926
Saccharomyces cerevisiae	J01355	ITS region, unknowns	
Schizosaccharomyces pombe	Z32848	RPC-11 (*Rhizopogon smithii*)	AF158017
Sphaerosporella brunnea	U38586	RPC-13 (*Rhizopogon rubescens*)	AF158018
Spiromastix warcupii	U17911	Mitochondrial large subunit, ML5–6 region, unknowns	
Talaromyces helicus	U15489		
Tricharina ascophanoides	U38580	MFC-4 (*Amanita francheti*)	AF156915
Tricharina gilva	U38574	MFC-14 (*Amanita muscaria*-like)	AF156907
Tricharina groenlandica	U38576	MFC-13 (*Amanita* sp.)	AF156909
Tricharina ochroleuca	U38578	MFC-10 (*Boletoid*-4)	AF156919
Trichothecium roseum	U69891	MFC-19 (*Cortinarioid*-2)	AF156910
Tuber gibbosum	U42690	MFC-12 (*Inocybe* sp.)	AF156920
Underwoodia columnaris	U42685	MFC-18 (*Rhizopogon subcaerulescens*)	AF156908
Verpa conica	U42671	MFC-2 (*Russula brevipes*)	AF156913
Wilcoxina mikolae	U38570	RPC-11 (*Rhizopogon smithii*)	AF156911
Wilcoxina rehmii	U38566	RPC-12 (*Suilloid*-1)	AF156912
Auricularia auricula-judae	L20278	MFC-6 (*Thelephoroid*-5)	AF156918
Boletus rubinellus	L20279	MFC-11 (*Thelephoroid*-6)	AF156914
Cantharellus cinnabarinus	U87989	MFC-17 (*Thelephoroid*-7)	AF156917
Craterellus tubaeformis	U87991	MFC-1 (*Tomentella sublilacina*)	AF156916
Cryptococcus neoformans	L14068		

All sequences of reference taxa in the 28S dataset were downloaded from GenBank. Sequences for reference taxa in the ML5/6 database are also available from GenBank, and are listed in Bruns *et al*. (1998). Sequences we obtained from the unknown mycorrhizal types have been deposited under the accession numbers listed.

mycorrhizae was feasible using existing primers. Fungal-specific fragments spanning both ITS spacers and the 5′ end of the 28S gene were amplified using the primers ITS1F and TW14 (GCTATCCTGAGGGAAACTTC). The primers Ctb6 (GCATATCAATAAGCGGAGG) and TW13 (GGTCC-GTGTTTCAAGACG) were then used in cycle-sequencing reactions to produce bidirectional sequences of ≈ 650 bases within the 28S gene, which were then combined with sequences from GenBank (Table 1) by hand to form a single dataset with independently aligned Basidiomycota and Ascomycota partitions. The ITS and 28S datasets were analysed by parsimony (heuristic search with 10 random addition replicates and default settings) using PAUP 3.1 (Swofford 1992). Owing to the computational load of parsimony analyses, the ML5-6 dataset was instead analysed by neighbour-joining under the 2-parameter model of Kimura using a test version of PAUP* 4.0. Types that were placed phylogenetically, but not matched to species, were referred to monophyletic groups, given an 'oid' ending and labelled in the numerical sequence used in previous studies at Point Reyes (Gardes & Bruns 1996a; Horton & Bruns 1998). For example, we called three unmatched types in the Thelephorales: Thelephoroid-5, -6 and -7, because they differed from four previously unmatched types in this group (Gardes & Bruns 1996a; Horton & Bruns 1998).

Statistical analyses

To test whether the abundance of particular MFC fungi was biased toward organic or mineral soil layers, we compared the biomass of a given fungus, found in the top vs. the middle layer for each core in which the fungus was found, by the Wilcoxon sign-rank test (Table 2). Overall, nearly equal mycorrhizal biomass occurred in the top and middle layers, and therefore this comparison should not be biased by overall EM distributional trends.

For the most frequent bioassay fungi, we tabulated the number of seedlings where a particular fungus was present vs. the number where the fungus was absent, stratified by plot and dilution. We then tested the null hypothesis of homogeneity of presence vs. absence over the three soil layers, controlling for plot and dilution, using the Cochran-Mantel-Haenszel strategy for $2 \times r$ tables as implemented in SAS 6.12 (Stokes *et al*. 1995). The test statistic was Q_{GMH} (general association), with depth classes as nominal variables.

Results

Molecular identification

Thirty-seven distinct fungal ITS RFLP types were found in this study (Table 2). We will henceforth refer to these 37 types as species because there is a general correspondence between ITS RFLP types and species (see Gardes & Bruns 1996a; Karen *et al*. 1997). Phylogenetic trees inferred from ML5-6 data provided well-supported family or generic placements for nine unmatched basidiomycetous species (Table 2; tree not shown). In addition, the nuclear 28S sequences provided family to generic placements for six unmatched species in the Ascomycota and one species in the Basidiomycota (Fig. 1). The ITS sequences from two unknown species with putative suilloid morphologies were compared with sequences from large datasets for the genera *Suillus* (Kretzer *et al*. 1996) and *Rhizopogon* (Grubisha 1998). One unknown was found to be closely related to *R. smithii* and the other to *R. rubescens*. Sequences and phylogenetic placements were not obtained for a total of eight types, either because the target regions could not be amplified or because the type contributed negligible biomass. Three of these types putatively belong to the Ascomycota because the ITS region could be amplified with ITS1F/ITS4 but not with ITS1F/ITS4B (Gardes & Bruns 1993), and because they had minimal mantles. None of these unidentified types were dominant species.

Seedling status and morphotype discrimination

Of the 226 seedlings that survived to harvest, only 36 were nonmycorrhizal. None of the control (steamed soil only) seedlings formed ecto- or ectendomycorrhizae, based on the presence of root hairs on root tips, although one control seedling was infected by an unidentified root pathogen. This fungus was not seen on any other seedlings. Colonization of fine roots from the mature forest approached 100%.

While our morphotyping was usually accurate, ITS RFLP analysis did occasionally indicate the need to split single morphotype samples or combine multiple morphotype samples from a single core/layer. In the RPC, accurate morphotyping of *R. subcaerulescens* and *Tomentella sublilacina* was confirmed by perfect ITS RFLP agreement within each set of 20 random samples. Other morphotypes, however, turned out to be species conglomerates composed of multiple related species, such as the six suilloid species other than *R. subcaerulescens* or the four *Tuber* species. Similarly, the four *Amanita* species of the MFC could not be consistently discriminated from one another. Had the several species in each of these three groups been treated as single entities, our interpretation of the abundance and spatial distribution of both mycorrhizae and propagules would have been significantly altered. Thus, the molecular methods provided a significant gain in precision.

The *Phialophora*-like fungus never formed a mantle, but differed from uninfected roots by the lack of root hairs.

Table 2 The mycorrhizal fungi colonizing *Pinus muricata* on roots from a mature stand and potted seedlings in the same soil

Type rank*	ML5/6 sequence	28S sequence	ITS RFLP match	Consensus	ΣMass (mg)	Frequency†	% T:M:B‡	*P*-value§
Mature forest community (MFC)								
1	Thelephorales	—	*Tomentella sublilacina*	*Tomentella sublilacina*	392.1	12 | 5	80 : 17 : 3	0.18
2	Russulaceae	—	*Russula brevipes*	*Russula brevipes*	272.6	6 | 2	36 : 56 : 8	0.69
3	Russulaceae	—	*Lactarius rufus*	*Lactarius rufus*	214.1	7 | 3	7 : 60 : 33	0.13
4	Amanitaceae	—	*Amanita francheti*	*Amanita francheti*	115.5	8 | 4	8 : 47 : 45	0.03
5	—	*Phialophora*-like	—	*Phialophora*-like	101.0	4 | 2	94 : 6 : 0	—
6	Thelephorales	—	—	Thelephoroid-5	77.4	1 | 1	0 : 0 : 100	—
7	—	—	*Amanita pantherina*¶	*Amanita pantherina*	43.3	5 | 2	20 : 30 : 50	0.89
8	—	—	*Cenococcum geophilum*	Cenococcum geophilum	25.6	1 | 1	32 : 41 : 27	—
9	—	—	—	Unknown-1	17.5	2 | 2	0 : 0 : 100	—
10	boletoid group	—	—	Boletoid-4	11.3	2 | 1	0 : 0 : 100	—
11	Thelephorales	—	—	Thelephoroid-6	8.0	1 | 1	0 : 0 : 100	—
12	Cortinariaceae	—	*Inocybe* sp.	*Inocybe* sp.	5.0	1 | 1	0 : 100 : 0	—
13	Amanitaceae	—	—	*Amanita* sp.	3.7	2 | 2	0 : 65 : 35	—
14	Amanitaceae	—	*Amanita muscaria***	*Amanita muscaria*-like	2.8	1 | 1	0 : 100 : 0	—
15	—	—	—	Unknown-2	1.4	1 | 1	0 : 100 : 0	—
16	—	Cantharellaceae	—	Cantharelloid-2	1.3	1 | 1	0 : 0 : 100	—
17	Thelephorales	—	—	Thelephoroid-7	0.7	1 | 1	0 : 100 : 0	—
18	suilloid group	—	*Rhizopogon subcaerulescens*	*Rhizopogon subcaerulescens*	0.7	2 | 1	0 : 0 : 100	—
19	Cortinariaceae	—	—	Cortinarioid-2	0.0	1 | 1	100 : 0 : 0	—
20	—	—	—	Unknown-3	0.0	1 | 1	0 : 100 : 0	—
Resistant propagule community (RPC)								
1	—	—	*Rhizopogon subcaerulescens*	*Rhizopogon subcaerulescens*	434.2	78 | 5	12 : 54 : 33	17.2; 0.001
2	—	—	*Tomentella sublilacina*	*Tomentella sublilacina*	129.5	37 | 5	65 : 16 : 19	25.3; 0.001
3	—	—	*Rhizopogon ochraceorubens*	*Rhizopogon ochraceorubens*	67.0	28 | 5	0 : 21 : 79	35.5; 0.001
4	—	—	*Rhizopogon olivaceotinctus*	*Rhizopogon olivaceotinctus*	52.3	20 | 4	5 : 55 : 40	8.3; 0.016
5	—	*Phialophora*-like	—	*Phialophora*-like	15.9	18 | 2	17 : 56 : 28	3.5; 0.174
6	—	—	*Cenococcum geophilum*	*Cenococcum geophilum*	91.3	14 | 2	29 : 43 : 28	2.7; 0.263

Table 2 *Continued*

Type rank*	ML5/6 sequence	28S sequence	ITS RFLP match	Consensus	∑Mass (mg)	Frequency†	% T:M:B‡	*P*-value§
7	—	—	Similar to *Rhizopogon olivaceotinctus*††	*Rhizopogon olivaceotinctus*-like	34.7	10 | 4	20 : 40 : 40	
8	—	*Tuber*-like	—	*Tuber* sp.-3	35.0	8 | 1	0 : 63 : 38	
9	—	*Tuber*-like	*Tuber californicum*	*Tuber californicum*	51.2	7 | 4	0 : 71 : 29	
10	—	*Wilcoxina*-like	—	*Wilcoxina* sp.	6.9	6 | 1	33 : 66 : 0	
11	suilloid group	—	ITS sequence—see text	*Rhizopogon smithii*	29.9	6 | 2	33 : 33 : 33	
12	suilloid group	—	—	Suilloid no. 1	6.9	5 | 2	0 : 60 : 40	
13	—	—	ITS sequence—see text	*Rhizopogon rubescens*	73.7	4 | 3	25 : 25 : 50	
14	—	—	—	Unknown-4	6.1	4 | 2	0 : 25 : 75	
15	—	—	Similar to *Tuber* sp.-3††	*Tuber* sp.-2	41.9	4 | 2	0 : 75 : 25	
16	—	—	—	Unknown-5	0.6	2 | 1	0 : 100 : 0	
17	—	—	—	Unknown-6	3.6	2 | 1	0 : 50 : 50	
18	—	*Tuber*-like	—	*Tuber* sp.-4	1.8	1 | 1	0 : 0 : 100	
19	—	*Tricharina*-like	—	*Tricharina* sp.	0.5	1 | 1	0 : 0 : 100	
20	—	—	—	Unknown-7	5.7	1 | 1	0 : 100 : 0	
21	—	—	—	Unknown-8	0.0	1 | 1	0 : 100 : 0	

*Rank order: by biomass for MFC; by frequency (number of seedlings) for RPC.

†Frequency, given in number of cores or seedlings, and by number of plots following the divider.

‡Percentage of mycorrhizae found in the top, middle, and bottom layers (by biomass for the MFC; by frequency for the RPC).

§Tests of homogeneity of biomass (MFC) or frequency (RPC) across soil layers. Wilcoxon paired-rank probabilities are given for the MFC, and the Mantel–Haenszel general association statistic and probability are given for the RPC.

¶*Amanita pantherina* and *A. gemata* have identical internal transcribed spacer restriction fragment length polymorphisms (ITS RFLPs) for *Hin*fI and *Alu*I, so this type may represent either or both taxa.

**This ITS type had one band that was shifted from the pattern of the single fruit-body which was assayed. However, the pattern is quite distinct from other *Amanita* species surveyed, and very similar to *A. muscaria*.

††These ITS types have RFLP patterns identical to the indicated type for one restriction enzyme, but differ slightly in the pattern produced by a second enzyme; the morphologies of these types were indistinguishable from those with which they share one RFLP pattern.

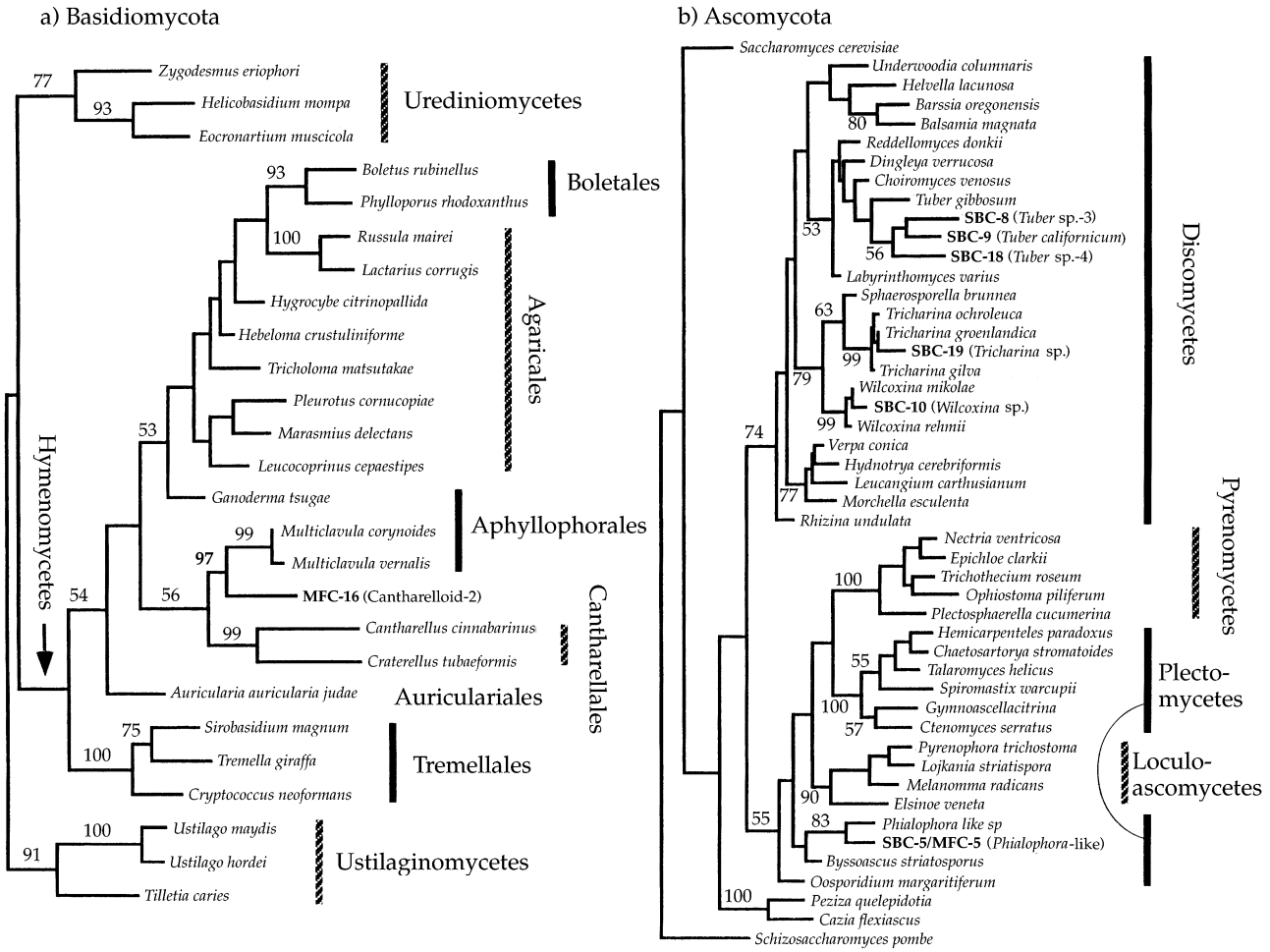

Fig. 1 Nuclear large-subunit sequence analysis. Sequences from the 5′ 680 bases of the 28S derived from unknown mycorrhizal types are designated by mature forest community (MFC) or resistant propagule community (RPC) and are numbered following Table 2. Bootstrap support from 100 replicates is provided above branches. (a) Basidiomycota. The single parsimony tree is shown. This analysis utilized 588 aligned positions. *Ustilago* species and *Tilletia carries* were designated as an outgroup. (b) Ascomycota. One of 105 most-parsimonious trees is shown. *Schizosaccharomyces pombe* was used as the outgroup. Only the first 344 positions were included as characters in this analysis owing to short sequences for some taxa. However, analyses of 600 aligned positions for the subset of longer sequences produced identical placements of the unknown mycorrhizae, higher bootstrap support for many groupings and more realistic relationships of some major groups (e.g. a monophyletic Plectomycetes; data not shown).

On the other hand, this fungus showed variable, but often quite intense, intracellular proliferation (A. Kretzer, personal communication). The presence of 'background' ITS RFLP patterns belonging to this fungus, together with expected RFLP patterns of mantle-forming fungi (such as *R. ochraceorubens*) in some extracts, suggests dual colonization as has been noted previously by Jumpponen *et al*. (1998). No other species in the present study exhibited this behaviour. The species we are calling *Phialophora*-like is similar in sequence to a pathogenic group of fungi related to *Gaeumannomyces* (Bryan *et al*. 1995), but some *Phialophora* strains increase plant survival and nutrient uptake (Jumpponen *et al*. 1998). While dark-septate fungi, with which *Phialophora* is grouped, can be very common (Stoyke *et al*. 1992; Horton *et al*. 1998; Jumpponen *et al*. 1998), their general impacts on plants are unclear owing to varying conditions in experimental studies to date and problematic taxonomy (Jumpponen *et al*. 1998).

Mature forest vs. resistant propagule communities

Inspection of Fig. 2 immediately reveals the striking lack of similarity between the fungal community colonizing the roots of the mature trees, *in situ*, and the resistant propagule community that colonized seedlings potted into air-dried soil. Only *T. sublilacina* was a dominant fungus in both assays. The other dominant fungi in the MFC did not colonize potted seedlings under our experimental

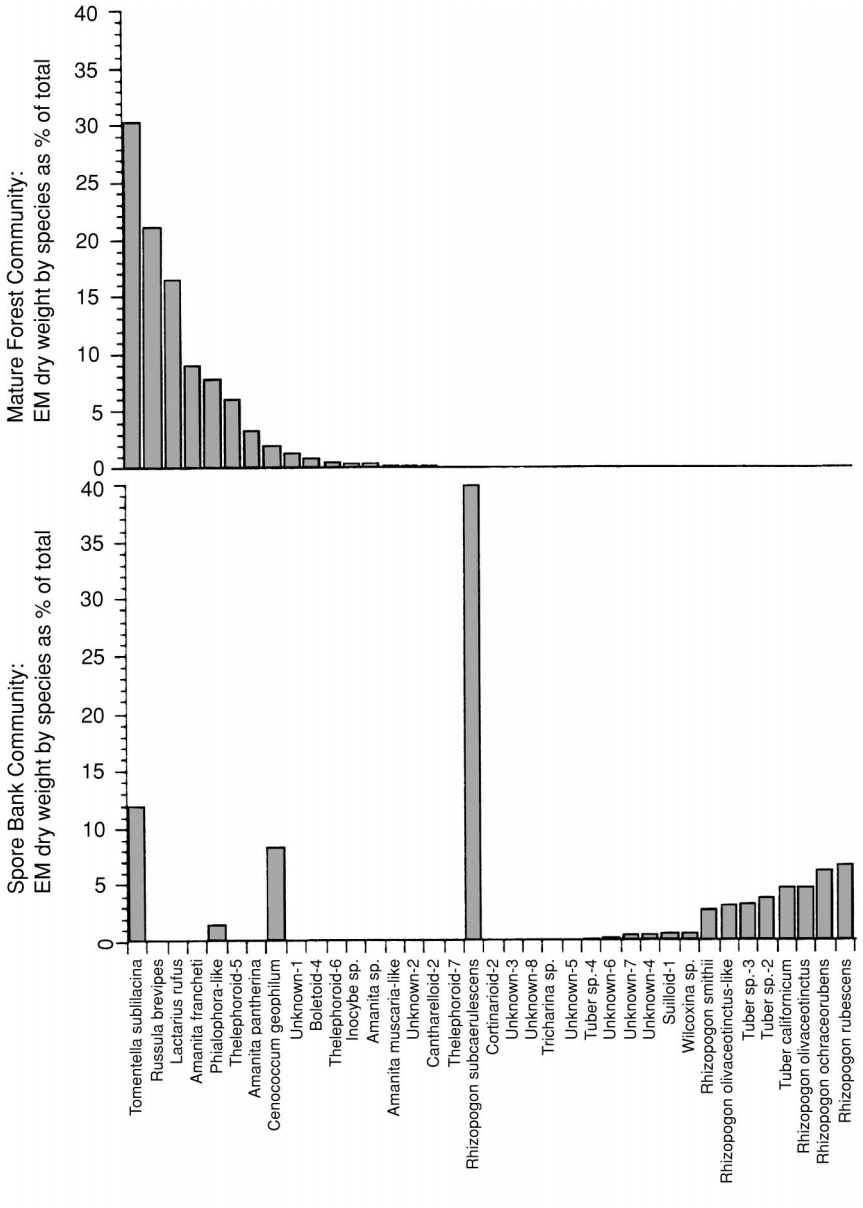

Fig. 2 Comparison of fungal communities colonizing mature forest roots vs. bioassay seedlings. Proportion of the total mycorrhizal biomass (dry weight) in each community accounted for by each fungus is shown.

conditions. Similarly, all the other dominant seedling colonizers, except *R. subcaerulescens*, were completely absent from sampled roots of the mature forest.

The lack of correlation between the two communities is also demonstrated in Table 2, which lists mature forest fungi in rank order by biomass, and seedling bioassay fungi in order by frequency. We note that biomass and frequency are only weakly correlated in the RPC. For example, *Cenococcum geophilum* was a dominant species in biomass within the RPC, ranking third overall, but was infrequent. We suggest that frequency, which depends primarily on colonization events and therefore the number of propagules, should be less influenced by the artificial nature of the potted conditions than biomass, which depends primarily on hyphal proliferation after colonization, and we therefore use frequency for comparisons and statistical tests within the RPC. However, biomass is the preferred measure in the MFC because it gives a good indication of the species that were vegetatively dominant at a particular site.

Spatial distribution

High biomass rankings were achieved by high frequency in some species and by high-density clumps in other species. For example, *Amanita francheti* was encountered frequently but never dominated the biomass of the core in which it was found. In contrast, Boletoid 4 and

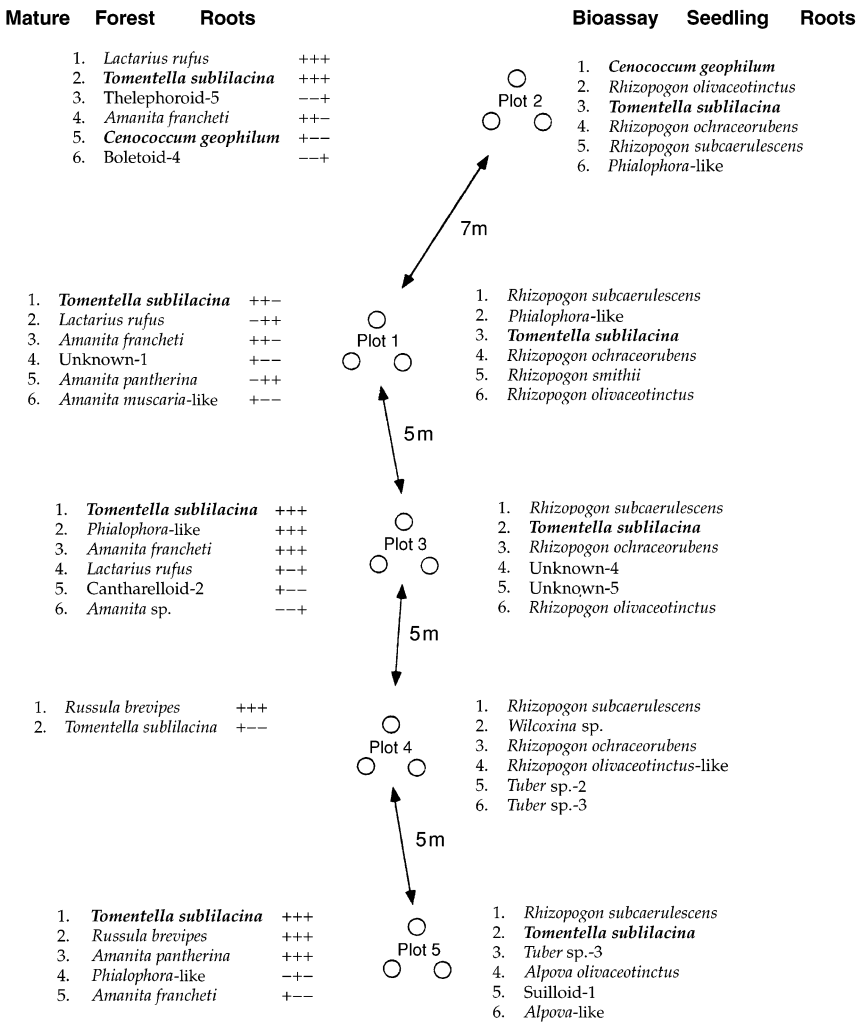

Fig. 3 Sampling scheme and rank orders of the top six mycorrhizal fungi found in each core or plot on mature roots, and on seedlings grown in the same soil sample. The open circles show the positions of individual soil cores. Each triangle of three cores comprised a plot. For mature forest community (MFC) mycorrhizae, presence or absence of a given type in each of the three cores from that plot is indicated by the three '+' and '−' signs following each taxon. Because soil samples were pooled across the three soil cores for seedling planting, the resistant propagule community (RPC) frequencies on the right side represent the entire plot, not individual soil cores.

C. geophilum were found in only a single core each, but dominated the biomass of that core. *Russula brevipes* dominated the biomass of several cores. In addition, *Ru. brevipes*, *Lactarius rufus* and *A. pantherina* were found in clustered (i.e. adjacent) cores (Fig. 3), rather than having scattered spatial distributions. *T. sublilacina* was ubiquitous, rather than clustered, but was also often dominant.

Although the seedling bioassay was an indirect measure of propagule frequency, our data suggest that several species in the RPC had unevenly distributed, localized propagule loads: *Wilcoxina* and *Tuber* sp.-3 occurred in single plots with high density (six to eight seedlings). The clumped distribution of *Wilcoxina* suggests colonization by below-ground chlamydospores rather than by airborne ascospores. Similarly, the *Phialophora*-like fungus and *Cenococcum* colonized 14–18 seedlings each, but from only two plots in each case (Table 2). All but one *Cenococcum*-colonized seedling came from plot 2, which coincides with the single, high-density occurrence of *Cenococcum* on mature forest roots (Fig. 3), and is probably explained by localized sclerotia production.

Soil vertical distribution

In terms of total combined mycorrhizal biomass in the MFC, 40% occurred in the top (mostly organic) layer, 38% in the middle (entirely mineral soil) layer and 22% in the bottom layer. The percentage of each species' total biomass found in each layer is given in Table 2. The percentages are highly skewed toward the organic layer in *T. sublilacina* and the *Phialophora*-like fungus, and toward the mineral soil layers in *Ru. brevipes*, *L. rufus* and *A. francheti*. When we tested whether these skewed percentages occurred by chance, using biomass on a core-by-core basis, the Wilcoxon rank-sum test was significant only for *A. francheti* (Table 2). The lack of significance in the other species may be caused by small sample sizes rather than by the absence of a biological pattern. Species

richness was considerably greater in mineral as opposed to organic layers, as 12 of the 14 types found in two or fewer cores were only found in the mineral horizons. Six of these were found only in the bottom layer, while five were found only in the middle layer. We tested the significance of these trends again by the Wilcoxon rank-sum test, in this case comparing the total biomass (combined across cores) in one layer against another, using each of the rare species as a replicate. These 14 rare species as a group were significantly more abundant in the middle layer vs. the top layer ($P = 0.034$), and were also significantly more abundant in the bottom layer vs. the top layer ($P = 0.036$). As the bottom layer had the least mycorrhizal biomass overall, this result is striking. These results show that coring depth can significantly influence EM species richness estimates.

In the RPC, homogeneity of occurrences across soil layers could not be rejected for *C. geophilum* and the *Phialophora*-like fungus. In contrast, the null hypothesis of homogeneity, while controlling for plot and dilution, was rejected for all other commonly occurring fungi (Table 2). Inspection of the percentages of occurrences by layer shows that the lack of homogeneity could be attributed to fewer occurrences in organic layer pots for all these fungi except *T. sublilacina*, which occurred more commonly in the organic layer pots.

We note that the washing step may have removed spores from the organic layer, so that, except in the case of *T. sublilacina*, the apparent middle and bottom bias in the bioassay must be interpreted with caution.

Dilution effects

The soil dilution series provided some insight into the relative abundance of resistant propagules of the different species. Nonmycorrhizal seedlings were found 24 times in pots containing inoculum diluted 100-fold, eight times in inoculum diluted 10-fold and only four times in undiluted soil, indicating that at least the 100-fold dilution treatment succeeded in diminishing inoculum potential. The number of seedlings colonized by a particular fungus at the time of harvest probably does not equate to the number of initial colonization events, as a result of competition and other forces. Spores of the mature forest dominants were almost certainly present, but did not result in detectable seedling colonization, thus illustrating the difficulty of directly estimating spore numbers in this way. However, for those species that did colonize seedlings, we can ask whether their relative colonization increased or decreased with dilution, thus providing an estimate of propagule numbers relative to other seedling colonizers. Based on this conservative interpretation, we suggest that *R. subcaerulescens*, *R. olivaceotinctus* and the *Phialophora*-like fungus had larger numbers of viable propagules than the other fungi, because these species accounted for 79% of all fungi in the pots containing inoculum diluted 100-fold. In contrast, *T. sublilacina* and *Cenococcum*, although common overall, accounted for only 5% of the fungi in 100-fold dilutions, suggesting more limited propagule loads of these fungi. *R. subcaerulescens* was the only fungus whose proportion of colonized seedlings dramatically increased in the pots containing inoculum diluted 100-fold.

Discussion

Common patterns among Californian MFCs

The dominance of the MFCs by the Russulaceae, Thelephorales and Amanitaceae at the present site echoes results obtained at the two sites studied previously (Gardes & Bruns 1996a; Horton & Bruns 1998). At all three sites, members of the Russulaceae and Thelephorales accounted for three of the four most abundant species, while a member of the Amanitaceae ranked either fourth, fifth or sixth. Dominance by the Russulaceae and Thelephoraceae in mature forests has also been found in an *Arctostaphylos*-dominated shrub community and a ponderosa pine community (Horton *et al*. 1999; Stendell *et al*. 1999); these latter sites are, respectively, ≈ 50 and 400 km distant from Point Reyes. Thus, a general pattern appears to be emerging, at least among Californian EM communities.

The three Point Reyes sites were fairly distinct when compared at the species level because the dominant members of the Russulaceae and Amanitaceae often differed. For example, *Russula amoenolens* and *Ru. xerampelina*-like fungi were dominant at two of the sites, but were not found at the current site, where *Ru. brevipes* and *Lactarius rufus* (Russulaceae) were instead the dominant species. Similarly, *Amanita francheti* was common at this site and at one other site, but at the third site it was not detected. This result further reinforces findings of strong spatial patchiness of dominant EM fungi, both within and between sites (Gardes & Bruns 1996a; Karen & Nylund 1997; Flynn *et al*. 1998; Gehring *et al*. 1998; Stendell *et al*. 1999).

Autecology of selected taxa in the MFC

Russula species are usually categorized as late-stage EM fungi (Deacon & Fleming 1992; Keizer & Arnolds 1994). In the Point Reyes communities, they are not abundant fruiters (Gardes & Bruns 1996a), but are dominant colonizers of mature roots (Gardes & Bruns 1996a; Horton & Bruns 1998), and did not colonize bioassay seedlings in the present study. *Ru. brevipes* and *L. rufus* were similar in that they appeared in sets of adjacent cores (i.e. had

clumped distributions) and dominated the cores in which they were found (see Fig. 3). Two possible explanations for these clumped patterns are microsite preferences or large clonal territories. The dominance of individual cores suggests strong vegetative capture of territory which, combined with the low reproductive effort in this ecosystem, suggests a fit with the competitive category of the R/S/C model. This hypothesis leads to the testable predictions that these species have large clones, are long lived and occupy a resource-rich environment.

Amanita species are also considered to be late-stage fungi (Deacon & Fleming 1992; Keizer & Arnolds 1994), but their patterns of occurrence and reproductive ecology in the Point Reyes communities are different from *Russula* and *Lactarius* species. In contrast to the Russulaceae, they are abundant fruiters in the Point Reyes forests (Gardes & Bruns 1996a), despite the fact that they did not colonize our bioassay seedlings. Compared with the Russulaceae, *Amanita* mycorrhizae were less clumped, more frequent and did not dominate the biomass of cores in which they were found. Perhaps these species are weaker vegetative competitors, but better dispersers, than the *Russula* species, a trade-off that can lead to coexistence of plant species under some conditions (Tilman 1994).

Tomentella sublilacina was the most frequent and abundant mycorrhizal fungus on mature trees in this study. It also ranked within the top three species in terms of biomass at the two sites studied previously (Gardes & Bruns 1996a; Horton & Bruns 1998) and it often dominated cores in which it was found. It is clearly an excellent competitor in mature forest settings, but in contrast to the other species discussed, it was also dominant in the RPC. The apparent organic layer preference of *T. sublilacina* mycorrhizae has been noted previously in a California ponderosa pine forest (Stendell *et al*. 1999) and in another *Tomentella* species in Canadian jack pine forests by Visser (1995).

It is clearly a 'multistage' fungus (Danielson & Pruden 1989) with excellent competitive abilities, but it is unclear what mechanism allows it to persist in the mature forest. Considering the apparent organic layer preference of *T. sublilacina* mycorrhizae, it is interesting that its basidiospores are produced on small, crust-like (i.e. resupinate) fruitbodies that coat pieces of litter and wood. The prevalence and responsiveness of its propagules in our bioassay suggests that it may re-establish frequently by spore, even in the mature forest. This idea is at odds with the nearly exclusive colonization by vegetative spread, thought to be common for dominant fungi in mature forest settings (Deacon & Fleming 1992). Perhaps this species is adapted to small-scale disturbance of the organic layer, such as drying, rodent diggings, nutrient enrichment or abundant root flushes (Bruns 1995), thereby employing a strategy like that of ruderal plants by occupying the most-disturbed microhabitat of the mature forest. This suggestion prompts the testable prediction that *T. sublilacina* has small, short-lived clones relative to other mature-forest dominants, and that it is stimulated by small-scale disturbance.

Rare species of the MFC had significantly greater biomass in each of the mineral soil layers than in the organic layer and occurred mostly in the deepest layer. Other studies have also found greater diversity in mineral soil, possibly because it is a less-disturbed habitat (Visser 1995; Stendell *et al*. 1999).

Autecology of selected taxa in the RPC

Unlike the MFC, the RPC was dominated by a variety of hypogeous fungi. These species fruit below ground and are often eaten and dispersed by rodents (Maser *et al*. 1978). *Rhizopogon* species (Basidiomycota) and the *Tuber* species (Ascomycota) are phylogenetically independent examples of this strategy. Most species in the RPC had not been found as mycorrhizae in this or in the two previous studies of MFCs within Point Reyes. *R. subcaerulescens* was the only exception; its mycorrhizae were both infrequent and low in abundance at two of the three sites studied to date (Gardes & Bruns 1996a). Thus, its uniformity in the bioassays is unlikely to be caused by residual vegetative mycelium. It was also very abundant and frequent on naturally regenerating pine seedlings that established within the former nonectomycorrhizal shrub community following the Point Reyes fire (Horton *et al*. 1998). In this latter setting, the homogeneity must be attributed to spore accumulation and persistence over time (Miller *et al*. 1992) because no vegetative mycelium could have been present.

Horton *et al*. (1998) previously suggested that *R. subcaerulescens* and *R. ochraceorubens* might be poor competitors. This suggestion was based on the fact that these species were much more abundant on postfire seedlings in the arbuscular mycorrhizal shrub community, which had low EM fungal diversity, than in the forest community, which had a much higher EM fungal diversity. Our study provides additional support for this theory. First, we showed that inoculum for *Rhizopogon* species was both abundant and widespread at our forest site; thus their scarcity on wild forest seedlings was unlikely to be the result of a scarcity of propagules. Second, we showed that the frequency of *R. subcaerulescens* increased with soil dilution; this suggests that it becomes more successful as its competitors are diluted out.

With the possible exceptions of *T. sublilacina* and the *Phialophora*-like fungus, the species of the RPC, including the ectendomycorrhizal *Wilcoxina*, agree well with the ruderal model in plants. They did not compete well in the stable habitat of the mature forest, but they have persistent

propagule banks and respond rapidly to disturbance. They have also been shown to persist as mycorrhizae for longer periods in habitats where competitors are reduced (Danielson & Pruden 1989).

Bioassay results can only be interpreted in terms of ecological strategies if similar patterns occur in the field; fortunately this appears to be the case in the Point Reyes pine forests. After the 1995 Point Reyes fire, the fungal community colonizing 1–1.5-year-old wild seedlings in our same plots was very similar to the fungal community colonizing our similarly aged bioassay seedlings; the field seedlings were dominated by *R. subcaerulescens*, *R. olivaceotinctus*, two *Wilcoxina* species and *T. sublilacina*, just as in our bioassay (Baar *et al*. 1999). The major differences were the addition of *Hebeloma* species in the field seedlings, and the reduction of *R. ochraceorubens* and the *Phialophora*-like fungus in the field, relative to our bioassays (Baar *et al*. 1999). However, elsewhere in Point Reyes, *R. subcaerulescens*, *R. ochraceorubens* and *Tuber* species were also common on postfire seedlings. Thus, members of the resistant propagule community increased dramatically in colonization success following disturbance.

Conclusions

The present study has documented striking differences in the colonization strategies of fungal species, and differences in soil layer preferences were also detected among some species. These differences may help to explain the long-term coexistence of these species in this ecosystem. Refinement of our understandings of the colonization, resource specialization and competitive strategies of these species will require additional information regarding size and persistence of individuals and the differential roles of spores vs. mycelial spread. This information will shed further insight into the coexistence of species at a given stage of forest regeneration.

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The authors are interested in plant and fungal community structure and their reciprocal impacts, as mediated by the mycorrhizal symbiosis. Highly specialized, nonphotosynthetic, myco-heterotrophic plants are a special interest because they represent an extreme in the continuum of fungal–plant community interactions.
