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Ectomycorrhizal Specificity Patterns in a Mixed *Pinus contorta* and *Picea engelmannii* Forest in Yellowstone National Park

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We used molecular genetic methods to test two hypotheses, (i) that host plant specificity among ectomycorrhizal fungi would be common in a closed-canopy, mixed *Pinus contorta-Picea engelmannii* forest in Yellowstone National Park and (ii) that specificity would be more common in the early successional tree species, *P. contorta*, than in the invader, *P. engelmannii*. We identified 28 ectomycorrhizal fungal species collected from 27 soil cores. The proportion of *P. engelmannii* to *P. contorta* ectomycorrhizae was nearly equal (52 and 48%, respectively). Of the 28 fungal species, 18 composed greater than 95% of the fungal community. No species was associated exclusively with *P. contorta*, but four species, each found in only one core, and one species found in two cores were associated exclusively with *P. engelmannii*. These fungi composed less than 5% of the total ectomycorrhizae. Thus, neither hypothesis was supported, and hypothesized benefits of ectomycorrhizal specificity to both trees and fungi probably do not exist in this system.

Ectomycorrhizal (EM) mutualisms (interactions between fungi and plant roots) provide plants with increased access to resources, such as water, nitrogen, and phosphorus (1, 18, 21, 31). They also protect plants from disease (3, 17), from chemical extremes, such as high pH, and from heavy-metal contamination (10, 11) and can facilitate establishment of pioneer plants in harsh environments, such as mine tailings (13, 38). While EM fungi are functionally similar in their roles as mycorrhizae (4), individual species may differ in physiological functions, e.g., the ability to degrade litter (16, 26); in responses to environmental conditions, such as soil temperature and moisture (4, 12); and in the specificity of interactions with host plants (28).

EM specificity is thought to influence ecosystem function and to benefit both plant and fungal partners (reviewed by Molina et al. [28]). For example, specificity could enhance carbon transfer to the fungal partner (15) and could benefit an early-successional plant by protecting portions of its root system from mycorrhizal parasitism by invading tree species. Specificity could also partition soil resources and provide "exclusive avenues" for nutrient transfer from the soil to the plant host, and because EM associations are required by many plants for survival, specificity may limit the ability of some plants to migrate and establish and thus influence the rates and directions of ecosystem change. Therefore, assessment of EM specificity patterns is critical to our understanding of ecosystem function, though it is yet unclear whether this phenomenon is common in nature.

Early specificity experiments indicated that most EM fungi could establish symbioses with most EM plants (27). Closer examination of specificity patterns reveals that the interaction phenomenon is complex and that combinations of specificity and nonspecificity occur simultaneously to influence ecosystem function and successional processes (28). Fruiting body assessments and long-term fungal community collections suggest a range of specificity patterns from generalist to specialist for both fungal species and vascular plant hosts (reviewed in reference 28). Bills et al. (7) reported that in mixed spruce and hardwood forest communities in the northeastern United States, only 8 of 54 fungal species were shared by hardwoods and spruce while 19 were associated only with spruce. In greenhouse experiments, Molina et al. (29, 30) found that a given fungus associated with only one of the two tree species tested, and soil bioassays and seedling outplantings of *Pseudotsuga menziesii* associated primarily with one genus of fungi (5, 9, 33).

These data show that EM specificity occurs in culture and may occur in nature. However, correlating laboratory studies with field analyses can be problematic, as field conditions can alter specificity patterns indicated by culture experiments. Termed "ecological specificity" by Harley and Smith (21), this phenomenon was a major justification for assessing specificity in the field. Furthermore, because it is often difficult to determine which plant species a given fungus associates with, few studies have addressed the issue of specificity by direct assessment of below-ground diversity in a natural setting. These studies usually require the tracking of single hyphae through the soil (e.g., reference 37), though studies of this type are difficult due to the fragile nature of individual hyphae. The use of molecular methods has alleviated this problem and made possible accurate in situ identifications of both fungi (19) and plants (14) that form EM in the field, enabling us to assess specificity patterns in mixed-tree-species forests.

Only a few studies have used molecular methods to assess EM specificity patterns in the field. The first indicated that a single plant species can associate exclusively with a single fungus, often across a broad geographic range (15), and the second involved mycotrophic orchids (36). Both plants were special cases involving achlorophyllous, epiparasitic plants that rely upon common mycorrhizal connections with neighboring trees for fixed carbon (8, 39). Another study, of two conifer species (22), concluded that multiple-host fungi dominated on mycorrhizal roots and colonized the roots of competing plant

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hosts. A third study (23) assessed patterns of EM sharing between an angiosperm and a conifer and concluded that sharing of EM fungi by *Arctostaphylos* sp. and *P. menziesii* facilitated the establishment of the conifer in sites dominated by the angiosperm. Because of the potential importance of EM specificity to ecosystem function, and the conflicting results of laboratory and field experiments, our objective was to use molecular methods to test for specificity in the field.

In this study, we tested the hypotheses that a closed-canopy forest would exhibit a high degree of EM specificity and that the early-successional tree species in a mixed stand would exhibit greater specificity than the invading tree species (25, 26, 28). We performed this test in a successional system in which *Pinus contorta* Douglas ex Louden (lodgepole pine) establishes after stand-replacing fire and is invaded and eventually replaced by *Picea engelmannii* Parry ex Engelmann (Engelmann spruce) and at a stage of forest development in which both tree species are codominant.

MATERIALS AND METHODS

EM were collected from three replicate mixed *P. contorta-P. engelmannii* 10-m by 10-m blocks located along the western shore of Yellowstone Lake, approximately 100 m north of the Pumice Point turnout. The soils were obsidian sand with a 2- to 5-cm-thick litter layer and sparse-to-thick *Vaccinium scoparium* Cov (grouse whortleberry) undergrowth. This system experiences natural fire disturbance triggering a successional series approximately every 300 years, and stand-replacing fires occur regularly at various spatial scales throughout the park (32). The series begins with *P. contorta* establishing first after a fire, and canopy closure with pure *P. contorta* occurs after 50 to 100 years. Later successional trees, in this case *P. engelmannii*, begin to establish after approximately 150 years and dominate after approximately 300 years.

We sampled in sites in which *P. contorta* stems were larger (28-cm mean diameter at breast height) than those of *P. engelmannii* (17-cm mean diameter at breast height) ($P < 0.0001$) and *P. contorta* trees/trees outnumbered *P. engelmannii* by approximately three to one ($P < 0.0001$). As a consequence, soil-coring sites were selected so that cores were taken from plots containing approximately equal numbers of both tree species, and coring plots were situated equidistant between individuals of the two species.

We sampled in late June, after bud break, approximately one-third of the way through the Yellowstone growing season. Because spatial variation in the EM fungal community could account for more variation than specificity patterns (24), we performed a preliminary study to determine the spatial scale at which we should sample. We took 12 cores, each 8 cm in diameter and 25 cm in depth (two plots of three cores each from two stands separated by 7 km), and observed that, at this scale, overlap among the blocks was too low for statistical analysis (data not shown). In the present study, we sampled for specificity on a smaller spatial scale using cores collected from three blocks separated by 20 to 100 m. Each block comprised three 5- by 5-m plots, with three cores taken from each plot (total number of cores, 27).

The cores were kept on ice until they were processed, soaked overnight in sterile water, and sieved to separate the EM tips from soil and rocks. The tips were sorted by morphotype for color and shape (2). Root tips were confirmed as mycorrhizal microscopically (22), and then individual morphotypes were subsampled from each core for PCR analysis using the following protocol: 1 to 3 individual mycorrhizae/core were sampled if the tip total within a core for that morphotype was less than 5, 3 to 5 were sampled if there were 5 to 10 tips of a particular morphotype, and 5 to 10 were sampled if there were more than 10 individuals of a given morphotype. To ensure that we found all of the diversity present in the EM community, we divided the morphotypes into subgroups for restriction fragment length polymorphism (RFLP) analysis. For example, an amber type would be separated into morphotypes with either single or multiple bifurcations. Split morphotypes of the same genetic type were later recombined for quantification of total EM of each species in the system (22).

DNA was extracted from individual EM by a cetyltrimethylammonium bromide extraction method described by Cullings (14) and amplified by PCR. To identify fungi forming EM, fungal DNA was amplified from individual EM with fungus-specific primers (ITS1f and ITS4b) for the nuclear ribosomal internal transcribed spacer (19). Thermocycling was conducted as follows: initial denaturation at 95°C for 95 s followed by nine cycles of denaturation at 94°C, primer annealing for 55 s at 55°C, and extension for 45 s at 72°C; nine additional cycles with 2 min at 72°C extension; nine cycles with 3 min at 72°C extension; and a final 10-min extension step at 72°C. Identifications of fungi forming individual EM were made by RFLP comparisons of restriction digests of amplified fungal EM DNAs to those of DNA amplified from reference fungal fruiting bodies (19) using two restriction enzymes, *Alu*I and *Hin*fI. Restriction fragment sizes were determined by graphing the distance traveled on the gel and comparing it to a fragment size marker (DNA Marker VIII; Boehringer Mannheim Corp., Indianapolis, Ind.). Patterns that were nearly identical were run on the same gel to confirm their differences. Trees forming individual EM were identified to the genus level. Plant DNA was amplified using the plant-specific primer combination 28KJ-28C and using the PCR amplification program described above (14). The amplified plant DNA was digested with *Hin*fI and *Rsa*I, and the restriction patterns of EM were compared to those of DNA amplified from reference needles collected at the site.

Fruiting bodies (mushrooms) for fungal species identification were collected over 2 years (1995 and 1996) both inside and outside the core sampling plots.

A contingency table and chi-square test (26 degrees of freedom) were used to test the hypothesis that the subset of fungi present was dependent upon the tree species. The contingency table is designed for just such a test (35). In our initial surveys, we discovered that cores taken within a few centimeters of each other could fail to detect the same fungal species. Thus, one core could sample the middle of a dense patch of EM of a given fungal species while an adjacent core might strike only the edge of the same patch, resulting in a much lower EM quantification. Therefore, we evaluated only the presence or absence of a fungal species on each tree species in a core rather than the abundance or biomass of individual EM root tips. The sizes and the mean difference in the number of individuals of each tree species (*P. contorta* and *P. engelmannii*) were analyzed by Student's *t* test.

RESULTS

We analyzed approximately 726 individual EM. These samples were distributed nearly equally between *P. engelmannii* and *P. contorta* (52 and 48%, respectively). The distribution of EM was patchy, so the numbers of species/core and of individual EM/core were variable (mean number of EM species/core, 2.8 [standard error, 1.5]; mean number of individual EM/core, 290 [standard error, 230]). We detected 18 RFLP patterns (Table 1), which accounted for greater than 95% of the total EM in the system. Ten additional RFLP types were detected in numbers too small to assess specificity and were therefore omitted from the analysis. (The numbers which follow the genus names are RFLP genetic type codes that link EM genetic types to fruiting body species.) Only five genera were detected in 5 or more cores of 27 sampled: *Russula* strain 38, *Inocybe* strain 11, *Cortinarius* strain 10, *Cortinarius* strain 11, *Hygrophorus* strain 50, and *Suillus tomentosus*. These fungal species composed 15, 12, 10, 10, 9, and 6% of the total EM root tips, respectively (Table 1).

There was no significant difference between groups of fungi detected on *P. contorta* and *P. engelmannii*, and all fungal species detected in two or more cores were found on both *P. contorta* and *P. engelmannii*, as were several of those found in only a single core. We observed this pattern in all cores in which each non-host-specific fungal species was detected. Some species that were found in only one core (e.g., *Cortinarius* strain 23, *Inocybe* strain 65, and *Hygrophorus* strain 49) were observed only on roots of *P. engelmannii*, but these data alone are insufficient to confirm that these fungi specifically colonize this host.

DISCUSSION

Our results provide little support for the hypothesis that specificity exists in a late-successional forest stand and none for the hypothesis that specificity would be more common in an early-successional tree species. In fact, *Cortinarius* strain 13 was the only fungal species detected in two cores that exhibited specificity, and this species was detected only on *P. engelmannii*. Fruiting body surveys suggest that *Cortinarius* spp. associate with a wide range of host species and that some species fruit only in association with *Picea* (27). Specificity is hypothesized to increase the competitive abilities of plant species by providing access to an exclusive pool of nutrients (28). However, this fungal species was relatively rare, composing less than 3% of the total number of individual EM sampled. Nevertheless, *Cortinarius* strain 13 also may benefit from the ex-

TABLE 1. Specificities, abundances, and RFLP patterns of the 18 most abundant EM fungus species

EM fungus species[a]	EM association			EM fungus fragment lengths (bp)	
	Host tree[b]	No. of cores[c]	No. of EM[d]	*Alu*I	*Hin*fI
Russula 38	LpP, ES	18	900	490, 300, 80	404, 360, 120, 50
Inocybe 11	LpP, ES	9	700	470, 140, 50[e]	320[e], 45
Cortinarius 10	LpP, ES	6	600	470, 140, 50[e]	320, 300, 125
Cortinarius 9	LpP, ES	6	600	410, 145, 80	320, 282, 115
Hygrophorus 50	LpP, ES	6	525	400, 130, 80, 70	320[e], 125
S. tomentosus	LpP, ES	5	365	690, 100, 70	242, 147, 70
Russula 43	LpP, ES	4	110	500, 300	420, 380
Inocybe 15	LpP, ES	4	500	670, 140, 50	404, 330, 120
Cortinarius 16	LpP, ES	4	40	410, 330, 120	320, 70, 140
Russula 39	LpP, ES	4	510	505, 290	395, 285, 120
Chroogomphus 35	LpP, ES	2	200	410, 400	404[e]
Cortinarius 13	ES only	3	160	580, 170, 70	404, 350, 130
Cortinarius 17	LpP, ES	1	250	404, 160, 100[e]	330, 190, 150, 120
Inocybe 65	ES only	1	75	480, 180, 60	360, 120
Cortinarius 23	ES only	1	75	600, 150, 80	404, 330, 130
Lactarius 42	LpP, ES	1	50	510, 282, 70	400, 320, 125
Russula 36	LpP, ES	1	115	505, 170, 130, 70	404, 260, 130, 110
Hygrophorus 49	ES only	1	75	395, 180, 95, 67	320, 310, 120

[a] Numbers following genus names indicate RFLP codes for cross reference to our fruiting body database. Many of the species, or genetic entities, used for analysis are cryptic species of red-capped *Russula* and small *Cortinarius*. Both are extremely difficult to identify to the species level reliably (6); thus, genetic-type codes are used here.

[b] LpP, lodgepole pine (*P. contorta*); ES, Engelmann spruce (*P. engelmannii*).

[c] Number of cores in which each fungal species was detected ($n = 27$).

[d] Total number of individual EM tips of the species in the system.

[e] Double; two bands with nearly indistinguishable sequence lengths were observed.

clusive association; it has been hypothesized that specialization could provide more efficient transfer of carbon from the plant to the fungal associate (15, 20). Experiments utilizing ^{14}C-labeled substrate are needed to test this hypothesis.

Overall, our results indicate that specificity is rare in this system. Dominance by broad-host-range fungi was reported in a *P. menziesii-Pinus muricata* forest in Marin County, Calif. (22). Like *P. contorta* forests of Yellowstone, this system is subject to frequent fires (32). In both cases, one tree species in each system (*P. muricata* in Marin County and *P. contorta* in Yellowstone) reestablishes after fire via release of seed from serotinous cones. Thus, these EM fungi may benefit from the ability to associate with both tree species in each system (22). A somewhat contrasting result was found in a successional system involving the invasion of a conifer, *P. menziesii*, into sites dominated by an angiosperm, *Arctostaphylos* (manzanita). Although the two species shared EM, some level of specificity was found on the part of the early-successional *Arctostaphylos* (23). We think that future studies should be designed to account for the full range of life history strategies and phylogenetic diversity of plant species in natural ecosystems.

Low specificity can affect ecosystem function in several ways. Hyphal connections shared among host plants may promote exchange of nutrients and photosynthate between plants of the same or different species, and therefore between overstory and understory plant populations (28). For example, there is a net flow of fixed carbon to shaded *Pseudotsuga* from unshaded *Betula* in seedlings planted in the field, via common EM connections (34). The ability of a plant to form EM with many fungal species may also increase the plant's ability to obtain fungal associates and hence nutrients and photosynthate, thereby enhancing its fitness (21). Low specificity, however, could be a disadvantage to existing plant species if newly establishing plants could utilize the existing mycota and effectively parasitize existing mycorrhizal associations (28). *P. engelmannii* individuals, being smaller and establishing in the shade of *P. contorta*, could benefit from the interaction in this way. However, although we detected little or no specificity on the species level, different fungal genes may associate specifically with a particular plant or plant species (22), restricting the avenues of carbon transfer. Development of individual genet-level DNA markers to determine whether hyphal connections exist between tree species, coupled with experiments utilizing ^{14}C-labeled substrate to track the net transfer of fixed carbon through the system, will be required to test this hypothesis.

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