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# Population, habitat and genetic correlates of mycorrhizal specialization in the 'cheating' orchids *Corallorhiza maculata* and *C. mertensiana*

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**Abstract**

**Unlike photosynthetic plants, several distantly related nonphotosynthetic plants are highly specialized toward their mycorrhizal fungi. It is unknown whether this specialization varies geographically or is influenced by the environment. We have investigated these questions in the nonphotosynthetic orchids *Corallorhiza maculata* and *C. mertensiana* by amplifying fungal internal transcribed spacer (ITS) fragments from widespread mycorrhiza samples and then discriminating putative fungal species using ITS restriction fragment length polymorphisms (RFLPs). Three fungal species were found across 27 plants representing seven populations of *C. mertensiana*; 20 species were found across 104 plants and 21 populations of *C. maculata*. All fungi belonged to the Russulaceae, an ectomycorrhizal family. Partitioning of Simpson's diversity showed that 48% of the variance in occurrences of fungal species coincided with population boundaries in *C. mertensiana*, vs. 68% in *C. maculata*. This differentiation coincided with geography but not habitat in *C. mertensiana*. In contrast, likelihood ratio tests showed strong associations between fungal occurrence and both habitat and phenotype in *C. maculata*. For example, *C. maculata* populations growing under oaks had no fungi in common with nearby populations growing under conifers, and those above 2000 m had no fungi in common with those below 2000 m. However, plant genetic differentiation may underlie some of these patterns. *C. mertensiana* and *C. maculata* never shared fungal species, even when growing intermixed at the same site, demonstrating genetic control that was independent of habitat. Similarly, intermixed normal and pale-coloured variants of *C. maculata* had no fungal species in common. These results demonstrate fine-scale genetic influences and geographical mosaicism in a mycorrhizal interaction.**

*Keywords*: ectomycorrhiza, fungal ITS, genetic variation, geographic mosaic, myco-heterotrophy

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## Introduction

Specificity, defined as the phylogenetic breadth of the associations formed by a particular species (Thompson 1994), is an important ecological and evolutionary attribute of any symbiosis. However, a simple taxonomic list of associations found in a particular species may mask significant complexity, especially structuring of specificity among or within populations (Thompson 1994). Specificity can potentially be influenced by extrinsic factors such as geography, season and habitat, and by intrinsic, i.e. genetic, factors. For example, parasites often jump to novel hosts following expansion of host or parasite geographical ranges (Feder *et al.* 1990). Seasonal changes in associations are particularly likely in less intimate interactions, such as pollinator activities which follow seasonal flowering phenologies. Switches in associations during ontogeny often occur in intimate parasites or grazers, especially those with distinct developmental phases, such as rust fungi and aphids which alternate between various plant hosts at each developmental stage (Petersen 1974; Dixon & Kundu 1994). Individuals from a single

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species that occupy diverse habitats may form different associations in different habitats for many reasons (Rowan & Knowlton 1995). Finally, genetic variation in traits determining specificity can create a 'geographical mosaic' of individuals and populations with differing intrinsic specialization (Thompson 1994). All of these sources of variation can potentially affect the evolution of specificity.

The degree of specificity has critical ramifications for the mycorrhizal symbiosis, because it determines which of the many conceivable plant–fungal interactions (linkages) actually occur at a site (Molina *et al*. 1992). These linkages, in turn, control plant access to soil resources (Read 1991), fungal access to carbon (Jones *et al*. 1991), and interplant connections (Brownlee *et al*. 1983; Cullings *et al*. 1996; Simard *et al*. 1997; Taylor & Bruns 1997; Horton & Bruns 1998; Horton *et al*. 1999). These connections influence plant competition (Perry *et al*. 1989; Simard *et al*. 1997), succession (Janos 1980; Amaranthus & Perry 1994), nutrient cycling (Bending & Read 1995), and plant community structure (Bever 1994; Francis & Read 1994; Bever *et al*. 1997). Specificity is regarded as low in arbuscular mycorrhizae from both plant and fungal perspectives (Smith & Read 1997) and is generally low in ectomycorrhizal plants toward their fungi (Molina *et al*. 1992). However, specialization of ectomycorrhizal fungi towards plants varies from low (associations across plant orders) to high (restriction to a single genus) (Borowicz & Juliano 1991; Molina *et al*. 1992).

Specificity in orchid mycorrhizae has been controversial for many years (Curtis 1937; Curtis 1939; Clements 1988). Assessment of specificity by *in vitro* determination of the range of fungi which support germination and growth of orchid seeds has lead some to conclude that specificity is low (Hadley 1970). In contrast, studies of the fungi associated with orchids in the wild demonstrate variable but often quite high specificity (Warcup 1971; Warcup 1981; Masuhara *et al*. 1993; Perkins & McGee 1995; Zelmer & Currah 1995; Taylor & Bruns 1997). Controversy concerning specificity has continued, in part, due to difficulties in identifying mycorrhizal fungi based solely upon vegetative morphology.

All orchids that have been studied form endogenous mycorrhizal structures reminiscent of arbuscular mycorrhizae (Smith & Read 1997). However, unlike arbuscular mycorrhizal fungi, which belong to the Glomales of the Zygomycota, all orchid fungi recorded belong to the Basidiomycota (Burgeff 1959), and most belong to the form-genus *Rhizoctonia*. The *Rhizoctonia* species associated with orchids include opportunistic soil pathogens (Adams 1988) and free-living saprophytes (Burgeff 1959) as well as fungi with poorly known trophic niches. The latter category includes *Sebacina* (Warcup 1988; Roberts 1993) and *Tulasnella* (Worrall *et al*. 1997). This array of fungal trophic niches contrasts with the obligately mycorrhizal niche of the fungi associated with arbuscular or ectomycorrhizal plants. Thus, orchid mycorrhizae are unique anatomically, taxonomically and functionally.

The Orchidaceae contains a disproportionate number of myco-heterotrophic species which have lost the capacity to photosynthesize (Furman & Trappe 1971; Dressler 1993; Leake 1994). The few orchids that have been studied acquire sugar from associated fungi, rather than providing it (Smith & Read 1997), which may explain the evolutionary propensity of orchids for the loss of photosynthesis. As the reciprocal plant–fungal exchange upon which mutualistic mycorrhizal interactions are based has been short-circuited by orchids, the entire family appears to consist of mycorrhizal 'cheaters'. Many nonphotosynthetic orchids have abandoned interactions with *Rhizoctonia* species in favour of other Basidiomycete species (Furman & Trappe 1971), possibly due to greater carbon availability (Taylor & Bruns 1997). Fungal associations in several of these myco-heterotrophic orchids have been described as specific (Kusano 1911; Campbell 1962; Campbell 1963; Campbell 1970; Terashita & Chuman 1987; Zelmer & Currah 1995), but this has been confirmed by widespread sampling in only one case (Taylor & Bruns 1997).

We have previously documented marked specialization in the nonphotosynthetic, cheating orchids *Corallorhiza maculata* and *Cephalanthera austinae*: each associates exclusively with fungi from a single family (Taylor & Bruns 1997). In contrast, mutualistic ectomycorrhizal plants usually form associations across tens of Basidiomycete families (Molina *et al*. 1992). We have argued that the specificity in these orchids resembles that frequently seen in more conventional parasites (Taylor & Bruns 1997). However, this specificity and parasitism may represent only an extreme in the continuum of mycorrhizal interactions, rather than a clearly separable phenomenon. Whether geographical mosaics are characteristic of mycorrhizal interactions, as they appear to be in phytophagous insects and other interactions (Thompson 1994), is a question of broad significance. This question is most easily addressed by the study of myco-heterotrophic plants due to their apparently high specificity. It is unknown whether specificity in myco-heterotrophic plants is influenced by the environment or whether it varies genetically within a species. This information is also critical to understanding the evolution of specialization in these plants.

To address these questions, we have conducted hierarchical sampling of *Corallorhiza maculata* and its sister species, *Corallorhiza mertensiana*, as follows. First, we assessed seasonal variation in fungal symbionts by sequential sampling of several orchids in a single population. Second, we tested for habitat and plant phenotype effects on

fungal species composition by sampling multiple orchid populations which were (a) growing in contrasting habitats or (b) contained orchids with differing phenotypes growing in the same habitat at the same site. Third, we analysed population structuring of specificity across all samples by partitioning the Simpson measure of species diversity for the fungal symbionts into within and among population components. Fourth, we mapped the geographical distributions of fungal species across a wider set of samples. The results show that specificity is spatially structured and strongly associated with habitat differences in one of the two orchids, but also suggest genetic control at a fine scale.

## Materials and methods

### *Orchid sample collection*

In general, small portions of the underground rhizomes (not whole plants) of the spotted coral root, *Corallorhiza maculata* (Rafinesque) Rafinesque, and the western coral root, *Corallorhiza mertensiana* Bongard, were collected when flower spikes were visible, from May to August. Data on floral morphology were collected for only some individuals because many flower spikes were pre- or postbloom at the time of sampling.

In order to analyse fungal associations in a hierarchical fashion, we attempted to sample several individuals which clearly belonged to the same population within a site and then travel a minimum of several tens of kilometres before sampling at a new site. Flowering adults were sampled randomly within a site. However, due to the low population densities of these species, we were usually reluctant to sample more than 2–6 individuals at a site. We assigned plants to the same population if they were growing within 2 km of each other, and if there were no major habitat breaks or barriers between individuals. The closest populations were 11 and 12, which were 8 km apart. Our designation of population boundaries was, by necessity, arbitrary, due to lack of information concerning gene flow or demographics in these plants. It should be noted, however, that high selfing rates and complex morphological variation have been documented (Freudenstein 1997).

In the seasonal study, four sequential samples were taken from a single large individual of *C. maculata* in June, September and December 1993, and again in April 1994. In addition, single samples from five other plants were collected in May, July, September, and December 1993 and April 1994. This sampling was all within Wildcat Regional Park (population 4).

To test whether the species of fungal symbionts were correlated with the dominant overstorey tree species, we sampled random orchid individuals from two populations (5 and 9) growing in conifer-dominated sites and from three populations growing under pure oak canopies (populations 1, 3 and 4) and compared the fungal associates. These populations are all located in the Coast Ranges of Northern California at similar elevations.

Three orchid populations occurring above 2000 m in the Sierra Nevada mountains (populations 2, 6 and 10) were compared with two nearby Sierra Nevada populations growing below 2000 m (populations 8 and 13) to test for an association between elevation and specificity. This elevation cut-off was determined partly by the range of sites where populations could be located. However, among the populations sampled, it also coincides with a transition from pine and Douglas fir (*Pseudotsuga menziesii*)-dominated forests at the low-elevation sites to true fir (*Abies* spp.) forests at the high-elevation sites.

Floral variation was used to estimate genetic correlates of specificity at two levels. In both cases we compared the fungal symbionts of plants growing close together in the same habitat in order to control for possible habitat influences. First, we tested for differences in the fungal associations of *C. maculata* and *C. mertensiana* where they grew together in four populations ranging from northern California to mid-Washington (populations 7, 12, 15 and 17). *C. mertensiana* has been considered a subspecies of *C. maculata* in some treatments, but was given species rank in the monograph of Freudenstein (Freudenstein 1997), and was consistently identifiable by labellum shape, mentum length and inflorescence colour at our study sites. Fourteen of these individuals were not sampled randomly; rather, they were sampled in *C. maculata*/*C. mertensiana* pairs where the two orchids were growing close together in order to maximize the chances that they would have the same fungal symbiont (i.e. no statistical association between plant species and fungal species). Second, fungal symbionts in normally coloured red/brown individuals of *C. maculata* var. *occidentalis* were compared to those of co-occurring *C. maculata* individuals with pale yellow flowers within a single site (population 24). Plants with the pale yellow colouring are less common than red/brown individuals, but are found intermittently across most of the range of *C. maculata* and have sometimes been referred to as var. *flavida*. However, colour variation does not appear to be a reliable systematic character in *C. maculata* (Freudenstein 1997). The pale individuals at this site corresponded most closely to var. *occidentalis* according to the more informative character of lip shape (Freudenstein 1997).

To estimate the geographical distribution of symbiotic interactions with each fungal species (defined based on molecular variation, see below), we sampled additional orchid individuals in locations where population sampling was unfeasible, and mapped the occurrence of each fungal species across every orchid sample.

Within one to four days of harvest, samples were rinsed in tap water and scrubbed with a brush, then blotted dry. Portions to be used for molecular identification were cleaned, frozen and stored at −20 °C. Approximately 70% of the *C. maculata* samples presented here were included in a previous study (Taylor & Bruns 1997), but analyses of habitat, phenotype and population partitioning of fungal species diversity have not been performed previously.

Ectomycorrhizae formed by surrounding photosynthetic trees were not harvested systematically, but were inadvertently collected along with orchid material on several occasions. They were sorted by morphology under a dissecting microscope (Agerer 1991) then lyophilized.

### *Molecular identification of fungal symbionts*

Our molecular approach to fungal identification was to amplify the highly variable internal transcribed spacer (ITS) region of the nuclear ribosomal repeat using fungal-specific primers (Gardes & Bruns 1993), then digest this amplicon with three different restriction enzymes to obtain species-specific restriction fragment length polymorphism (RFLP) banding patterns (Gardes & Bruns 1996a; Karen *et al*. 1997). These patterns were then compared with reference patterns from identified fungal fruitbodies to identify mycorrhizal symbionts at the species level. A conserved fungal mitochondrial ribosomal region was amplified and sequenced from samples for which ITS–RFLP matches were not found in order to obtain family-level placements of all fungal symbionts encountered (Bruns *et al*. 1998).

As we knew that at least *C. maculata* was associated with diverse fungal species within the Russulaceae (Taylor & Bruns 1997), we collected and dried *Russula* fruitbodies from numerous locations in order to maximize the chances of finding fruitbodies that were ITS–RFLP matches to orchid symbionts. Fruitbodies of fungi in the Russulaceae were also kindly provided by the Harry D. Thiers Herbarium of San Francisco State University (SFSU) and by Thomas R. Horton, who also shared *Russula* molecular data. Many of the fruitbodies we collected, as well as those deposited at SFSU, were identified by Harry D. Thiers.

Mycorrhizal tissue 2–15 mm distal to growing meristems of previously frozen *Corallorhiza* rhizomes was selected for DNA extraction by the following modification of a protocol provided by Kerry O'Donnell (USDA Northern Regional Research Center, Peoria, IL, USA). In general, separate extractions from two distant (i.e. 4–20 cm apart) rhizome branches were carried out for each orchid individual. For each extraction, three to five 1 mm-thick cross-sections were deposited in 1.5 mL Eppendorf tubes. The tissue was frozen and thawed three times in 400 μL of extraction buffer (25 mM EDTA, 250 mM NaCl, 0.5% SDS, 200 mM Tris), then ground using a 1.5-mL sterile plastic pestle (Konte) attached to a power drill. Samples were extracted with an equal volume of chloroform. The aqueous phase was moved to a new tube, then treated with the Gene Clean® DNA purification kit according to the manufacturer's instructions. This protocol employs binding of DNA to glass particles under high salt conditions to remove contaminants. We found that 5 μL of glass milk and two washes of the bound DNA resulted in consistent PCR amplification. DNA was released from the glass milk into 100 μL of TE buffer or $H_2O$ and diluted 10–50-fold prior to PCR amplification. DNA was extracted from fungal fruitbodies and lyophilized ectomycorrhizae following the CTAB miniprep of Gardes and Bruns (Gardes & Bruns 1996b).

Initially, the Basidiomycete-specific primer ITS4B (Gardes & Bruns 1993) was used along with the general fungal primer ITS1F (Gardes & Bruns 1993) to amplify the fungal internal transcribed spacer (ITS) region of the nuclear ribosomal repeat from *Corallorhiza* mycorrhizae and fungal fruit bodies. As all orchid fungi that have been recorded belong to the Basidiomycetes, the discrimination against Ascomycete fungi provided by ITS4B seemed desirable. However, later trials showed that ITS4B also discriminates against some 'lower' Basidiomycetes including *Rhizoctonia* species known to associate with orchids. For this reason, we used the universal primer ITS4 rather than ITS4B to amplify samples collected late in the study, and reanalysed many of the samples collected early in the study using ITS1F/ITS4 as well.

The primer pair ML5/ML6 (White *et al*. 1990) was employed to amplify fungal mitochondrial ribosomal large subunit fragments for sequencing spanning the corresponding region (Bruns *et al*. 1998). Amplification reactions of 25 and 50 μL were carried out with final concentrations of 200 μM for each dNTP, 0.5 μM for each primer, 50 mM KCl, 10mM Tris-HCl (pH 8.3), 2.5 mM MgCl, 0.1 mg/mL gelatin, 0.25 units of *Taq* DNA polymerase (Boehringer Mannheim). Amplifications consisted of 35 cycles in a Techne PHC-2 thermocycler and employed a 1.5-min initial denaturation at 95 °C prior to thermocycling, and a 35-s denaturation at 95 °C followed by a 55-s annealing step at 53 °C for each cycle. Elongation was carried out at 72 °C for 45 s in the initial 13 cycles, 2 min in the next 13 cycles, and 3 min for the final nine cycles for all amplifications. The last cycle was followed by extension at 72 °C for 10 min. Reactions with no template DNA were performed along with each amplification to ensure the absence of contaminants in the reagents. Amplification was attempted from both the DNA extracts from each orchid individual.

Aliquots of 7–9 μL of the PCR products were digested overnight in a total volume of 15–20 μL. It was not necessary to clean PCR products prior to digestion. Buffers and digestion temperatures were according to the manufacturer's instructions. Digested products were electrophoresed in minigels of 2% NuSieve® Agarose/1% Agarose (ultrapure electrophoresis grade) at 150–200 mV for 1.5–2 h, stained with 1 μg/mL ethidium bromide for 15 min, destained in water for 10 min, and visualized on a UV transilluminator. Either Polaroid 665 film or an Eagle-Eye Transvue digital image capture apparatus was used to record the gel images. Samples were grouped by ITS–RFLP pattern, using two or three of the following restriction enzymes: *Alu*I, *Hin*fI and *Mbo*I (or its isoschizmer, *Dpn*II). Samples were categorized as belonging to the same RFLP type only if pattern identity was confirmed on a single gel, usually in adjacent lane comparisons. Each of the different ITS–RFLP patterns obtained from orchid samples was then compared with patterns from identified fruitbodies of fungi in the Russulaceae. Further details of the ITS–RFLP analysis for fungal typing are provided in Gardes & Bruns 1996a.

In the case of unmatched ITS–RFLP patterns, double-stranded fungal amplicons of the ML5–6 region were cycle-sequenced using the Rhodamine DyeTerminator kit (Perkin-Elmer) and 0.5× reaction sizes with the primers ML5 and ML6. Fluorescent fragments were cleaned by ethanol precipitation then electrophoresed in 4.25% acrylamide gels on an ABI 373 or 377 automated sequencer. ML5–6 sequences were added to the database for this region (Bruns *et al*. 1998) and analysed by neighbour-joining using PAUP 4.0 beta (Swofford 1999).

### *Data analysis*

We tested the null hypothesis of homogeneity of fungal species distributions across habitats (tree, elevation) and phenotype by determining the likelihood ratio ($G^2$) and two sided probabilities of the observed $r \times c$ contingencies shown in Table 1. Each habitat and phenotype factor (row variables) was subject to an independent contingency test with counts for each fungal species found in the test populations as column variables. Each test employed several populations belonging to each factor level, but a given population was never used for more than one test. Due to many low cell counts, the probabilities of the likelihood test statistics were evaluated by the exact permutation procedures implemented in StatXact 3.1 (Cytel Software, Boston, MA, USA). A measure of the degree of association between each factor and fungal occurrence, the Phi contigency coefficient, was also calculated using exact methods. The rows and columns were unordered, but the row and column totals are treated as fixed in these exact probability calculations.

To determine whether population boundaries were correlated with variation in fungal symbionts, we partitioned the Simpson measure of species diversity into within and among population components. This analysis included all the sampled populations, and was conducted separately for *C. maculata* and *C. mertensiana*. A version of Simpson's dominance, $1/\lambda$, is a popular index of within-community species diversity that incorporates richness and evenness attributes. We have employed a similar measure, sometimes called the Simpson's diversity index, $1 - \lambda$, which has the useful mathematical

**Table 1** Tests of association between habitat, phenotype and fungal symbiont species. Separate contingency table likelihood ratio ($G$) tests were performed for each pair of rows below the test indicated. All comparisons are between different habitats or phenotypes within *Corallorrhiza maculata* except the 'species test.' Cells give counts for each fungal ITS–RFLP type found in the corresponding orchid/habitat. '—' indicates fungal species absent from both categories in a given comparison; these empty cells were not included in the calculations of the likelihoods or the contingency coefficients. The number of populations of each category used in each test are provided. The exact probabilities given in the last column are two tailed and apply to both the null model of homogeneity across rows/columns and to the Phi coefficient

| | A | B | C | D | E | H | I | J | L | M | N | O | U | v | w | x | No. of populations | $G$ | Phi | $p$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tree test | | | | | | | | | | | | | | | | | | | | |
| 1. Oak | 0 | — | — | 0 | — | 14 | 4 | 5 | 0 | — | 1 | — | 1 | — | — | — | 3 | 39.30 | 1 | < 0.0001 |
| 2. Conifer | 7 | — | — | 2 | — | 0 | 0 | 0 | 3 | — | 0 | — | 0 | — | — | — | 2 | | | |
| Elevation test: | | | | | | | | | | | | | | | | | | | | |
| Above 2000 m | 0 | 11 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 3 | 19.87 | 1 | 0.0002 |
| Below 2000 m | 5 | 0 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 2 | | | |
| Species test: | | | | | | | | | | | | | | | | | | | | |
| *C. maculata* | 2 | — | — | — | 1 | — | — | — | 3 | 1 | — | 3 | — | 0 | 0 | 0 | 4 | 27.73 | 1 | 0.0001 |
| *C. mertensiana* | 0 | — | — | — | 0 | — | — | — | 0 | 0 | — | 0 | — | 7 | 2 | 1 | — | | | |
| *C. maculata* variety test: | | | | | | | | | | | | | | | | | | | | |
| normal *occidentalis* | 6 | — | 0 | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | 11.46 | 1 | 0.0119 |
| albino *occidentalis* | 0 | — | 3 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | |

property that the total variance can be partitioned into additive components for any hierarchical organization. Simpson's diversity is defined as $1 - \Sigma x_i^2$, where $x_i$ is the frequency of species *i*. Several workers have noted that Simpson's diversity is precisely analogous to Nei's measure of gene diversity, $H_T$ (Patil & Taillie 1982; Lande 1996). Nei has shown how gene diversity can be partitioned into within- and among-population components, and proposed the summary statistic $G_{ST}$, which is the proportion of total variation accounted for by differences among populations, and is identical to Wright's $F_{ST}$ for diploid, random-mating populations (Nei 1973). We estimated the among-population component of Simpson's diversity, which we will call $S_{ST}$ (= $G_{ST}$), in *C. maculata* and *C. mertensiana* using the shareware PopGene (Yeh *et al*. 1997) by treating each fungal species in each orchid sample as an allele at a single haploid locus.

## Results

### *Efficacy of PCR-based identification*

The identification of orchid mycorrhizal fungi via ITS typing was found to be quite effective relative to the traditional approach of fungal isolation and morphological identification. Two major hurdles are encountered in the traditional approach. First, many orchid mycorrhizal fungi are slow growing or unculturable, and are often over-run by faster-growing surface contaminants or other endophytic fungi when fungal isolation is undertaken. Thus, the proportion of orchid samples which yield legitimate mycorrhizal isolates is often quite low (Harvais 1974; Ramsay *et al*. 1986) (actually zero in the case of *Corallorhiza maculata*; Taylor & Bruns 1997). Second, taxonomic placement of vegetative cultures is problematic (Andersen 1990; Currah 1991; Andersen 1996) and few isolates produce sexual states, which would permit definite identification (Marchisio *et al*. 1985; Ramsay *et al*. 1986; Currah *et al*. 1990, but see Warcup 1981). Of the 150 orchid samples that were originally collected, 132 (88%) could be amplified by PCR. All but one failure could be attributed to decay of the tissue due to delayed sample processing. As described below, the fungi in all of these samples were identified to family level, and most to species level.

All fungal symbionts found in this study belong to a single family: the Russulaceae, and all of our molecular analyses supported this conclusion. Some apparent primer pair biases were encountered when the ITS region was amplified, and we therefore used both the ITS1F/ITS4B pair, which is specific to basidiomycetes, and the more general fungal-specific pair, ITS1F/ITS4. Of the 132 samples, 10% were amplified only with the primer pair ITS1F/ITS4, 37% with only ITS1F/ITS4B and 53% with both primer pairs. The identifications corresponded perfectly for all samples where both primer pairs were used. Furthermore, independent amplification of each of the two DNA extracts from an individual orchid was achieved for 80% of the samples, and the resulting patterns were identical in all but one case. The mitochondrial target (ML5/6) was amplified and sequenced successfully in all samples where this was attempted, and the sequences unambiguously belonged to the Russulaceae. Thus, three different PCR targets provided congruent fungal identifications.

A total of 179 fruitbodies of Russulaceous fungi, representing 80 identified species, as well as a number of unidentified collections, were subject to ITS–RFLP analysis in a search for matches to the *Corallorhiza* symbionts. Not all enzymes were used with every fruitbody because many patterns could be eliminated as potential matches with one or two enzyme digests. The ITS was found to be useful for species-level mycorrhizal matching in the Russulaceae, as has been observed for other Basidiomycete fungi (Gardes & Bruns 1996a, b; Karen *et al*. 1997), as most species surveyed had distinct RFLP patterns. However, in several cases, different taxa had identical patterns, and in 11 of the 13 cases where multiple fruitbodies from one taxon were surveyed, varying ITS patterns were encountered. These inconsistencies are probably due to misidentifications of the fruitbodies in this taxonomically problematic genus. For this reason, we use the RFLP types as our operational taxonomic units, rather than replacing them with names based on putative species matches. However, intraspecific ITS variation, which has been documented previously in other Basidiomycetes (Vilgalys & Sun 1994; Karen *et al*. 1997; Taylor & Bruns 1997), may also explain some of the inconsistencies.

### *Diversity and mycorrhizal status of fungal associates*

Symbiont diversity was greater in *C. maculata* than in *C. mertensiana*. *C. maculata* had 20 fungal associates (ITS-types), 12 of which were matched to fruitbodies, while *C. mertensiana* was associated with only three species, two of which were matched to fruitbodies (Table 2). The remaining unmatched types were found to belong to the Russulaceae based on ML5–ML6 sequence analysis presented previously (Taylor & Bruns 1997), and additional ML5–ML6 sequences obtained in the present study (Table 2; phylogenetic tree not shown). Despite the overall diversity in *C. maculata*, a single fungal entity, ITS type A, was found in about 33% of the samples. Fruitbody matching for this type illustrates some of the difficulties encountered. The RFLP pattern matched one collection of *R. graveolens*, but mismatched another. It also matched two out of four collections of *R. elaeodes*, one collection of *R. eccentrica*, two out of three collections

**Table 2** Geographic distribution and ITS–RFLP patterns for fungal species associated with *Corallorhiza maculata* and *C. mertensiana*

| | | Fungal distribution† | | ITS restriction fragment size | | |
|---|---|---|---|---|---|---|
| RFLP type | Matching fruitbodies* | *C. maculata* | *C. mertensiana* | *Hin*fI | *Alu*I | *Mbo*I |
| A | *R. semirubra* HDT 54406 (3/3) *R. xerampelina* TRH228 *R. elaeodes* HDT 52991 (2/4) *R. graveolens* HDT54290 (1/4) | 5, 7, 8, 9, 11, 12, 13, 14, 20, 22, 24, 26, 28 | — | 425, 350, 115 | 510, 380‡, 290 | 360, 290, 220 |
| B | *Gymnomyces abietis* SNF74 | 2, 6, 10 | — | 345, 240, 155, 115 | 495, 375‡, 285 | 585, 180 |
| C | Russulaceae no. 7 GenBank AF176827 | 24 | — | 345, 240, 155, 115 | 500, 370‡, 285 | 340, 330, 180 |
| D | Russulaceae no. 2 | 9, 11 | — | 345, 215, 160, 115 | 495, 355‡, 260 | 565, 190 |
| E | *Russula* sp. LT37 | 7, 12, 23 | — | 345, 160, 155, 115 | 495, 355‡, 260 | 290, 270, 185 |
| F | Russulaceae no. 8 GenBank AF176826 | 21 | — | 345, 235, 150, 115 | 485, 365‡, 280 | 580, 185 |
| G | Russulaceae no. 5 | 31 | — | 355, 245, 160, 115 | 510, 380‡, 290 | 305, 295, 215 |
| H | *R. flaviceps* LT51 | 3, 4 | — | 430, 350, 125 | 515, 385‡, 295 | 460, 210, 175 |
| I | *Russula* sp. DED5585 | 3, 4 | — | 420, 350, 115 | 515, 385‡, 295 | 455, 215, 165 |
| J | *R. californiensis* HDT54442 | 1, 4, 25 | — | 400, 260, 115, 90 | 510, 375‡, 280 | 260, 250, 200, 100 |
| K | Russulaceae no. 9 GenBank AF176828 | 25 | — | 380, 350, 115 | 490, 380‡, 295 | 350, 260, 225 |
| L | *R. murrillii* HDT53368 | 7, 9, 14 15, 16 | — | 350, 250, 175, 115 | 510, 385‡, 290 | 625, 210 |
| M | Russulaceae no. 3 | 12 | — | 410, 345, 115 | 370, 310, 195 | 340, 265, 215 |
| N | *R. amoenolens* SNF63 | 4 | — | 360, 345, 115 | 520, 370‡, 280 | 325, 225, 200 |
| O | *Russula* sp. SNF288 | 12, 16, 17 | — | 400, 125, 115 | 340, 265, 170 | 405, 330, 280 |
| P | *Russula* sp. LT40 | 27 | — | 385, 340, 115 | 475, 365‡, 280 | 345, 260, 215 |
| Q | *R. integra* HDT54375 | 9 | — | 400, 360, 115 | 520, 390‡, 300 | 355, 270, 240 |
| R | Russulaceae no. 4 | 14 | — | 345, 170, 155, 115 | 485, 355‡, 270 | 455, 220, 170 |
| T | Russulaceae # 10 GenBank AF176829 | 25 | — | 365, 225, 115 | 450, 425, 270 | 405, 255, 185 |
| U | *Lactarius* sp. LT80 | 4 | — | 450, 300, 125, 115 | 525, 225, 135, 125 | 410, 335, 180, 145 |
| v | *R. simillima* HDT 54346 | — | 7, 12, 15, 29, 30 | 440, 370, 115 | 525, 385‡, 290 | 310, 220, 180, 110 |
| w | *R. occidentalis* HDT 54284 | — | 15, 17, 18, 19, 20, 30 | 415, 355, 115 | 510, 380‡, 285 | 335, 205, 160, 100 |
| x | Russulaceae no. 11 GenBank AF176825 | — | 17, 19, 20, 29, 30 | 405, 355, 115 | 515, 380‡, 285 | 335, 220, 165, 100 |

*GenBank Accession nos are provided for new ML5–6 sequences.

†The locations by county (in California except as listed) of the populations for each species are: 1, Monterey; 2, Mono; 3, San Mateo; 4, Santa Clara; 5, Marin; 6, Alpine; 7, Sonoma; 8,El Dorado; 9, Mendocino; 10, Sierra; 11, Glenn; 12, Tehama; 13, Plumas; 14, Humboldt; 15, Lane, OR; 16, Lewis, WA; 17, Pierce, WA; 18, Pierce, WA; 19, King, WA, 20, Whatcom, WA; 21, Uinta, UT; 22, Daggett, UT; 23, Cache, UT; 24, Lincoln, WY; 25, Lincoln, WY. Additional counties where individual samples were collected are: 26, Siskiyou, CA; 27, Tuolumne, CA; 28, Clark, WA; 29, Skagit, WA; 30, Snohomish, WA; 31, Summit, OH. Restriction fragment sizes are approximate, and suffer greater error for smaller fragments. Fragments smaller than 80 bp were not scored.

‡This fragment is submolar, and varies in intensity in different PCR reactions/digestions. Matched fruitbodies in the personal collection of Bruns and Taylor (LT and SNF) will be deposited in the UC Herbarium; those designated HDT (Harry D. Thiers) and DED (Dennis E. DesJardin) belong to the Harry D. Thiers Herbarium, San Francisco State University.

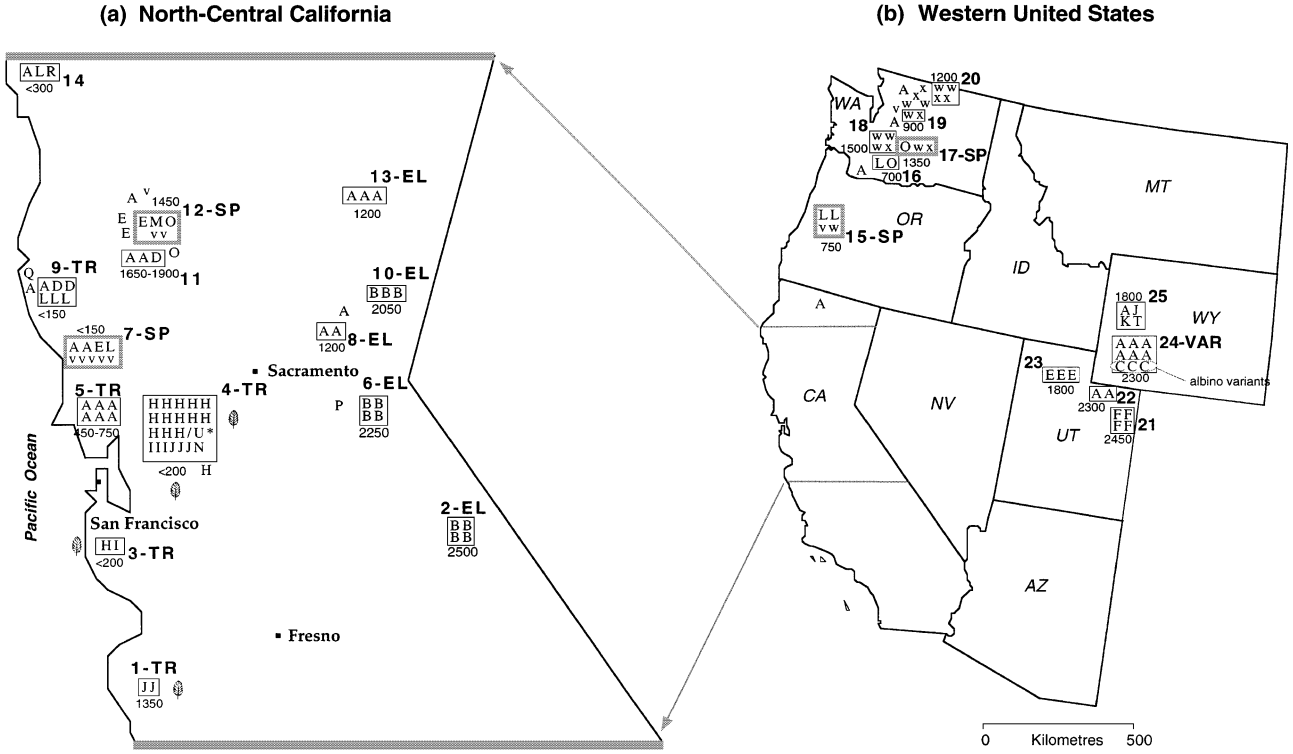


**Fig. 1** The distribution of fungal symbionts and their incidence within populations reveal geographical, habitat, and populational patterning of the associations of *Corallorhiza maculata* and *C. mertensiana*. Each letter shows the fungal symbiont ITS–RFLP type (as listed in Table 2) found in a single orchid individual. Upper-case letters indicate symbionts of *C. maculata*, while lower-case letters are used for symbionts of *C. mertensiana*. Populations of *C. maculata* or *C. mertensiana* are bounded by thin lines and numbered in bold, and sites containing both species are enclosed in thicker, grey lines. Populations used for habitat and phenotype tests are indicated: TR, tree test; EL, elevation test; SP, species test; VAR, *C. maculata* variety test. Individual samples, outside populations, are not enclosed by boxes. Site elevations are given in metres above or below each population box, and the populations growing in oak-dominated sites are highlighted with a leaf. Sample sites are only approximately positioned on the map, and distances between sites are not to scale, especially for closely spaced sites. *H/U in population 4 indicates a single individual which displayed the type H pattern in one extract, and type U (the anomalous *Lactarius* association) in the second extract.

of *R. xerampelina*, and three out of three collections of *R. semirubra*. These taxa all belong to subsection Viridantinae, and are thus similar morphologically and are probably closely related.

Despite very limited sampling, we found tree root ectomycorrhizae with ITS types matching those of the resident orchid at two sites. One of these was type A, the most common *C. maculata* symbiont.

### *Partitioning of variation in fungal associations*

All nine seasonal samples taken from six *C. maculata* plants were colonized exclusively by *Russula* type H, which was the dominant fungus in this population as a whole (Fig. 1). Thus, there was no evidence for seasonal turnover in the species of fungal symbiont at this site in this orchid.

From the map of fungal associates shown in Fig. 1, it can be seen that fungal distributions were not geographically uniform. In *C. maculata*, some fungi were widespread and dominant, others dominant but geographically restricted, and yet others were localized as well as minor where they occurred. For example, type A was a widespread and often dominant symbiont, type F was found in every sample from population 21, but was encountered nowhere else, and types N and U each infected only a single individual at sites where other fungi were dominant (Fig. 1). Another portion of the individual plant with type U was associated with the dominant fungus for that site, type H. In *C. mertensiana*, the distribution provides some suggestion of dominance of type v in the South, with replacement by types w and x in the North. There were no rare types in this orchid.

When we treated the fungal species found in each orchid as analogous to communities, total Simpsons' diversity in *C. mertensiana* was 0.66 while total diversity in *C. maculata* was 0.85, in agreement with the broader array of symbionts in *C. maculata*. Population level structuring

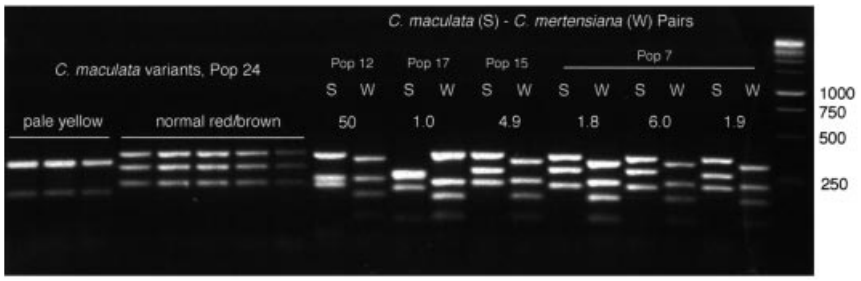


**Fig. 2** Lack of overlap in fungal ITS–RFLP patterns obtained from intermixed *Corallorhiza mertensiana*/*C. maculata* individuals and intermixed floral variants of *C. maculata*. *MboI* digest of fungal ITS fragments amplified with ITS1F and ITS4B directly from DNA extracted from orchid mycorrhizae. The source populations are numbered following Fig. 1 and Table 2. Orchid species are abbreviated within lanes on the right side of the gel: S, the spotted coral root, *C. maculata*; W, the western coral root, *C. mertensiana*. Distances in metres between the paired orchid species are indicated below the species abbreviations. Samples from the common red/brown and uncommon pale yellow morphs of *C. maculata* are shown on the left of the gel.

of specificity was apparent in both orchids, as 48% of total Simpson's diversity in *C. mertensiana* and 68% of total diversity in *C. maculata* could be attributed to the among-population component, $S_{ST}$. This population differentiation in fungal symbionts cannot be explained solely by the natural distributions of *Russula* species, as all of the study sites support numerous species in the Russulaceae (Arora 1986; Thiers 1994; Thiers 1997; D. L. Taylor and T. D. Bruns, personal observations). For example, we collected fruitbodies from six *Russula* species within 100 m of an orchid in population 5, none of which turned out to match type A, even though type A was the sole symbiont in every orchid sampled over a much wider area at this site.

There was zero overlap in fungal species between *C. maculata* plants in the test Coast Range conifer populations and the oak populations, obviously resulting in a maximum coefficient of association, and a very low probability of homogeneity across these sites (Table 1). Thus, forest composition is strongly associated with the occurrence of particular fungal species as orchid symbionts (which is not necessarily the same as the occurrence of these fungi outside orchid symbioses). There was also a perfect association between fungal species composition and elevation, again, producing a very low probability ($P < 0.0002$) of homogeneity in the likelihood test. This pattern may also be related to forest composition.

*C. maculata* and *C. mertensiana* had no overlap in fungal symbionts across the four populations where they grew together, so that the association between fungal species and plant species was also perfect (Phi = 1). While the first two tests suggested a habitat influence over specificity, this latter result suggests a strong genetic control that is not confounded with habitat effects, because the plants were completely intermixed and often growing in close proximity (Fig. 2). There was also no overlap in fungal symbionts across samples where the two orchid species grew in isolation (Fig. 1).

Even more strikingly, the two variants of *C. maculata* that grew intermixed at site 24 did not share fungal symbionts. While the sample size for this comparison was small, homogeneity of fungi across the two variants again had a low probability. The same pattern of lack of overlap of fungi in pale yellow vs. red/brown flowered plants has been seen again in a recently sampled, distant site (D. L. Taylor, unpublished data).

## Discussion

Based on extensive sampling, we conclude that both *Corallorhiza maculata* and *C. mertensiana* associate exclusively with fungi in the Russulaceae. Relative to photosynthetic ectomycorrhizal plants which may associate with thousands of species from tens of families (Molina *et al*. 1992), *C. maculata* and *C. mertensiana* are highly specialized. This degree of specialization mirrors that found in the nonphotosynthetic orchid *Cephalanthera austinae* (Taylor & Bruns 1997), and several nonphotosynthetic species in the dicotyledonous Monotropoideae (Cullings *et al*. 1996). At least some photosynthetic orchids may be equally specialized (Warcup 1971; Masuhara & Katsuya 1994; Perkins *et al*. 1995). The population differentiation and strong statistical associations between fungal species occurrence and habitat or genotype are therefore the most striking results of this study.

### *Environmental and geographical correlates of specificity*

The lack of seasonal changes in the associations of *C. maculata* is not surprising. The densely infected rhizomes

of *C. maculata* are perennial, which should allow the migration of the fungus from older areas to younger tissues. This phenology contrasts with that of many orchid species in which roots, bulbs or corms are annual, and mycorrhizal structures must be re-infected from the soil (Rasmussen 1995). Seasonal or developmental turnover in the species of fungal symbionts seem much more likely in these orchids.

The geographical variation in associations (Fig. 1) was expected given the wide sampling area, and could be directly caused by limited geographical ranges of particular fungal species, or indirectly caused by geographical variation in the ways the plants and fungi interact. The distribution of the three fungi found in *C. mertensiana* varied clinally, which may explain the population partitioning of diversity discussed below, and suggests that the geographical ranges of the fungal symbionts may have been important in this orchid. Geographic variation in *C. maculata* was much more complex.

Strong population structuring of the symbiosis was evident in both orchids based on the proportion, $S_{ST}$, of total Simpson's diversity attributable to among-population divergence. Symbiont species richness and population partitioning of diversity were both greater in *C. maculata* than in *C. mertensiana*. The greater $S_{ST}$ value in *C. maculata* is not explained simply by the greater fungal richness, as $S_{ST}$ is a ratio and is little affected by the absolute diversity. On the other hand, there are several caveats concerning the partitioning of fungal diversity in this study. First, the estimate of fungal diversity at each study site is only a crude approximation due to the small sample sizes, implying that estimates of $S_{ST}$ might change significantly with additional sampling. Second, the fact that we intentionally sampled several individuals in a small area and then travelled a considerable distance before sampling again, may have exaggerated the similarity within populations and the differences between populations. Finally, *C. mertensiana* was sampled over a narrower geographical and habitat range than was *C. maculata*, but this difference does not account for the contrasting symbiont species richness in the two orchids, because associations in *C. maculata* were also more diverse than in *C. mertensiana* at the four sites where they were sampled together (see Figs 1 and 2, Table 1).

Intensive sampling of a few sites, especially population 4, revealed greater fungal richness than was found in similar sites where only one or two samples were collected. However, additional fungal species were only found at low frequencies in the course of additional sampling of population 4, and the estimate of Simpson's diversity at this site was fairly stable beyond five samples (data not shown). We therefore conclude that, while different sampling schemes would probably provide different estimates of richness, Simpson's diversity, and its population partitioning, the demonstration that much of the fungal diversity falls among rather than within orchid populations, is robust. Strong population differentiation in specificity has not been recorded in the mycorrhizal symbiosis, to our knowledge, and is also less common than the converse pattern of greater within than between population variation in other symbioses (Thompson 1994). Our data suggest several factors that may have contributed to this structuring of specificity.

We compared the fungal species compositions of populations that differed primarily in a single, obvious habitat parameter to evaluate possible habitat influences over specificity. For both parameters tested, overstorey tree species and elevation, there was no overlap in fungal species across habitats (Table 1). The statistical significance of these patterns was not due to random differences between paired populations, as each test was based upon multiple populations scattered over hundreds of kilometres (Fig. 1, Table 1). Furthermore, there were no sampled plants outside the test populations that countered the patterns found in the test populations. For example, the unique type B fungus found at high elevations in the Sierra Nevada was absent from all low elevation sites, not only the ones that were included in the analyses testing for elevation effects on fungal species occurrences.

However, all of our habitat tests must be interpreted with caution because we do not know whether plant genotypes or other factors that could affect specificity also vary between habitats. In fact, the overstorey tree test was clearly confounded with genetic variation because most of the plants at the conifer sites conformed to the spotted, wide-lipped var. *occidentalis*, while the populations under oaks were fixed for a spotless, narrower-lipped form that is intermediate between var. *occidentalis* and var. *maculata* in lip shape (J. V. Freudenstein, personal communication). This habitat–phenotype association suggests that ecotypic differentiation exists within *C. maculata*.

### *Genetic correlates of specificity*

Chloroplast RFLP studies have shown that *C. mertensiana* and *C. maculata* are very closely related, and suggest that *C. mertensiana* may be derived from a paraphyletic *C. maculata* (Freudenstein & Doyle 1994a; Freudenstein & Doyle 1994b). Because the geographical range of *C. mertensiana* is almost entirely within that of *C. maculata*, and they occur together at many sites, we were able to exclude habitat effects while examining genetic influences on specificity by comparing fungi in the two orchids at sites where they co-occurred. There was no overlap in *Russula* species between these orchids, showing that orchid genetic differences at the level of sibling species simultaneously control the acceptance and exclusion of closely related fungi. Even differences in microhabitat

preferences at these sites seem unlikely, as the two species were completely intermixed and often growing very close together (Fig. 2). This level of genetic control over mycorrhizal interactions has not been recorded previously, although it may well occur in other specialized myco-heterotrophs such as members of the Monotropoideae.

A very similar result was obtained in our comparison of the two varieties of *C. maculata* found growing together in population 24, suggesting that genetic controls over specificity are important even at the varietal level. Colour variants of *C. maculata* often grow together (Coleman 1988), and such variation was observed in several of the populations with wider ranges of fungal species. Genetic differentiation in specialization at the population level is rare in panmictic, widely dispersing species (Thompson 1994). However, differing, genetically controlled specialization in genetically isolated populations or host races has been found repeatedly in conventional parasites (Price 1980; Theron & Combes 1995) and phytophagous insects (Jaenike 1990; Feder & Bush 1991; Craig *et al*. 1993; Feder *et al*. 1994; Brown *et al*. 1996; Menken 1996), and is nearly always associated with incipient or cryptic speciation (Thompson 1994). Whether the oak-associated floral variant, or other uniquely specialized *C. maculata* populations, represent host races or incipient species remains to be determined. We are currently analysing molecular variation in these plants, which should help to distinguish genetic vs. habitat influences over specificity.

The fact that the sibling *Corallorhiza* species target fungi in the same family could be interpreted as conservative specialization, at a coarse phylogenetic scale. Yet, the fact that the sibling species do not share any fungal species and that variation in fungal associations within *C. maculata* has at least some genetic component tells us that specialization is dynamic and rapidly evolving, at the finer phylogenetic scale of fungal species.

### *Implications for the mycorrhizal symbiosis*

The present results are the first demonstration, to our knowledge, of strong geographical patterning in a mycorrhizal interaction. This geographical patterning probably encompases variation in species ranges, habitat influences, and genotypes of one or both interactants. These results reinforce the view that most interactions vary over time and space in a 'geographical mosaic' (Thompson 1994). The narrow specialization in these nonphotosynthetic orchids has made geographical patterns easy to detect. However, ectomycorrhizal fungi range from broad to narrow specificity toward their plant hosts (Molina *et al*. 1992), suggesting that fungal geographical variation in specificity might also exist.

Temperate genera of the Russulaceae, including *Russula*, *Lactarius*, and *Gymnomyces*, are all thought to be ectomycorrhizal (Singer 1986). We showed that *Russula* type A simultaneously formed endogenous mycorrhizae with *C. maculata* and ectomycorrhizae on roots of a nearby tree at two sites. Thus, these *Corallorhiza* species appear to be myco-heterotrophic (Leake *et al*. 1994) epiparasites that extract sugar from a fungus which in turn acquires carbon from its mutualistic association with a photosynthetic host. Ectomycorrhizal epiparasitism was also inferred from a larger set of samples of orchids, fungi and tree roots in the nonphotosynthetic orchid *Cephalanthera austinae* (Taylor & Bruns 1997).

## Conclusions

A great deal is known about the specialization of parasitic fungi and phytophagous insects on their plant hosts (Jaenike 1990; Futuyma 1991; Thompson 1994), and some important phylogenetic–evolutionary generalizations are beginning to emerge (Janz & Nylin 1998). However, situations where fungi are hosts have received almost no attention, and parasitism by plants has not been intensively studied (but see Jakucs & Vetter 1992; Overton 1997; Norton & Carpenter 1998; Salle *et al*. 1998). It is therefore of general significance that the patterns of extreme but dynamic specialization so frequent in conventional parasites are also emerging in plants that cheat their mycorrhizal fungi (Cullings *et al*. 1996).

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## References

Adams GC (1988) *Thanatephorus cucumeris* (*Rhizoctonia solani*), a species complex of wide host range. *Advances in Plant Pathology*, **6**, 535–552.

Agerer R (1991) Characterization of ectomycorrhiza. In: *Methods in Microbiology*, Vol. 23. *Techniques for the Study of Mycorrhiza.* (eds Norris JR, Read DJ, Varma AK), pp. 25–73. Academic Press Ltd., London, England, UK.

Amaranthus MP, Perry DA (1994) The functioning of ectomycorrhizal fungi in the field: linkages in space and time. *Plant and Soil*, **159**, 133–140.

Andersen TF (1990) A study of hyphal morphology in the form-genus *Rhizoctonia*. *Mycotaxon*, **37**, 25–46.

Andersen TF (1996) A comparative taxonomic study of *Rhizoctonia* sensu lato employing morphological, ultrastructural and molecular methods. *Mycological Research*, **100**, 1117–1128.

Arora D (1986) Mushrooms demystified. *A Comprehensive Guide to the Fleshy Fungi*. Ten Speed Press, Berkeley.

Bending GD, Read DJ (1995) The structure and function of the vegetative mycelium of ectomycorrhizal plants: V. Foraging behaviour and translocation of nutrients from exploited litter. *New Phytologist*, **130**, 401–409.

Bever JD (1994) Feedback between plants and their soil communities in an old field community. *Ecology*, **75**, 1965–1977.

Bever JD, Westover KM, Antonovics J (1997) Incorporating the soil community into plant population dynamics: the utility of the feedback approach. *Journal of Ecology*, **85**, 561–574.

Borowicz VA, Juliano SA (1991) Specificity in host–fungus associations: Do mutualists differ from antagonists? *Evolutionary Ecology*, **5**, 385–392.

Brown JM, Abrahamson WG, Way PA (1996) Mitochondrial DNA phylogeography of host races of the goldenrod ball gallmaker, *Eurosta solidaginis* (Diptera: Tephritidae). *Evolution*, **50**, 777–786.

Brownlee C, Duddridge JA, Malibari A, Read DJ (1983) The structure and function of mycelial systems of ectomycorrhizal roots with special reference to their role in forming inter-plant connections and providing pathways for assimilate and water transport. *Plant and Soil*, **71**, 433–443.

Bruns TD, Szaro TM, Gardes M *et al*. (1998) A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. *Molecular Ecology*, **7**, 257–272.

Burgeff H (1959) Mycorrhiza of Orchids. In: *The Orchids: a Scientific Survey* (ed. Withner CL), pp. 361–395. The Ronald Press Company, New York.

Campbell EO (1962) The mycorrhiza of *Gastrodia cunninghamii* Hook. f. *Transactions of the Royal Society, New Zealand*, **1**, 289–296.

Campbell EO (1963) *Gastodia minor* Petrie, an epiparasite of Manuka. *Transactions of the Royal Society, New Zealand*, **2**, 73–81.

Campbell EO (1970) Morphology of the fungal association in three species of *Corallorhiza* in Michigan. *Michigan Botanist*, **9**, 108–113.

Clements MA (1988) Orchid mycorrhizal associations. *Lindleyana*, **3**, 73–86.

Coleman RA (1988) The coral root orchids of California. *Fremontia*, **16**, 21–22.

Craig TP, Itami JK, Abrahamson WG, Horner JD (1993) Behavioral evidence for host-race formation in *Eurosta solidaginis*. *Evolution*, **47**, 1696–1710.

Cullings KW, Szaro TM, Bruns TD (1996) Evolution of extreme specialization within a lineage of ectomycorrhizal epiparasites. *Nature*, **379**, 63–66.

Currah RS (1991) Taxonomic and developmental aspects of the fungal endophytes of terrestrial orchid mycorrhizae. *Lindleyana*, **6**, 211–213.

Currah RS, Smreciu EA, Hambleton S (1990) Mycorrhizae and mycorrhizal fungi of boreal species of *Platanthera* and *Coeloglossum* (Orchidaceae). *Canadian Journal of Botany*, **68**, 1171–1181.

Curtis JT (1937) Non-specificity of orchid mycorrhizal fungi. *Proceedings of the Society for Experimental Biology and Medicine*, **36**, 43–44.

Curtis JT (1939) The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. *American Journal of Botany*, **26**, 390–398.

Dixon AFG, Kundu R (1994) Ecology of host alternation in aphids. *European Journal of Entomology*, **91**, 63–70.

Dressler RL (1993) *Phylogeny and Classification of the Orchid Family*. Dioscorides Press, Portland.

Feder JL, Bush GL (1991) Genetic variation among apple and hawthorn host races of *Rhagoletis pomonella* across an ecological transition zone in the mid-western USA. *Entomologia Experimentalis et Applicata*, **59**, 249–266.

Feder JL, Chilcote CA, Bush GL (1990) The geographic pattern of genetic differentiation between host associated populations of *Rhagoletis pomonella* (Diptera: Tephritidae) in the eastern USA and Canada. *Evolution*, **44**, 570–594.

Feder JL, Opp SB, Wlazlo B *et al*. (1994) Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. *Proceedings of the National Academy of Sciences of the USA*, **91**, 7990–7994.

Francis R, Read DJ (1994) The contributions of mycorrhizal fungi to the determination of plant community structure. *Plant and Soil*, **159**, 11–25.

Freudenstein JV (1997) A monograph of *Corallorhiza* (Orchidaceae). *Harvard Papers in Botany*, **10**, 5–51.

Freudenstein JV, Doyle JJ (1994a) Character transformation and relationships in *Corallorhiza* (Orchidaceae: Epidendroideae). I. Plastid DNA. *American Journal of Botany*, **81**, 1449–1457.

Freudenstein JV, Doyle JJ (1994b) Plastid DNA, morphological variation, and the phylogenetic species concept: the *Corallorhiza maculata* (Orchidaceae) complex. *Systematic Botany*, **19**, 273–290.

Furman TE, Trappe JM (1971) Phylogeny and ecology of mycotrophic achlorophyllous angiosperms. *Quarterly Review of Biology*, **46**, 219–225.

Futuyma DJ (1991) Evolution of host specificity in herbivorous insects: genetic, ecological, and phylogenetic aspects. In: *Plant–Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions* (eds Price PW, Lewinsohn TM, Fernandes GW, Benson WW). John Wiley & Sons, New York.

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Molecular Ecology*, **2**, 113–118.

Gardes M, Bruns TD (1996a) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. *Canadian Journal of Botany*, **74**, 1572–1583.

Gardes M, Bruns TD (1996b) ITS-RFLP matching for identification of fungi. In: *Methods in Molecular Biology*, Vol. 50. *Species Diagnostics Protocols: PCR and Other Nucleic Acid Methods*, Vol. 50 (ed. Clapp JP), pp. 177–186. Humana Press Inc., New Jersey.

Hadley G (1970) Non-specificity of symbiotic infection in orchid mycorrhiza. *New Phytologist*, **69**, 1015–1023.

Harvais G (1974) Notes on the biology of some native orchids of Thunder Bay, their endophytes and symbionts. *Canadian Journal of Botany*, **52**, 451–460.

Horton TR, Bruns TD (1998) Multiple-host fungi are the most frequent and abundant ectomycorrhizal types in a mixed stand of Douglas-fir (*Pseudotsuga menziesii* D. Don) and bishop pine (*Pinus muricata* D. Don). *New Phytologist*, **139**, 331–339.

Horton TR, Bruns TD, Parker T (1999) Mycorrhizal fungi associated with *Arctostaphylos* facilitate the establishment of *Psuedotsuga menziesii* during succession. *Canadian Journal of Botany*, **77**, in press.

Jaenike J (1990) Host specialization in phytophagous insects. *Annual Review of Ecology and Systematics*, **21**, 243–273.

Jakucs E, Vetter J (1992) Comparative studies on the lignocellulose

degrading ability of various fungus species. *International Journal of Mycology and Lichenology*, **5**, 217–235.

Janos DP (1980) Mycorrhizae influence tropical succession. *Biotropica*, **12**, 56–64.

Janz N, Nylin S (1998) Butterflies and plants: a phylogenetic study. *Evolution*, **52**, 486–502.

Jones MD, Durall DM, Tinker PB (1991) Fluxes of carbon and phosphorus between symbionts in willow ectomycorrhizas. *New Phytologist*, **119**, 99–106.

Karen O, Hogberg N, Dahlberg A, Jonsson L, Nylund J-E (1997) Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. *New Phytologist*, **136**, 313–325.

Kusano S (1911) Gastrodia elata and its symbiotic association with *Armillaria mellea*. *Journal of the College of Agriculture, Japan*, **9**, 1–73.

Lande R (1996) Statistics and partitioning of species diversity, and similarity among multiple communities. *Oikos*, **76**, 5–13.

Leake J, R (1994) The biology of myco-heterotrophic (saprophytic) plants. *New Phytologist*, **127**, 171–216.

Marchisio VF, Berta G, Fontana A, Marzetti Mannina F (1985) Endophytes of wild orchids native to Italy: their morphology, caryology, ultrastructure and cytochemical characterization. *New Phytologist*, **100**, 623–641.

Masuhara G, Katsuya K (1994) *In situ* and *in vitro* specificity between *Rhizoctonia* spp. & *Spiranthes sinensis* (Persoon) Ames. var. *amoena* (M. Bieberstein) Hara (Orchidaceae). *New Phytologist*, **127**, 711–718.

Masuhara G, Katsuya K, Yamaguchi K (1993) Potential for symbiosis of *Rhizoctonia solani* and binucleate *Rhizoctonia* with seeds of *Spiranthes sinensis* var. *amoena in vitro*. *Mycological Research*, **97**, 746–752.

Menken SBJ (1996) Pattern and process in the evolution of insect–plant associations: Yponomeuta as an example. *Entomologia Experimentalis et Applicata*, **80**, 297–305.

Molina R, Massicotte H, Trappe JM (1992) Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. In: *Mycorrhizal Functioning: an Integrative Plant-Fungal Process* (ed. Allen MF), pp. 357–423. Chapman & Hall, New York.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the USA*, **70**, 3321–3323.

Norton DA, Carpenter MA (1998) Mistletoes as parasites: Host specificity and speciation. *Trends in Ecology and Evolution*, **13**, 101–105.

Overton JM (1997) Host specialization and partial reproductive isolation in desert mistletoe (*Phoradendron californicum*). *Southwestern Naturalist*, **42**, 201–209.

Patil GP, Taillie C (1982) Diversity as a concept and its measurement. *Journal of the American Statistical Association*, **77**, 548–561.

Perkins AJ, McGee PA (1995) Distribution of the orchid mycorrhizal fungus, *Rhizoctonia solani*, in relation to its host, *Pterostylis acuminata*, in the field. *Australian Journal of Botany*, **43**, 565–575.

Perkins AJ, Masuhara G, McGee PA (1995) Specificity of the associations between *Microtis parviflora* (Orchidaceae) and its mycorrhizal fungi. *Australian Journal of Botany*, **43**, 85–91.

Perry DA, Margolis H, Choquette C, Molina R, Trappe JM (1989) Ectomycorrhizal mediation of competition between coniferous tree species. *New Phytologist*, **112**, 501–512.

Petersen RH (1974) The rust fungus life cycle. *Botanical Review*, **40**, 453–513.

Price PW (1980) Evolutionary Biology of Parasites. In: *Monographs in Population Biology, 15* (ed. May RM), Princeton University Press, Princeton.

Ramsay RR, Dixon KW, Sivasithamparam K (1986) Patterns of infection and endophytes associated with western Australian orchids. *Lindleyana*, **1**, 203–214.

Rasmussen HN (1995) Terrestrial orchids. *From Seed to Mycotrophic Plant*. Cambridge University Press, New York.

Read DJ (1991) Mycorrhizas in ecosystems. *Experientia (Basel)*, **47**, 376–391.

Roberts P (1993) Exidiopsis species from Devon including the new segregate genera *Ceratosebacina Endoperplexa Microsebacina* and *Serendipita*. *Mycological Research*, **97**, 467–478.

Rowan R, Knowlton N (1995) Intraspecific diversity and ecological zonation in coral-algal symbiosis. *Proceedings of the National Academy of Sciences of the USA*, **92**, 2850–2853.

Salle G, Tuquet C, Raynal-Roques A (1998) Biology of flowering parasitic plants. *Comptes Rendus Des Seances de la Societe de Biologie et de Ses Filiales*, **192**, 9–36.

Simard SW, Perry DA, Jones MD *et al*. (1997) Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature (London)*, **388**, 579–582.

Singer R (1986) *The Agaricales in Modern Taxonomy*. Koeltz Scientific Books, Koenigstein, FRG.

Smith SE, Read DJ (1997) *Mycorrhizal Symbiosis*. Academic Press, San Diego.

Swofford DL (1999) (pre-release) paup*: Phylogenetic Analysis Using Parsimony. Version 4. Sinauer Associates, Sunderland, MA.

Taylor DL, Bruns TD (1997) Independent, specialized invasion of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences of the USA*, **94**, 5410–5415.

Terashita T, Chuman S (1987) Fungi inhabiting wild orchids in Japan IV. *Armillariella–Tabescens* a new symbiont of *Galeola–Septentrionalis*. *Nippon Kingakukai Kaiho*, **28**, 145–154.

Theron A, Combes C (1995) Asynchrony of infection timing, habitat preference, and sympatric speciation of schistosome parasites. *Evolution*, **49**, 372–375.

Thiers HD (1994) The subgenus Compactae of Russula in California. *Mycologia Helvetica*, **6**, 107–120.

Thiers HD (1997) New species of Russula from California. *Mycotaxon*, **63**, 349–358.

Thompson JN (1994) The coevolutionary process. *University of* Chicago Press, Chicago.

Vilgalys R, Sun BL (1994) Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. *Proceedings of the National Academy of Sciences of the USA*, **91**, 4599–4603.

Warcup JH (1971) Specificity of mycorrhizal association in some Australian terrestrial orchids. *New Phytologist*, **70**, 41–46.

Warcup JH (1981) The mycorrhizal relationships of Australian orchids. *New Phytologist*, **87**, 371–381.

Warcup JH (1988) Mycorrhizal associations of isolates of *Sebacina vermifera*. *New Phytologist*, **110**, 227–232.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a Guide to Methods and Applications* (eds Innis MA, Gelfand DH, Sninsky JJ, White TJ), pp. 315–322. Academic Press, Inc., San Diego.

Worrall JJ, Anagnost SE, Zabel RA (1997) Comparison of wood decay among diverse lignicolous fungi. *Mycologia*, **89**, 199–219.

Yeh FC, Yang RC, Boyle TBJ, Ye Z-H, Mao JX (1997) POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Alberta.

Zelmer CD, Currah RS (1995) Evidence for a fungal liaison between *Corallorhiza trifida* (Orchidaceae) *and Pinus contorta* (Pinaceae). *Canadian Journal of Botany*, **73**, 862–866.

The authors wish to understand the evolutionary relationships between symbiotic specialization and biodiversity. Highly specialized, nonphotosynthetic, myco-heterotrophic plants are a particular focus because they engage in a type of interaction that differs fundamentally from the current model systems in the study of specialization. Plant and fungal community structures, and their reciprocal impacts as mediated by the mycorrhizal symbiosis, are a related area of inquiry.