Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid[1]

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Evidence is accumulating for specialized yet evolutionarily dynamic associations between orchids and their mycorrhizal fungi. However, the frequency of tight mycorrhizal specificity and the phylogenetic scale of changes in specificity within the Orchidaceae are presently unknown. We used microscopic observations and PCR-based methods to address these questions in three taxa of non-photosynthetic orchids within the *Hexalectris spicata* complex. Fungal ITS RFLP analysis and sequences of the ITS and nuclear LSU ribosomal gene fragments allowed us to identify the fungi colonizing 25 individuals and 50 roots. *Thanatephorus ochraceus* (Cera-tobasidiaceae) was an occasional colonizer of mycorrhizal roots and nonmycorrhizal rhizomes. Members of the Sebacinaceae were the primary mycorrhizal fungi in every *Hexalectris* root and were phylogenetically intermixed with ectomycorrhizal taxa. These associates fell into six ITS RFLP types labeled B through G. Types B, C, D, and G were found in samples of *H. spicata* var. *spicata*, while only type E was found in *H. spicata* var. *arizonica* and only type F was found in *H. revoluta*. These results provide preliminary evidence for divergence in mycorrhizal specificity between these two closely related orchid taxa. We hypothesize that mycorrhizal interactions have contributed to the evolutionary diversification of the Orchidaceae.

Key words: *Hexalectris spicata*; host-shift; ITS; myco-heterotrophy; mycorrhizal specificity; nLSU; Orchidaceae; *Rhizoctonia*; *Sebacina*.

Interactions between species, especially intimate symbioses, have played major roles in the adaptive evolution and diversification of life. In plants, interactions with herbivores, pathogens, pollinators, seed dispersers, and mutualistic soil microorganisms have been particularly influential. Mycorrhizal symbioses with soil fungi are ancient, possibly dating to the emergence of plants on land (Heckman et al., 2001) and are still critical to the mineral nutrition of most wild plants (Smith and Read, 1997). While mycorrhizal interactions have certainly influenced the evolution of nutrient uptake strategies in plants, most plants do not interact specifically with narrow taxonomic groups of fungi (Molina et al., 1992), making pairwise coevolution between plant and fungal species unlikely (Taylor, 2000). In stark contrast, some members of the species-rich Orchidaceae display marked mycorrhizal specificity toward particular taxonomic groups of basidiomycete fungi (reviewed in Taylor et al., 2002). We use ''specificity'' to refer to the relative phylogenetic diversity of fungal associates that interact with a particular plant (Thompson, 1994; Taylor and Bruns, 1999b). The mycorrhizal specificity in orchids suggests that mycorrhizal interactions, like pollination syndromes, may have played a role in the diversification of the Orchidaceae. However, we do not know how common mycorrhizal specialization is within the family, how often changes in specificity occurred during the evolution of the family, or at what phylogenetic scale changes in specificity occurred. Answers to these questions will help to generate a much more complete picture of the evolution of the Orchidaceae.

Mycorrhizal specificity in orchids is probably related to the unique mycorrhizal ecology of the family. Most autotrophic mycorrhizal plants supply sugars to their mycorrhizal fungi in return for nutrients scavenged from the soil (Smith and Read, 1997). This reciprocal exchange is thought to result in a mutualistic interaction between plant and fungus (Smith and Read, 1997). In contrast, certain plant lineages have evolved the capacity to acquire substantial quantities of sugar as well as nutrients from their associated fungi such that the net flow of carbon is from fungus to plant. This behavior is termed myco-heterotrophy (Leake, 1994). A uniting feature of the Orchidaceae is an unusual life history in which miniscule seeds that lack significant energy reserves are produced in great number and are dispersed by wind and water. This strategy is possible because orchids form mycorrhizal associations very close to the time of seed germination (Burgeff, 1959; Rasmussen and Whigham, 1993) and acquire carbon compounds from their mycorrhizal fungi (Smith and Read, 1997). The initial growth of orchid seedlings (protocorms) is myco-heterotrophic. It is possible that as orchid protocorms mature and become photoautotrophic, carbon exchange reverses directions, with carbon flowing from orchid to fungus. However, in the few adult photosynthetic orchids that have been examined, carbon flow from orchid to fungus has not been detected (Smith, 1966, 1967; Hadley and Purves, 1974; Purves and Hadley, 1974).

Some orchid species have given up photosynthesis entirely and rely upon fungal-derived energy sources throughout their life cycles. Plants that are completely dependent on this form

TABLE 1.   Fungi found in three members of the *Hexalectris spicata* complex.

Collection location and date	Sample number	Orchid species/Variety	Number of roots	Fungal ITS RFLPs
Union County, Florida 19 October 1995	1	*H. spicata* var. unknown	0	A[a]
Alachua County, Florida 14 February 1996	2	*H. spicata* var. *spicata*	4	B
Alachua County, Florida 5 January 2002	3	*H. spicata* var. *spicata*	2	G
Jackson County, Florida	4	*H. spicata* var. *spicata*	1	C, A[b]
	5	*H. spicata* var. *spicata*	1	C, A
	6	*H. spicata* var. *spicata*	1	C, A
	7	*H. spicata* var. *spicata*	1	C, A
	8	*H. spicata* var. *spicata*	1	C, A
	9	*H. spicata* var. *spicata*	1	C, A
11 July 1996	10	*H. spicata* var. *spicata*	1	C, A
Decatur County, Georgia	11	*H. spicata* var. *spicata*	2	D
11 July 1996	12	*H. spicata* var. *spicata*	1	D
	13	*H. spicata* var. *spicata*	2	D
	14	*H. spicata* var. *spicata*	2	D
	15	*H. spicata* var. *spicata*	2	D
	16	*H. spicata* var. *spicata*	1	D
	17	*H. spicata* var. *spicata*	1	D
	18	*H. spicata* var. *spicata*	1	D
	19	*H. spicata* var. *spicata*	2	D
Santa Cruz County, Arizona	20	*H. spicata* var. *arizonica*	2	E
10 June 1998	21	*H. spicata* var. *arizonica*	4	E
Oversight Canyon, Arizona	22	*H. spicata* var. *arizonica*	2	E
Pima County, Arizona	23	*H. revoluta*	7	F
Santa Cruz County, Arizona	24	*H. revoluta*	5	F
Cochise County, Arizona	25	*H. revoluta*	3	F

Notes: Each row provides the data from one orchid individual. The fungal ITS RFLP types are based on amplifications using ITS1F/ITS4. Separate DNA extractions, PCRs, and RFLP analyses were performed on multiple roots from most orchid individuals. ''Number of roots'' refers to the number of extracts from an individual that produced a distinct ITS amplicon.

[a] There were no roots in the sample from Union County; the fungus was amplified from rhizome tissue.

[b] The samples from Jackson County had fungal RFLP patterns predominantly attributable to type C, with weaker ''background'' patterns from type A.

of nutrition and have lost photosynthetic capabilities are described as fully myco-heterotrophic (Leake, 1994). The requirement for fungal-derived carbon as well as the mycorrhizal specificity of some orchids suggests that these plants are exceptionally dependent upon their fungal symbionts. Despite this dependence, we do not know whether or how this interaction has influenced the evolution of the Orchidaceae.

Certain phylogenetically distant species within the Orchidaceae are specialized toward different fungal groups (Warcup, 1981; Taylor et al., 2002), suggesting that specificity may have shifted during the diversification of major orchid lineages. If shifts in specificity have been frequent and occur at a fine phylogenetic scale (i.e., within species) in certain orchid lineages, then mycorrhizal interactions may have contributed to the phylogenetic diversification of orchids. At present, there is only limited evidence concerning the frequency or phylogenetic scale of shifts in mycorrhizal specificity within the Orchidaceae (Warcup, 1981; Taylor and Bruns, 1999b; Otero et al., 2002).

The extreme specificity in some orchids also implies that the protection of endangered orchids could depend upon effective conservation of their required fungi. Unfortunately, the mycorrhizal associations of relatively few North American orchids have been studied (but see Currah et al., 1987, 1988, 1990; Zelmer and Currah, 1995; Zelmer et al., 1996; Taylor and Bruns, 1997, 1999b).

Hexalectris is a genus of fully myco-heterotrophic orchids containing roughly seven species with the center of diversity in northern Mexico (Luer, 1975). Several species are quite rare and may be threatened by habitat loss. The most widespread species, *Hexalectris spicata*, occurs in diverse habitats: from swamps in Florida and Georgia to oak canyons rising out of the desert in southern Arizona (Luer, 1975). There are several distinct floral variants within this complex, although species boundaries are not yet certain (Catling and Engel, 1993; Coleman, 2000, 2002). We are not aware of any information on the mycorrhizal associations of these orchids. The goals of the present work were to determine the degree of specificity of *Hexalectris spicata* and to test whether mycorrhizal specificity varies geographically or across morphologically distinct members of the complex. To assess specificity, we needed a clear understanding of the identities and phylogenetic relationships of the fungal symbionts found in *Hexalectris*.

MATERIALS AND METHODS

Collection, fungal isolation, and anatomical observation—We sampled three floral variants within the *Hexalectris spicata* complex at its eastern and western distribution limits. *Hexalectris spicata* var. *spicata* (Walter) Barnhart is the most widespread member of the complex, ranging from a northeastern limit in Virginia to a western limit in Arizona and through northern Mexico (Catling and Engel, 1993). We sampled this taxon at sites separated by tens to hundreds of kilometers in Florida and Georgia (Table 1). It has large, open flowers and is thought to outcross (Catling and Engel, 1993). We did not succeed in locating a second outcrossing form, *H. spicata* var. *spicata* (Walter) Barnhart forma *albolabia* Brown, which occurs in the southeastern United States (Luer, 1975). We sampled the closed-flowered autopollinating variety

H. spicata var. *arizonica* (S. Watson) Catling & Engel in the Chiracahua mountains of southern Arizona; it is also found at several sites in southern Texas and into Mexico (Catling and Engel, 1993). We also collected individuals in the Santa Rita mountains that regularly flower earlier than typical *H. spicata* var. *spicata* and have dramatically out-rolled petals. Ronald Coleman identified our samples as *Hexalectris revoluta* Correll (Coleman, 2000, 2002). This taxon is very similar to *H. spicata*, and it is unclear whether *H. revoluta* or any of the several varieties of *H. spicata* represent distinct species.

Representative roots and rhizomes from all Florida and Georgia samples and several Arizona individuals were hand-sectioned, and the distribution of fungal hyphae in these organs was observed using a compound microscope at 100 to 1000-fold magnification. Fungal isolations were performed as in Taylor and Bruns (1997) by sterilizing fresh root segments in 20% household bleach for 5 min, rinsing in three changes of sterile water, decorticating root sections, and moving liberated pelotons (hyphal coils within orchid root cells) onto modified Marx-Norkrans medium (Marx, 1969). Roots and rhizomes from each plant were scrubbed under tap water and portions to be used for molecular analysis were frozen within 4 d of harvest.

Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis, and sequencing—DNA was extracted from frozen orchid tissue by the SDS/GeneClean (Bio 101, La Jolla, California, USA) method (Taylor and Bruns, 1999b). To discriminate among fungal taxa colonizing *Hexalectris* roots, we amplified the highly variable fungal internal transcribed spacer (ITS) region of the nuclear ribosomal repeat directly from mycorrhizal orchid tissue using the PCR, then performed restriction digests of the resulting amplicons. Fungal species usually display unique ITS RFLP patterns (Gardes and Bruns, 1996a,b; Karen et al., 1997). The fungal ITS region was amplified using the primers ITS 1F and ITS 4B (Gardes and Bruns, 1993) or ITS 1F and ITS 4 (White et al., 1990). Each ITS amplicon was digested using the restriction enzymes Alu I, Hinf I, and Mbo I (New England Biolabs, Beverly, Massachusetts, USA) in separate reactions (see Taylor and Bruns, 1997 for details). The resulting fragments were separated on gels containing 2% agarose and 1% high resolution agarose (Sigma-Aldrich, St. Louis, Missouri, USA) to visualize the fungal RFLP patterns from each root sample. Each ITS amplicon with a unique RFLP pattern was then sequenced in two directions using a Big Dye cycle sequencing kit (PE Applied Biosystems BigDye kit, Foster City, California, USA), again using the primers ITS 1F, and ITS 4. In some cases, the internal sequencing primers ITS 2 and ITS 3 were also used.

The 5′ end of the fungal nuclear large subunit (nLSU) ribosomal gene was amplified using the fungal-specific primer combination ITS 1F and cNL2F (GTTTCCCTTTTAACAATTTCAC). The primers Ctb6 (GCATATCAATA-AGCGGAGG) and cNL2F were employed to sequence a 5′ fragment of the nLSU gene. A larger portion of the nLSU gene was amplified and sequenced from various fruitbodies and fungal isolates using the primers Ctb6 and TW14 (White et al., 1990) for initial amplification, with the addition of TW13 and cTW13 (White et al., 1990) as internal primers in sequencing reactions. All new sequences from this study have been deposited in GenBank under accession numbers AY243515 to AY243533.

Phylogenetic analysis of sequence data—The *Hexalectris* fungal nLSU sequences were subjected to BLAST searches (Altshul et al., 1997), and all close matches were added to the nLSU alignment of Taylor and Bruns, (1999a), along with an array of additional GenBank accessions that provide a broader taxonomic representation of the Basidiomycota. We also sequenced 600–900 bases of the nLSU from several well-characterized culture-collection orchid isolates. The alignment spans positions 75–912 in the *Sacharomyces cerevisiae* sequence (accession Z73326) and contains 990 positions due to insertion of gaps. We excluded 42 extremely variable bases spanning *S. cerevisiae* positions 599–601 from analysis because the homology of positions within this region was highly suspect.

Several different phylogenetic analyses of the nLSU alignment using PAUP*4.0b10 (Swofford, 2000) were performed because of variation in available sequence lengths and rates of sequence evolution across lineages. Our first goal was to estimate the phylogenetic breadth of *Hexalectris* fungi within

the Basidiomycota. We therefore included sequences representing the three major classes within the Basidiomycota. Because the sequences we obtained from most of the *Hexalectris* fungal types extended 400 or fewer bases into our nLSU alignment, we restricted this first analysis to alignment positions 1–385.

The data set displayed high levels of sequence variation and markedly differing evolutionary rates among lineages, both of which pose problems such as "long-branch attraction" for most phylogenetic methods (Swofford et al., 1996). Maximum likelihood is more reliable than parsimony or distance methods under these conditions (Kuhner and Felsenstein, 1994; Huelsenbeck, 1995; Bruno et al., 2000; Swofford et al., 2001). However, processing power was insufficient to carry out searches under the maximum likelihood (ML) criterion. We therefore used the neighbor-joining (NJ) algorithm to search for minimum evolution trees under various DNA substitution models, as well as maximum parsimony, then used maximum likelihood to compare the resulting trees. Support for the tree topology was assessed by 1000 NJ bootstrap replicates.

All *Hexalectris* associates fell within one of the three classes of the Basidiomycota, the Hymenomycetes. Hence, to better estimate the relationships of the *Hexalectris* fungi, we then analyzed a selected set of hymenomycete taxa by maximum likelihood and parsimony. Taxa with sequences ending prior to position 610 were excluded, and positions 1–610 were included. The hymenomycete taxa *Tulasnella*, the Cantharellaceae, and *Hydnum repandum* had extreme rate acceleration and were also excluded from further analyses. A starting tree was generated by neighbor-joining and used to estimate model parameters via ML (substitution probability matrix, gamma shape parameter with four rate categories, proportion invariant sites), followed by tree bisection-reconnection (TBR) branch swapping under the likelihood criterion with the parameters fixed. Support was again assessed by performing 1000 NJ bootstrap replicates. We also inferred trees under the parsimony criterion with transversions weighted 4 : 1 over transitions (frequencies estimated by ML), heuristic search, accelerated character transformation, "collapse" and "multrees" options in effect, TBR branch swapping, and 100 random addition replicates. Support was assessed via 500 bootstrap replicates using the same settings.

Each *Hexalectris* fungal ITS sequence was also subjected to a BLAST search, the highest scoring matches were downloaded from GenBank, and a 631 character ITS sequence alignment was created using clustal X (Thompson et al., 1997), then improved by eye. Heuristic searches were employed in maximum-likelihood (various models) and parsimony (2 : 1 transversion to transition weights) analyses. The nLSU and ITS alignments are available at the web site http://mercury.bio.uaf.edu/~lee_taylor.

RESULTS

Underground anatomy and fungal isolation—All *Hexalectris spicata* samples had two grossly similar, but distinct, underground organs. The first were swollen, tuberous organs, often bearing the inflorescence terminally and additional swollen appendages protruding at right angles. Following Luer (1975), we interpret this first organ as a rhizome because it had nodes with vestigial, decomposing scale leaves and scattered vascular bundles. Some of the lateral organs were additional rhizomes, while others had a similar oblong shape and were as large as 1.5 cm in diameter, but had a central vascular bundle and lacked leaf scars. Only these latter organs were heavily colonized by fungi. We suggest that these organs are highly modified roots. The fungi formed hyphal coils (pelotons) in root cortical cells that are typical of tolypophagous orchid mycorrhizae (Burgeff, 1959). Peloton formation is the primary criterion used to establish the mycorrhizal status of an orchid fungus (Rasmussen, 1995). The vast majority of intact pelotons contained narrow hyphae (2–3 μm diameter). Much wider hyphal fragments (6–10 μm) were occasionally observed in roots and in rhizomes of samples from Florida

and Georgia. In roots, some of these wide hyphae were partially coiled, while others entered and exited cortical cells without coiling. In rhizomes, pelotons were not observed, suggesting that rhizomes are not mycorrhizal. The collection from Union County, Florida included only rhizome material and lacked roots and mycorrhizae.

Although pelotons were not seen in rhizomes, uncoiled hyphae were occasionally seen, and multiple fast-growing isolates were obtained from the rhizome material of sample 1 from Union County. These isolates had vegetative features typical of fungi related to *Thanatephorus* and *Ceratobasidium* in the *Rhizoctonia* group, namely, wide hyphae with restrictions as branch points and a septum just distal to the branch, and swollen monilioid cells (sometimes aggregated in sclerotia). The ITS RFLP patterns obtained directly from the rhizome of this plant matched those obtained from the isolates (see next section). Similar fast-growing *Rhizoctonia* fungi with broad hyphae were isolated from the roots of several samples, but isolation from pelotons in the heavily colonized roots of other plants failed completely. A slow-growing fungus with fine hyphae and ITS RFLP type D was isolated from sample 12; this RFLP pattern was also seen in the direct PCRs from roots at this site.

The ITS RFLP analyses—The ITS RFLP technique is widely used to discriminate fungal species directly from ectomycorrhizal roots (reviewed in Horton and Bruns, 2001) and has also been applied to orchid mycorrhizae (Taylor and Bruns, 1997, 1999b; Sen et al., 1999; McKendrick et al., 2000, 2002). In addition to revealing fungal diversity by discriminating among species, ITS RFLPs can identify fungi when they match patterns from identified fungal fruitbodies or cultures (Gardes and Bruns, 1996b). In the absence of fruitbody RFLP matches, several ribosomal gene regions can be sequenced to provide taxonomic placements of unknown mycorrhizal fungi at various phylogenetic levels (see Horton and Bruns, 2001). All of these approaches have been utilized in the present study.

First, fungal diversity was estimated by generating fungal ITS RFLP patterns separately from multiple roots from each orchid individual. When we attempted fungal amplification using ITS 1F together with the basidiomycete-selective primer ITS 4B (Gardes and Bruns, 1993), sample 1 (rhizome), and samples 4–10 (roots) from Jackson County yielded weak ITS amplification, and no product was obtained from the other samples. All ITS 1F/ITS 4B amplicons, including the fungal isolate from sample 1, displayed an RFLP pattern labelled type A, which is identical to the pattern obtained from a culture collection isolate of *Thanatephorus ochraceus* (originally described as *T. pennatus*, Currah, 1987; Roberts, 1998). Discrete fungal ITS amplicons were obtained from 50 DNA extracts representing each of the 25 plants with roots and approximately 90% of the roots from which DNA was extracted, using the broad-spectrum fungal primer ITS 1F together with the universal primer ITS 4.

A different picture emerged from analysis of the ITS 1F/ITS 4 amplicons. All of the orchids from Jackson county displayed a dominant RFLP pattern, labeled type C, that did not match *Thanatephorus ochraceus*. However, all these samples displayed a "background" RFLP pattern that matched *Thanatephorus ochraceus* (Fig. 1). We use the term "background" to refer to bands that contain less DNA than the primary bands, as judged by the intensity of ethidium bromide staining.
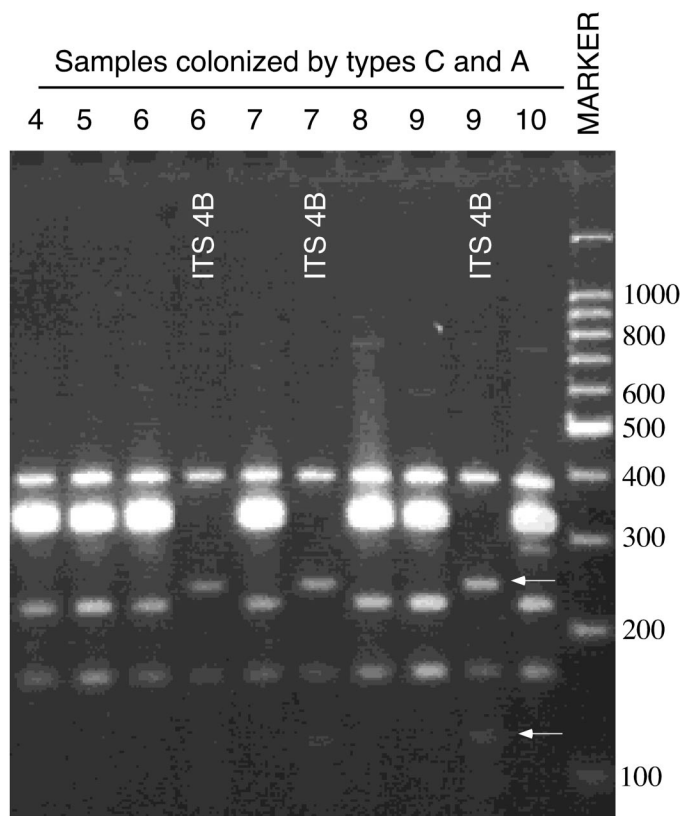

Fig. 1. Multiple colonization of *Hexalectris* roots. The gel shows Hinf I fungal ITS RFLP patterns obtained from *Hexalectris* roots from the Jackson County site; numbers above each lane correspond to the samples listed in Table 1. Most lanes show digests of fragments produced with the "universal fungal" primers ITS 1F and ITS 4 and reveal the background *T. ochraceus* pattern along with the brighter pattern of *Sebacina*-like type C. The lanes marked ITS 4B were amplified with the more selective primers ITS 1F and ITS 4B. Note that the pattern for type C disappears, leaving only the *T. ochraceus* pattern. One band is shifted and one new band appears for the *T. ochraceus* pattern in ITS 4B lanes because the amplification product is about 130 bases longer when using ITS 4B in place of ITS 4.

This result suggests that these plants were colonized by two fungi, one of which could not be amplified with the primer ITS 4B.

The remaining ITS 1F/ITS 4 amplicons yielded RFLP fragments of equal intensity, the weights of which summed to the size of the undigested ITS amplicons, consistent with colonization by a single fungus. Five additional RFLP patterns, labeled types B, D, E, F, and G were observed in these samples (Table 1). Types B, D, and G were each found at single sites in Florida and Georgia, while type E was found in samples of *H. spicata* var. *arizonica* from several sites in Arizona, and type F was found in samples of *H. revoluta* from several sites in Arizona. The RFLP patterns for types B through G were similar to one another, but did not match any patterns obtained from an array of culture collection orchid isolates (data not shown).

Ribosomal gene sequence analyses—To identify the *Hexalectris* fungi, the 5′ end of the nuclear large subunit (nLSU) ribosomal gene region was targeted because of the following features: it is relatively conserved (Hillis and Dixon, 1991), it has been sequenced in a wide diversity of Basidiomycetes, and
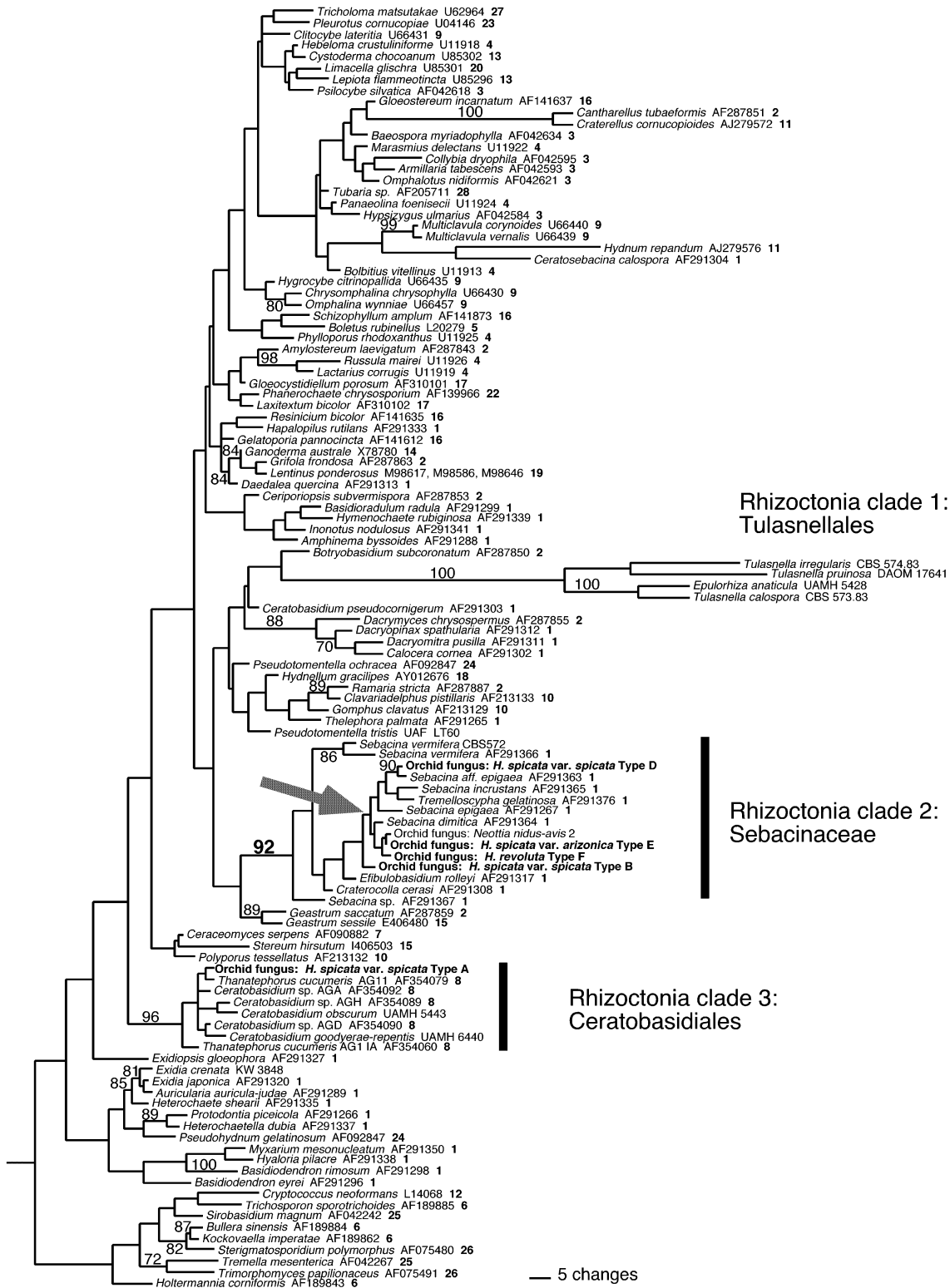
Rhizoctonia clade 1:
Tulasnellales

Rhizoctonia clade 2:
Sebacinaceae

Rhizoctonia clade 3:
Ceratobasidiales

_____ 5 changes

Fig. 2. Most *Hexalectris* fungal associates are closely related members of the Sebacinaceae (see grey arrow). The tree resulted from neighbor-joining analysis of positions 1–385 of the nLSU data set. Significant bootstrap support (>70%) from 1000 neighbor-joining replicates is given above or below branches. The extreme rate accelerations that have occurred in *Tulasnella*, *Hydnum*, and the *Cantharellaceae* can be seen clearly. The polyphyly of the three major clades of "rhizoctonia" fungi (Ceratobasidiaceae, Sebacinaceae, Tulasnellales) is also apparent. The tree is rooted to the Ustilaginiomycetes plus Urediniomycetes, which were removed from the figure due to space limitation. Following each taxon are either the sequence accession from public databases and the reference (bold), or, for new sequences determined in this study, the culture collection, herbarium, or sample code. The culture collections and herbaria are as follows: CBS, CentralBureau voor Schimmelcultures; DAOM, National Mycological Herbarium, Canada; UAMH, University of Alberta Microfungus Garden and Herbarium, Edmonton, Canada; KW, personal collection of Kenneth Wells; RJB, personal collection of Robert J. Bandoni; UAF, Museum of the University of Alaska, Fairbanks, Alaska, USA. References for the sequences downloaded from GenBank are as follows: 1 = Weiss and Oberwinkler, 2001; 2 = Hibbett et al., 2000;

it has assisted in the placement of several unknown ectomy-corrhizal fungi (Chapela et al., 1994; Taylor and Bruns, 1999a; McKendrick et al., 2002). The 5–6 region of the mitochondrial large (ML) subunit is also useful for the placement of unknown fungi into genera and families (Bruns et al., 1998; Kristiansen et al., 2001). However, fungal ML5–6 sequences from several *Hexalectris* samples did not have close relatives in the database and so provided little insight into the fungal identities (data not shown). Fungal-specific primers designed to amplify portions of the nLSU from mixed plant-fungal DNAs have not been reported, to our knowledge. The primer used here, cNL2F, has three mismatches in positions that are conserved across diverse plant species. One of these mismatches is at the final 3′ base, suggesting that this primer should not amplify plant nLSU genes. ITS 1F and cNL2F efficiently amplified a fragment of approximately 1100 bp spanning ITS 1, the 5.8S gene, ITS 2, and about 400 bp of the 5′ end of the nLSU gene directly from *Hexalectris* root DNA extracts. This amplification allowed 5′ sequences of the nLSU gene to be obtained from samples representing all but two of the *Hexalectris* fungal ITS RFLP types.

In our NJ analyses of all taxa and positions 1–385 of the nLSU (Fig. 2), the most complex model, general-time-reversible (GTR + G + I), where each of the 12 possible base changes has an independent probability, with allowance for both rate variation across sites and invariant sites, produced a significantly more likely tree under the ML criterion than any simpler model. The NJ tree is not well resolved or reliable (several of the deep branches are counter to the weight of systematic evidence and to our further analyses, described later, using longer sequences). This result is not surprising since the data set has characteristics known to make phylogenetic inference difficult (Swofford et al., 1996), namely, a large number of taxa relative to the sequence length, highly diverged (i.e., saturated) sequences, and dramatically unequal rates of base substitution. Nevertheless, this analysis indicated that *Hexalectris* fungal types B, D, E, and F are closely related and fall within the Sebacinaceae, while type A is closely related to *Thanatephorus* (as expected from the ITS RFLP match). Neighbor-Joining bootstrap support for these relationships was strong (92% and 96%), despite the poor support for other well-defined clades.

Figure 3 shows the ML tree obtained from analysis of a restricted dataset with longer sequences comprising selected hymenomycete taxa. Again, the most complex available model (GTR + G + I) in NJ analyses resulted in the most-likely tree with −ln = 12 618. However, an incomplete heuristic ML search starting from the NJ tree found a tree with a higher likelihood of −ln = 12 469. A heuristic search under the parsimony criterion retrieved 27 shortest trees of length 2718 with consistency indexes of 0.207 and rescaled consistency indexes of 0.113. Despite some conflict, clades that received strong NJ bootstrap support were also supported in parsimony bootstrapping (data not shown), there was general agreement between the ML and MP topologies (see heavy branches in Fig. 3), and

most expected major groupings of Hymenomycetes were retrieved in this analysis. Most importantly, the Sebacinaceae forms a well-supported major clade near the base of the Hymenomycetes. The ML and MP analyses both suggested that the Sebacinaceae and *Geastrum* are allied to the Tremellales, but without significant bootstrap support. Again, the placement of *Hexalectris* fungus type D within the Sebacinaceae was strongly supported.

The nLSU sequence data also provided a clear explanation for the ITS PCR amplification results with different primer pairs described earlier. *Thanatephorus ochraceus* has two mismatches with the primer ITS4B, neither of which is at the 3′ end, so that weak amplification is not surprising. The *Hexalectris* symbionts and *Sebacina vermifera* have three and four mismatches with ITS4B, one of which is at the final 3′ position; this accounts for the lack of amplification of these fungi with ITS 4B. Neither *Ceratobasidium/Thanatephorus* accessions from GenBank nor the *Sebacina*-like fungi exhibited mismatches with the universal primer ITS 4.

The nLSU sequences allowed the identification of the *Hexalectris* types B, D, E, and F as members of the Sebacinaceae, but did not resolve relationships among the *Hexalectris* symbionts. Furthermore, we did not obtain nLSU sequences from types C or G. We therefore sequenced the faster-evolving ITS region from each ITS RFLP type. Types B–G all had highest scoring matches (90–94% identity) to *Tremellodendron pallidum* (Sebacinaceae) accession AF384862 in BLASTn searches of GenBank; type A had the highest scoring match (95% identity) to *Thanatephorus cucumeris* AG4 accession AY089956.

Analyses of Sebacinaceae ITS sequences showed that the associates of *Hexalectris* are phylogenetically intermixed with the associates of *Neottia nidus-avis* as well as ectomycorrhizae collected from woody hosts in Australia, while *Efibulobasidium* and *Sebacina vermifera* CBS 572 were distant relatives (Fig. 4). Relationships at the tips of the tree were well resolved, as indicated by significant parsimony bootstrap support values and agreement between topologies from maximum-likelihood and parsimony analyses (Fig. 4). However, deeper relationships were not well resolved, presumably because of the high levels of sequence variation within the sampled taxa. The GTR + G + I model best fit the ITS data and produced a tree with −ln = 3597 from an heuristic ML search (as a contrasting example, the HKY85 model with enforcement of a molecular clock produced a tree with a likelihood of −ln = 3921). The ITS dataset had 634 characters, of which 382 were constant and 138 were parsimony-informative. The heuristic searches found four most parsimonious trees of 606 steps with consistency indexes (CIs) of 0.625 and rescaled consistency indexes (RCs) of 0.377; they had lower likelihoods under the GTR + G + I model than the best ML tree.

DISCUSSION

The major goals of this work were, first, to determine the degree of mycorrhizal specificity in *Hexalectris spicata*, and,

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3 = Moncalvo et al., 2000; 4 = Chapela et al., 1994; 5 = Berres et al., 1995; 6 = Fell et al., 2000; 7 = Larsson and Larsson, 1998; 8 = Gonzalez et al., 2001; 9 = Lutzoni, 1997; 10 = Humpert et al., 2001; 11 = Li et al., 1999; 12 = Fan et al., 1994; 13 = Johnson and Vilgalys, 1998; 14 = Moncalvo et al., 1995; 15 = Hibbett et al., 1997; 16 = Hallenberg and Parmasto, 1999; 17 = Larsson and Hallenberg, 2001; 18 = Koljalg and Renvall, 2000; 19 = Hibbet and Vilgalys, 1993; 20 = Drehmel et al., 1999; 21 = McKendrick et al., 2002; 22 = Thorn et al., 2000; 23 = Vilgalys and Sun, 1994; 24 = Koljalg and Larsson, 1998; 25 = Chen, 1998; 26 = Fell et al., 1999; 27 = Hwang and Kim, 1995; 28 = Aime, 1999.

Fig. 3. The Sebacinaceae comprise a distinct clade of basal hymenomycetes, which includes *Hexalectris* type D (see grey arrow). The tree was derived from maximum-likelihood analysis of positions 1–610 of the nLSU alignment. Branches that were also found in the majority-rule consensus of the 27 most-parsimonious trees are indicated by heavy lines. Parsimony bootstrap support levels from 500 replicates are shown above or below branches. The tree is midpoint rooted.
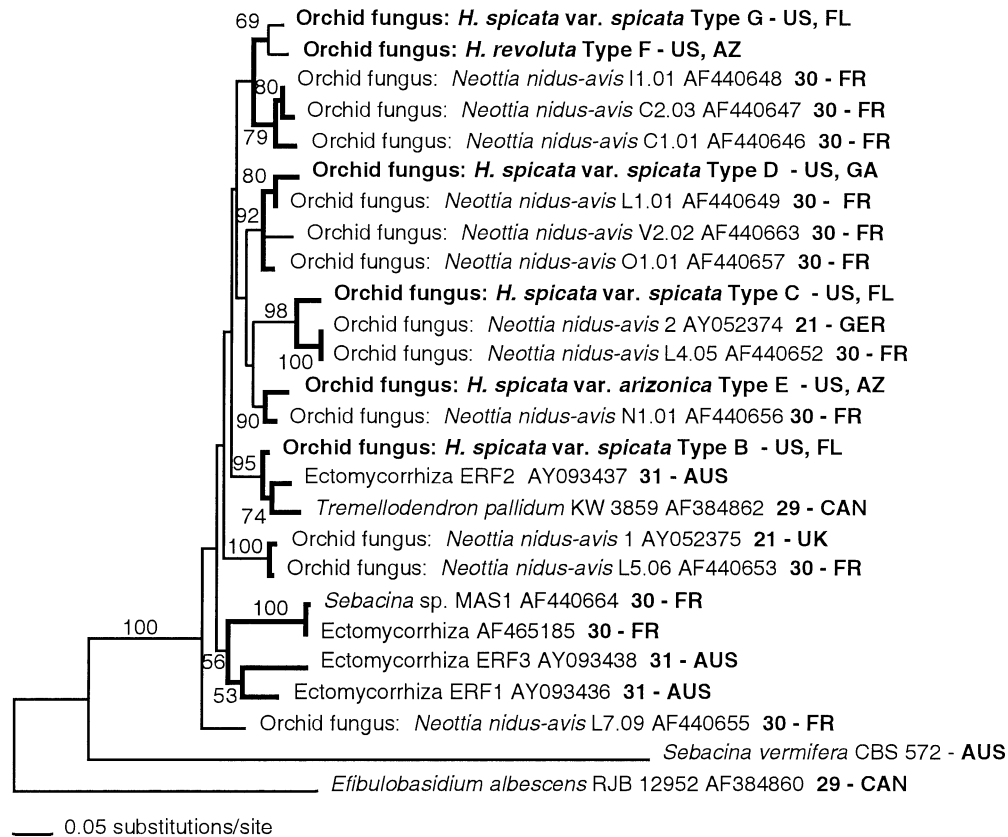
Fig. 4. The *Sebacina*-like associates from *Hexalectris* are phylogenetically intermixed with ectomycorrhizal fungi from the orchid *Neottia nidus-avis* in France and woody hosts in Australia. Maximum-likelihood tree derived from analyses of nuclear ITS sequences. Support from 1000 parsimony bootstrap replicates is provided above or below branches; heavy lines indicate branches also found in the parsimony strict consensus tree. The tree is rooted to *Efibulobasidium*. Information following the taxon names follows the format in Fig. 2, with the addition of the following localities: AUS, Australia; CAN, Canada; FR, France; GER, Germany; US, United States (AZ, Arizona; GA, Georgia; FL, Florida). References for the sequences downloaded from GenBank are as follows: 29 = Lim and Barbee, 2001; 30 = Selosse et al., 2002a, b; 31 = Glen et al., 2002.

second, to determine whether specificity is structured *within* this taxon. The issue of specificity in orchids has provoked controversy for more than 50 yr (Bernard, 1903; Curtis, 1939; Hadley, 1970; Warcup, 1981). The confused taxonomy of the fungi found in orchids has probably contributed to the controversy. Identification of the fungal symbionts of *Hexalectris* was not straightforward. The predominant fungal associates of *Hexalectris* were extremely difficult to isolate in pure culture. Furthermore, ML5–6 sequences did not help to identify these fungi, and related nLSU sequences were not available in GenBank until very recently.

The fungi that have been most frequently isolated from orchids are often described as "rhizoctonia" strains. *Rhizoctonia* is an ill-defined anamorphic (i.e., defined on asexual characters) form-genus within the Basidiomycota, that includes the well-known plant pathogen *Rhizoctonia solani* (teleomorph = *Thanatephorus cucumeris*). In much of the orchid mycorrhizal literature, the term "rhizoctonia" is used as if it were a natural grouping. Furthermore, it is usually assumed that orchid rhizoctonia fungi are saprophytes or opportunistic parasites by extrapolation from the ecology of *Rhizoctonia solani*. However, recent molecular phylogenetic and ultrastructural analyses show that "rhizoctonia" is deeply polyphyletic (Wells, 1994; Anderson, 1996; Muller et al., 1998), with orchid rhizoctonia strains falling into three distantly related lineages: the Ceratobasidiales, the Sebacinaceae, and the Tulasnellales (see

Fig. 2 and Taylor et al., 2002). Furthermore, the diversity and ecology of the fungi within the latter two lineages is poorly known at present.

Diversity and relationships of the fungi associated with Hexalectris spicata—We predicted that *Hexalectris spicata* would display a high degree of specificity because recent molecular phylogenetic studies of several nonphotosynthetic orchids as well as species in the Ericaceae have documented remarkable specificity (Taylor and Bruns, 1997, 1999b; Bidartondo and Bruns, 2001, 2002). *Hexalectris spicata* appears to fit this prediction: relative to typical photosynthetic plants, it has a very restricted range of mycorrhizal associates. The ITS type A is identical to that obtained from a culture collection isolate of *Thanatephorus ochraceus* and falls within the Ceratobasidiaceae (Fig. 2). The significance of the interaction between *Hexalectris* and *Thanatephorus* is unclear. This fungus occurred sporadically in orchid roots and at low abundance when it did occur, suggesting that it is not a critical mycorrhizal symbiont. Its occurrence in nonmycorrhizal rhizome tissue suggests, instead, the possibility of a pathogenic interaction with the orchid, which would fit the niche ascribed to these fungi in other settings (Adams, 1988). Each of the remaining six fungal ITS RFLP patterns from *Hexalectris* roots fall within the Sebacinaceae based on both nuclear nLSU and ITS sequence analyses (see Figs. 2–4). Hence, we conclude

that the *Hexalectris spicata* complex is primarily specialized toward fungi in the Sebacinaceae.

To better understand the dynamics of mycorrhizal specialization within the Orchidaceae, it is important to pinpoint the phylogenetic position of each group of fungi that is targeted by orchids. Determination of the relationship of *Sebacina*-like fungi to other major lineages of hymenomycetous Basidiomycetes has been problematic. It has most often been placed within one of the four heterobasidiomycetous families of "jelly" fungi—the Auriculariales, Dacrymycetales, Tremellales, and Tulasnellales (Wells, 1994). However, a recent detailed molecular systematic study of the Auriculariales by Weiss and Oberwinkler (2001) revealed that *Sebacina*, *Tremelloscypha*, *Efibulobasidium*, and *Craterocolla* formed a well-supported group quite separate from the Auriculariales, Dacrymycetales, and Tremellales. Our analyses show that the Sebacinaceae is also quite distant from the Tulasnellales (Fig. 2). Hence, the Sebacinaceae comprises a distinct major lineage near the base of the Hymenomycetes. Note that this "rhizoctonia" lineage is also quite distant from *Thanatephorus* (Ceratobasidiales; Figs. 2, 3).

Ecology of the fungal associates of Hexalectris spicata—We have proposed previously that nonphotosynthetic orchids are likely to associate with fungi that have access to large, persistent sources of carbon (Taylor and Bruns, 1997, 1999b; Taylor et al., 2002). Ectomycorrhizal fungi with direct connections to large photosynthetic trees via ectomycorrhizal fungi provide a striking example (Taylor and Bruns, 1997). We did not investigate the activities of the *Sebacina*-like fungi outside of their associations with *Hexalectris*. Until recently, there was little compelling evidence concerning the trophic activities of fungi in the Sebacinaceae, but saprophytic, mycoparasitic, and mycorrhizal activities have all been proposed. Recently, the *Sebacina*-like fungi that associate with the European nonphotosynthetic orchid *Neottia nidus-avis* have been shown to form ectomycorrhizae with surrounding woody hosts (Selosse et al., 2002a, b). Furthermore, sequences of *Sebacina*-like fungi have been obtained from ectomycorrhizal roots in Australia (Glen et al., 2002). The *Sebacina*-like associates of *Hexalectris* are phylogenetically intermixed with those of *Neottia nidus-avis* and the Australian fungi (Fig. 4), suggesting that the *Hexalectris* associates are also ectomycorrhizal. Consistent with this hypothesis, *Hexalectris spicata* inhabits forests dominated by oak (*Quercus*) and hickory (*Carya*), both of which are ectomycorrhizal. However, at least some members of the Sebacinaceae are saprotrophic (Weiss and Oberwinkler, 2001), and further investigation of the ecology of these fungi is warranted.

Patterns of specificity in Hexalectris spicata—Autotrophic plants such as ectomycorrhizal (EM) pines or AM grasses rarely show significant specialization toward subordinal taxa of mycorrhizal fungi (Molina et al., 1992). Yet *Hexalectris spicata*, along with other orchids and myco-heterotrophs, displays a considerable degree of mycorrhizal specificity.

Not only are some orchids mycorrhizal specialists, but different orchid lineages are specialized on distantly related taxa of Basidiomycetes (Warcup, 1971, 1981; Ramsay et al., 1986; Taylor and Bruns, 1997, 1999b; Taylor et al., 2002). This might be explained in two ways. If the common ancestor of these lineages was also a mycorrhizal specialist, then a number of switches among fungal taxa have occurred. Alternatively,

the common ancestor may have had broad associations, and each orchid lineage evolved specificity independently. In either case, specificity has changed dramatically during the evolution of these orchid lineages. This inference raises the critical questions of how often specialization has changed, at what point in lineage divergence changes occur, and whether they may contribute to ecological isolation between orchid populations and species.

The most striking possibility that emerges from our data on *Hexalectris* is that the two western floral variants may associate with different *Sebacina*-like fungi (Table 1). The samples of *H. spicata* var. *spicata* contained four different *Sebacina*-like taxa (B–D, G), while the other *Hexalectris* varieties were each associated with a single *Sebacina*-like type (E, F). It is impossible to disentangle geographic distance from plant genetic variation in comparing *H. spicata* var. *spicata*, which we sampled only in the eastern United States, with the other two floral variants, which we sampled only in the Southwest. However, the sampling sites for *H. spicata* var. *arizonica* and *H. revoluta* were geographically intermixed (Table 1). Furthermore, these taxa grow sympatrically within meters of one another (R. A. Coleman, University of Arizona Herbarium, personal communication), although we were unable to sample sympatric individuals. Hence, the fact that each variant was consistently associated with a particular *Sebacina*-like taxon is more likely to be related to genetic variation for specificity than to geographic distance. Because we only sampled one or two individuals per population, our results should be considered provisional. These populations each support only 5–20 flowering individuals, a factor that dissuaded us from further sampling.

Only a handful of other studies have sought to determine the phylogenetic scale of changes in specificity in orchids. A series of detailed studies have documented narrow specificity in an array of photosythetic terrestrial orchids from Australia (Warcup, 1971, 1981; Perkins and McGee, 1995; Perkins et al., 1995; Ramsay et al., 1986, 1987). These studies allow for some inferences concerning the evolutionary dynamics of specificity. Associations within tribes span the Sebacinaceae, Tulasnellales, and Ceratobasidiales, implying that specificity is not conserved at this broad phylogenetic scale. However, specificity was often conserved in the subtribes and genera that make up these tribes. For example, 31 species in five out of six genera of the Caladeniinae are specialized on the fungus *Sebacina vermifera* (Warcup, 1981). In a few cases, however, changes in specificity were detected within genera (Warcup, 1981). Intraspecific variation in specificity was not detected. However, these studies relied on fungal isolation and morphological characterization, meaning that closely related taxa, such as the *Sebacina*-like fungi associated with *Hexalectris*, may not have been distinguished.

The three floral forms of the *H. spicata* complex considered in this study may or may not be reproductively isolated. Regardless, they are clearly closely related, comprising either sister taxa or divergent intraspecific populations. The preliminary evidence for differences in *Sebacina*-like associates between the two western floral forms suggest that mycorrhizal specificity may have diverged in concert with recent phylogenetic divergence in this orchid lineage. This evidence is very similar to recent findings in the fully myco-heterotrophic orchid genus *Corallorhiza*. The closely related sister taxa *C. mertensiana* and *C. maculata* associate with nonoverlapping assemblages of fungi in the Russulaceae (Taylor and Bruns, 1999b). Even

more striking, the occurrence of particular russuloid fungi within *C. maculata* is strongly correlated with plant genotype, as revealed by neutral DNA markers (D. L. Taylor, T. D. Bruns, and S. A. Hodges, unpublished data). In addition, considerable specificity as well as differences in specificity between sister genera have recently been reported in several photosynthetic, epiphytic orchids (Otero et al., 2002). These emerging patterns of fine-scale diversification in mycorrhizal specificity mirror recent findings in certain nonphotosynthetic lineages of the Monotropoideae (Bidartondo and Bruns, 2002).

The roots of *Hexalectris spicata* are strikingly short and wide, a trend noted in diverse myco-heterotrophic plants (Leake, 1994). The virtual absence of leaves, the starch-packed rhizomes, and the modified roots of *Hexalectris* together suggest a state of advanced myco-heterotrophy (Leake, 1994), suggesting that these plants must depend heavily on their mycorrhizal fungi. *Hexalectris nitida*, *H. warnockii*, *H. revoluta* and *H. spicata* var. *arizonica* are on state sensitive plant lists. Though widespread, its habitats are threatened, and even *H. spicata* var. *spicata* has recently been listed as endangered in Florida. If the pattern of variation in specificity among floral forms and specificity toward single *Sebacina* taxa is upheld in additional studies and also occurs in other species of *Hexalectris*, conservation of these orchids may well require the protection of an array of *Sebacina*-like taxa. Because of our ignorance of the ecologies of these fungi and their resistance to laboratory manipulation, the only practical approach to achieving this goal would appear to be the protection of the habitats and successional sequences in which these orchids *and their fungi* are found. If similar specificity patterns continue to be found in other orchids, this conclusion may have broad relevance.

Divergence in pollination syndromes is thought to have contributed to the phylogenetic radiation of the Orchidaceae (van der Pijl and Dodson, 1966), as it has in other angiosperm lineages (Grant, 1949; Hodges and Arnold, 1994). Changes in pollination can lead to reproductive isolation, and, hence, speciation. Given the unique mycorrhizal ecology of orchids and the hints of rapidly evolving specificity, mycorrhizal interactions may have also contributed to orchid diversification. However, if changes in specificity promote speciation, the route to reproductive isolation must be less direct than is the case with changes in pollination.

LITERATURE CITED

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

AIME, M. C. 1999. Infrageneric concepts in the genus *Crepidotus* (Fr.) Stuade. http://www.ncbi.nlm.nih.gov/Genbank/index.html.

ALTSHUL, S. F., ET AL. 1997. Gapped BLAST and PSLBLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389–3402.

ANDERSEN, T. F. 1996. A comparative taxonomic study of *Rhizoctonia* sensu lato employing morphological, ultrastructural and molecular methods. *Mycological Research* 100: 1117–1128.

BERNARD, N. 1903. La germination des orchidées. *Comptes Rendus de l' Académie des Sciences* 138: 483–485.

BERRES, M. E., L. J. SZABO, AND D. J. MCLAUGHLIN. 1995. Phylogenetic relationships in auriculariaceous basidiomycetes based on 25S ribosomal DNA sequences. *Mycologia* 87: 821–840.

BIDARTONDO, M. I., AND T. D. BRUNS. 2001. Extreme specificity in epiparasitic Monotropoideae (Ericaceae): widespread phylogenetic and geographical structure. *Molecular Ecology* 10: 2285–2295.

BIDARTONDO, M. I., AND T. D. BRUNS. 2002. Fine-level mycorrhizal speci-

ficity in the Monotropoideae (Ericaceae): specificity for fungal species groups. *Molecular Ecology* 11: 557–569.

BRUNO, W. J., N. D. SOCCI, AND A. L. HALPERN. 2000. Weighted neighbor joining: a likelihood-based approach to distance-based phylogeny reconstruction. *Molecular Biology and Evolution* 17: 189–197.

BRUNS, T. D., 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* C. L. Withner [ed.], The orchids: a scientific survey, 361–395. Ronald Press, New York, New York, USA.

CATLING, P. M., AND V. S. ENGEL. 1993. Systematics and distribution of *Hexalectris spicata* var. *arizonica* (Orchidaceae). *Lindleyana* 8: 119–125.

CHAPELA, I. H., S. A. REHNER, T. R. SCHULTZ, AND U. G. MUELLER. 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science* 266: 1691–1694.

CHEN, C. J. 1998. Morphological and molecular studies in the genus *Tremella*. *In* A. Bresinsky, H. Butin, and H. O. Schwantes [eds.], Bibliotecha mycologica, vol. 174, 1–225. J. Cramer, Stuttgart, Germany.

COLEMAN, R. A. 2000. *Hexalectris revoluta* Correll (Orchidaceae). *Madrono* 47: 138.

COLEMAN, R. A. 2002. The wild orchids of Arizona and New Mexico. Comstock, Ithaca, New York, New York, USA.

CURRAH, R. S. 1987. *Thanatephorus pennatus* sp. nov. isolated from the mycorrhizal roots of *Calypso bulbosa* (Orchidaceae) from Alberta. *Canadian Journal of Botany* 65: 1957–1960.

CURRAH, R. S., S. HAMBLETON, AND A. SMRECIU. 1988. The mycorrhizae and mycorrhizal fungi of *Calypso bulbosa* (Orchidaceae). *American Journal of Botany* 75: 737–750.

CURRAH, R. S., L. SIGLER, AND S. HAMBLETON. 1987. New records and new taxa of fungi from the mycorrhizae of terrestrial orchids of Alberta. *Canadian Journal of Botany* 65: 2473–2482.

CURRAH, R. S., E. A. SMRECIU, AND S. HAMBLETON. 1990. Mycorrhizae and mycorrhizal fungi of boreal species of *Platanthera* and *Coeloglossum* (Orchidaceae). *Canadian Journal of Botany* 68: 1171–1181.

CURTIS, J. T. 1939. The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. *American Journal of Botany* 26: 390–398.

DREHMEL, D., J.-M. MONCALVO, AND R. VILGALYS. 1999. Molecular phylogeny of *Amanita* based on large-subunit ribosomal DNA sequences: implications for taxonomy and character evolution. *Mycologia* 91: 610–618.

FAN, M., B. P. CURRIE, B. P. GUTTELL, M. A. RAGAN, AND A. CASADEVALL. 1994. The 16S-like, 5.8S, and 23S-like rRNA's of the two varieties of *Cryptococcus neoformans*: sequence, secondary structure, phylogenetic analysis, and restriction fragment polymorphisms. *Journal of Medical and Veterinary Mycology* 32: 163–180.

FELL, J. W., T. BOEKHOUT, A. FONSECA, G. SCORZETTI, AND A. STATZELL-TALLMAN. 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *International Journal of Systematic and Evolutionary Microbiology* 50: 1351–1371.

FELL, J. W., H. ROEIJMANS, AND T. BOEKHOUT. 1999. Cystofilobasidiales, a new order for heterobasidiomycetous yeasts. *International Journal of Systematic Bacteriology* 49: 907–913.

GARDES, M., AND T. D. BRUNS. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118.

GARDES, M., AND T. D. BRUNS. 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., AND T. D. BRUNS. 1996b. ITS-RFLP matching for identification of fungi. *Methods in Molecular Biology* 50: 177–86.

GLEN, M., I. C. TOMMERUP, N. L. BOUGHER, AND P. A. O'BRIEN. 2002. Are Sebacinaceae common and widespread ectomycorrhizal associates of *Eucalyptus* species in Australian forests? *Mycorrhiza* 12: 243–247.

GONZALEZ, D., D. E. CARLING, S. KUNINAGA, R. VILGALYS, AND M. A. CUBETA. 2001. Ribosomal DNA systematics of *Ceratobasidium* and *Thanatephorus* with *Rhizoctonia* anamorphs. *Mycologia* 93: 1138–1150.

GRANT, V. 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* 3: 82–97.

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

HADLEY, G., AND S. PURVES. 1974. Movement of ¹⁴carbon from host to fungus in orchid mycorrhiza. *New Phytologist* 73: 475–482.

HALLENBERG, N., AND E. PARMASTO. 1999. Taxonomy of phlebioid fungi (Basidiomycota). http://www.ncbi.nlm.nih.gov/Genbank/index.html.

HECKMAN, D. S., D. M. GEISER, B. R. EIDELL, R. L. STAUFFER, N. L. KARDOS, AND S. B. HEDGES. 2001. Molecular evidence for the early colonization of land by fungi and plants. *Science* 293: 1129–1133.

HIBBETT, D. S., L. B. GILBERT, AND M. J. DONOGHUE. 2000. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* 407: 506–508.

HIBBETT, D. S., E. M. PINE, E. LANGER, G. LANGER, AND M. J. DONOGHUE. 1997. Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. *Proceedings of the National Academy of Sciences, USA* 94: 12 002–12 006.

HIBBETT, D. S., AND R. VILGALYS. 1993. Phylogenetic relationships of *Lentinus* (Basidiomycotina) inferred from molecular and morphological characters. *Systematic Botany* 18: 409–433.

HILLIS, D. M., AND M. T. DIXON. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology* 66: 411–446.

HODGES, S. A., AND M. L. ARNOLD. 1994. Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens. Proceedings of the National Academy of Sciences, USA* 91: 2493–2496.

HORTON, T. R., AND T. D. BRUNS. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology* 10: 1855–1871.

HUELSENBECK, J. P. 1995. Performance of phylogenetic methods in simulation. *Systematic Biology* 44: 17–48.

HUMPERT, A. J., E. L. MUENCH, A. J. GIACHINI, M. A. CASTELLANO, AND J. W. SPATAFORA. 2001. Molecular phylogenetics of *Ramaria* and related genera: evidence from nuclear large subunit and mitochondrial small subunit rDNA sequences. *Mycologia* 93: 465–477.

HWANG, S., AND J. KIM. 1995. Nucleotide sequence analysis of the 5S ribosomal RNA genes of the mushroom *Tricholoma matsutake. Journal of Microbiology* 33: 136–141.

JOHNSON, J., AND R. VILGALYS. 1998. Phylogenetic systematics of *Lepiota* sensu lato based on nuclear large subunit rDNA evidence. *Mycologia* 90: 971–979.

KAREN, O., N. HOGBERG, A. DAHLBERG, L. JONSSON, AND J.-E. NYLUND. 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.

KOLJALG, U., AND E. LARSSON. 1998. *Pseudotomentella ochracea* sp. nov., based on morphological and molecular data. *Folia Cryptogamica Estonica* 33: 53–56.

KOLJALG, U., AND P. RENVALL. 2000. Hydnellum gracilipes: a link between stipitate and resupinate Hymenomycetes. *Karstenia* 40: 71–77.

KRISTIANSEN, K. A., D. L. TAYLOR, R. KJOLLER, H. N. RASMUSSEN, AND S. ROSENDAHL. 2001. Identification of mycorrhizal fungi from single pelotons of *Dactylorhiza majalis* (Orchidaceae) using single-strand conformation polymorphism and mitochondrial ribosomal large subunit DNA sequences. *Molecular Ecology* 10: 2089–2093.

KUHNER, M. K., AND J. FELSENSTEIN. 1994. A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates. *Molecular Biology and Evolution* 11: 459–468.

LARSSON, E., AND N. HALLENBERG. 2001. Species delimitation in the *Gloeocystidiellum porosum-clavuligerum* complex inferred from compatibility studies and nuclear rDNA sequence data. *Mycologia* 93: 907–914.

LARSSON, K.-H., AND E. LARSSON. 1998. A molecular perspective on *Ceraceomyces sublaevis*. http://www.ncbi.nlm.nih.gov/Genbank/index.html.

LEAKE, J. R. 1994. Tansley review no. 69. The biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist* 127: 171–216.

LI, T. H., Y. Q. CHEN, AND L. H. HU. 1999. Partial 25S rDNA sequence of *Cantharellus* and its phylogenetic implication. *Mycosystema* 1: 25–31.

LIM, S.-R., AND M. L. BERBEE. 2001. Molecular phylogenetics of the Sebacinaceae. http://www.ncbi.nlm.nih.gov/Genbank/index.html.

LUER, C. A. 1975. The native orchids of the United States and Canada, 1st ed. New York Botanical Garden, Ipswitch, New York, USA.

LUTZONI, F. M. 1997. Phylogeny of lichen- and non-lichen-forming omphalinoid mushrooms and the utility of testing for combinability among multiple data sets. *Systematic Biology* 46: 373–406.

MARX, D. H. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* 59: 153–163.

MCKENDRICK, S. L., J. R. LEAKE, D. L. TAYLOR, AND D. J. READ. 2000. Symbiotic germination and development of myco-heterotrophic plants in nature: ontogeny of *Corallorhiza trifida* Chatel and characterisation of its mycorrhizal fungi. *New Phytologist* 145: 523–537.

MCKENDRICK, S. L., J. R. LEAKE, D. L. TAYLOR, AND D. J. READ. 2002. Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. *New Phytologist* 154: 233–247.

MOLINA, R., H. MASSICOTTE, AND J. M. TRAPPE. 1992. Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. *In* M. F. Allen [ed.], Mycorrhizal functioning: an integrative plant-fungal process, 357–423. Chapman and Hall, New York, New York, USA.

MONCALVO, J.-M., F. M. LUTZONI, S. A. REHNER, J. JOHNSON, AND R. VILGALYS. 2000. Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic Biology* 49: 278–305.

MONCALVO, J. M., H. H. WANG, AND R. S. HSEU. 1995. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia* 87: 223–228.

MULLER, W. H., J. A. STALPERS, A. C. VAN AELST, T. P. VAN DER KRIFT, AND T. BOEKHOUT. 1998. Field emission gun-scanning electron microscopy of septal pore caps of selected species in the *Rhizoctonia* s.1. complex. *Mycologia* 90: 170–179.

OTERO, J. T., J. D. ACKERMAN, AND P. BAYMAN. 2002. Diversity and host specificity of endophytic *Rhizoctonia*-like fungi from tropical orchids. *American Journal of Botany* 89: 1852–1858.

PERKINS, A. J., G. MASUHARA, AND P. A. MCGEE. 1995. Specificity of the associations between *Microtis parviflora* (Orchidaceae) and its mycorrhizal fungi. *Australian Journal of Botany* 43: 85–91.

PERKINS, A. J., AND P. A. MCGEE. 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.

PURVES, S., AND G. HADLEY. 1974. Movement of carbon compounds between the partners in orchid mycorrhiza. *In* F. E. Sanders, B. Mosse, and P. B. Tinker [eds.], Endomycorrhizas, 175–194. Academic Press, San Francisco, California, USA.

RAMSAY, R. R., K. W. DIXON, AND K. SIVASITHAMPARAM. 1986. Patterns of infection and endophytes associated with western Australian orchids. *Lindleyana* 1: 203–214.

RAMSAY, R. R., K. SIVASITHAMPARAM, AND K. W. DIXON. 1987. Anastomosis groups among rhizoctonia-like endophytic fungi in southwestern Australian *Pterostylis* species (orchidaceae). *Lindleyana* 2: 161–166.

RASMUSSEN, H. N. 1995. Terrestrial orchids: from seed to mycotrophic plant. Cambridge University Press, New York, New York, USA.

RASMUSSEN, H. N., AND D. F. WHIGHAM. 1993. Seed ecology of dust seeds *in situ*: a new study technique and its application to terrestrial orchids. *American Journal of Botany* 80: 1374–1378.

ROBERTS, P. 1998. *Thanatephorus ochraceus*: a saprotrophic and orchid endomycorrhizal species. *Sydowia* 50: 252–256.

SELOSSE, M. A., R. BAUER, AND B. MOYERSOEN. 2002a. Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. *New Phytologist* 155: 183–195.

SELOSSE, M. A., W. M. ELSS, J. L. JANY, AND A. TILLIER. 2002b. Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. *Molecular Ecology* 11: 1831–1844.

SEN, R., A. M. HIETALA, AND C. D. ZELMER. 1999. Common anastomosis and internal transcribed spacer RFLP groupings in binucleate *Rhizoctonia* isolates representing root endophytes of *Pinus sylvestris*, *Ceratorhiza* spp. from orchid mycorrhizas and a phytopathogenic anastomosis group. *New Phytologist* 144: 331–341.

SMITH, S. E. 1966. Physiology and ecology of orchid mycorrhizal fungi with reference to seedling nutrition. *New Phytologist* 65: 488–499.

SMITH, S. E. 1967. Carbohydrate translocation in orchid mycorrhizas. *New Phytolgist* 66: 371–378.

SMITH, S. E., AND D. J. READ. 1997. Mycorrhizal symbiosis, 2nd ed. Academic Press, San Diego, California, USA.

SWOFFORD, D. L. 2000. PAUP*4.0—Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, Massachusetts, USA.

SWOFFORD, D. L., G. J. OLSEN, P. J. WADDELL, AND D. M. HILLIS. 1996. Phylogenetic inference. *In* D. M. Hillis, C. Moritz, and B. K. Mable [eds.], Molecular systematics, 407–514. Sinauer Associates, Sunderland, Massachusetts, USA.

SWOFFORD, D. L., P. J. WADDELL, J. P. HUELSENBECK, P. G. FOSTER, P. O. LEWIS, AND J. S. ROGERS. 2001. Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. *Systematic Biology* 50: 525–539.

TAYLOR, D. L. 2000. A new dawn—the ecological genetics of mycorrhizal fungi. *New Phytologist* 147: 236–239.

TAYLOR, D. L., AND T. D. BRUNS. 1997. Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences, USA* 94: 4510–4515.

TAYLOR, D. L., AND T. D. BRUNS. 1999a. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. *Molecular Ecology* 8: 1837–1850.

TAYLOR, D. L., AND T. D. BRUNS. 1999b. Population, habitat and genetic correlates of mycorrhizal specialization in the 'cheating' orchids *Corallorhiza maculata* and *C. mertensiana*. *Molecular Ecology* 8: 1719–1732.

TAYLOR, D. L., T. D. BRUNS, J. R. LEAKE, AND D. J. READ. 2002. Mycorrhizal specificity and function in myco-heterotrophic plants. *In* M. G. A. van der Heijden and I. R. Sanders [eds.], The ecology of mycorrhizas, vol. 157, 375–414. Springer-Verlag, Berlin, Germany.

THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN, AND D. G. HIGGINS. 1997. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876–4882.

THOMPSON, J. N. 1994. The coevolutionary process. University of Chicago Press, Chicago, Illinois, USA.

THORN, R. G., J.-M. MONCALVO, C. A. REDDY, AND R. VILGALYS. 2000. Phylogenetic analyses and the distribution of nematophagy support a monophyletic Pleurotaceae within the polyphyletic pleurotoid-lentinoid fungi. *Mycologia* 92: 241–252.

VAN DER PIJL, L., AND C. H. DODSON. 1966. Orchid flowers: their pollination and evolution. Fairchild Tropical Garden and the University of Miami Press, Coral Gables, Florida, USA.

VILGALYS, R., AND B. L. SUN. 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, USA* 91: 4599–4603.

WARCUP, J. H. 1971. Specificity of mycorrhizal association in some Australian terrestrial orchids. *New Phytologist* 70: 41–46.

WARCUP, J. H. 1981. The mycorrhizal relationships of Australian orchids. *New Phytologist* 87: 371–381.

WEISS, M., AND F. OBERWINKLER. 2001. Phylogenetic relationships in Auriculariales and related groups: hypotheses derived from nuclear ribosomal DNA sequences. *Mycological Research* 105: 403–415.

WELLS, K. 1994. Jelly fungi, then and now. *Mycologia* 86: 18–48.

WHITE, T. J., T. BRUNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White [eds.], PCR protocols: a guide to methods and applications, 315–322. Academic Press, San Diego, California, USA.

ZELMER, C. D., AND R. S. CURRAH. 1995. Evidence for a fungal liaison between *Corallorhiza trifida* (Orchidaceae) and *Pinus contorta* (Pinaceae). *Canadian Journal of Botany* 73: 862–866.

ZELMER, C. D., L. CUTHBERTSON, AND R. S. CURRAH. 1996. Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms. *Mycoscience* 37: 439–448.