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# Mycorrhizal specificity in the fully mycoheterotrophic *Hexalectris* Raf. (Orchidaceae: Epidendroideae)

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## Abstract

**Mycoheterotrophic species have abandoned an autotrophic lifestyle and obtain carbon exclusively from mycorrhizal fungi. Although these species have evolved independently in many plant families, such events have occurred most often in the Orchidaceae, resulting in the highest concentration of these species in the tracheophytes. Studies of mycoheterotrophic species' mycobionts have generally revealed extreme levels of mycorrhizal specialization, suggesting that this system is ideal for studying the evolution of mycorrhizal associations. However, these studies have often investigated single or few, often unrelated, species without consideration of their phylogenetic relationships. Herein, we present the first investigation of the mycorrhizal associates of all species of a well-characterized orchid genus comprised exclusively of mycoheterotrophic species. With the employment of molecular phylogenetic methods, we identify the fungal associates of each of nine *Hexalectris* species from 134 individuals and 42 populations. We report that *Hexalectris warnockii* associates exclusively with members of the Thelephoraceae, *H. brevicaulis* and *H. grandiflora* associate with members of the Russulaceae and Sebacinaceae subgroup A, while each member of the *H. spicata* species complex associates primarily with unique sets of Sebacinaceae subgroup A clades. These results are consistent with other studies of mycorrhizal specificity within mycoheterotrophic plants in that they suggest strong selection within divergent lineages for unique associations with narrow clades of mycorrhizal fungi. Our results also suggest that mycorrhizal associations are a rapidly evolving characteristic in the *H. spicata* complex.**

*Keywords*: host parasite interactions, mycoheterotrophy, mycorrhizal specificity, orchid mycorrhizae, Russulaceae, Sebacinaceae

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## Introduction

The mycorrhizal symbiosis is one of the most common on Earth and is a diffuse mutualism between plants and fungi whereby plants gain mineral nutrition from fungi and fungi gain photosynthetically derived carbon from plants (Smith & Read 2008). As evolutionary theory predicts of mutualisms (Sachs & Simms 2006), the mycorrhizal mutualism has evolved into a heavily one-sided affair, if not parasitism, many times, resulting in over 400 fully (i.e., life-long) mycoheterotrophic plant species (MHPs) in at least 10 families (Leake 1994; Merckx & Bidartondo 2008). These 'cheaters' of the mycorrhizal mutualism (Taylor & Bruns 1997) are recognizable based on a loss of autotrophic capabilities via degeneration of photosynthetic genes (Cameron & Molina 2006; Barrett & Freudenstein 2008), photosynthetic pigments, and leaves and loss or reduction of roots (Leake 1994).

The species-rich Orchidaceae is particularly predisposed to mycoheterotrophy. Its species produce tiny seeds that lack the nutrient reserves necessary for early growth and development (Leake 1994). For this reason it is thought that nearly all of its ~25 000 species

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(Dressler 1981) are adapted to hosting mycorrhizal fungi that provide mineral nutrition and carbon during seed germination and at least until the development of photosynthetic tissue (Bernard 1904; Leake 1994). Among these, life-long mycoheterotrophy has evolved independently most often in this family (Freudenstein & Barrett 2009), resulting in over 100 described species (Leake 1994). Determining the identities of orchid-associated mycorrhizal fungi has been difficult due to the paucity of characters in the asexual stage (referred to as anamorphs), and high rates of failure associated with inducing sexual stages (known as teleomorphs) in cultures (Taylor *et al.* 2002).

Autotrophic orchids generally associate with a wide range of Agaricomycete members of the form-genus *Rhizoctonia* (Rasmussen 1995; Currah 1997; Bidartondo *et al.* 2004). However, full MHPs associate with fungi having access to large and persistent carbon sources (Leake 2005), such as ectomycorrhizal fungi (Björkman 1960; Furman & Trappe 1971; Taylor & Bruns 1997; McKendrick *et al.* 2000; Selosse *et al.* 2002b; Gebauer & Meyer 2003; Bidartondo *et al.* 2004; Leake 2005; Girlanda *et al.* 2006), and saprotrophic fungi in the tropics (Hynson & Bruns 2010). Studies of mycorrhizal specificity, defined as the phylogenetic diversity of the fungi that form mycorrhizal associations with a particular plant taxon (Thompson 1994; Taylor *et al.* 2003), have revealed a pattern of extreme specificity among many MHPs associated with ectomycorrhizae, where individual species specialize on members of a single fungal genus or subgeneric clade (Taylor & Bruns 1997, 1999; McKendrick *et al.* 2002; Selosse *et al.* 2002b; Taylor *et al.* 2003; Barrett *et al.* 2010).

Investigations of mycorrhizal specificity in orchid MHPs have generally focused on either a single- or a few species with distant or uncertain phylogenetic relationships and have never included an entire and well-characterized genus. *Hexalectris*, the crested coral root orchids, represents an excellent opportunity to study the evolution of mycorrhizal associations in orchid MHPs because it is monophyletic (Sosa 2007) and composed of nine fully MHP species whose delimitations have been clarified (Kennedy & Watson 2010). This genus is distributed throughout most of the eastern and southern U.S., and south throughout mountainous regions of Mexico and northern Guatemala (Goldman 2005; Kennedy & Watson 2010). The monophyletic *H. spicata* complex is comprised of six species, members of which have relatively similar floral morphologies (Goldman *et al.* 2002; Kennedy & Watson 2010).

In the present study we identified the fungal root associates of specimens from all species of *Hexalectris* by sequencing the nuclear ribosomal internal transcribed spacer region (ITS) and conducting phylogenetic analyses with these data in the context of homologous sequences from a wide variety of closely related fungi. The mycorrhizal status of each fungus was inferred based on the nutritional needs of MHPs and current knowledge of their function in other plant associations. We then evaluated the breadth of mycorrhizal associations within *Hexalectris* as a whole, within the *H. spicata* species complex, and within each species.

## Materials and methods

### *Sampling*

Roots, the sites of mycorrhizal colonization (Taylor *et al.* 2003), were sampled from 134 adult individuals representing 42 populations and all nine species of *Hexalectris* (Table 1): *H. warnockii* Ames & Correll, *H. grandiflora* (A. Rich. & Galeotti) L. O. Williams, *H. brevicaulis* L. O. Williams, *H. revoluta* Correll, *H. parviflora* L. O. Williams, *H. arizonica* (S. Watson) A. H. Kennedy & L. E. Watson, *H. colemanii* (Catling) A. H. Kennedy & L. E. Watson, *H. nitida* L. O. Williams, and *H. spicata* (Walter) Barnhart. Unless limited by availability, molecular data were collected from two roots per individual. Sampling for some species was limited due to rarity, distribution, small population size and the ephemeral and cryptic nature of their inflorescence (the only above ground organ). Voucher specimens were deposited in the Willard Sherman Turrell Herbarium, Miami University (MU).

### *Molecular methods*

Roots were gently scrubbed with a soft brush in a dilute detergent solution, surface sterilized in a 20% bleach solution for 5 min, and triple rinsed with $ddH_20$ (Taylor & Bruns 1997; Shefferson *et al.* 2005). Total DNAs were extracted using QIAGEN's DNeasy Plant Mini Kit (QIAGEN, Gaithersburg, MD, USA) from non-necrotic root sections containing pelotons (i.e., mycorrhizal structures unique in orchids and consisting of hyphal coils that surround the plasmalemma of root cortical cells) immediately following sterilization or after storage at −80° C in the AP1 DNA extraction buffer provided in the QIAGEN DNeasy Plant Kit.

A representative group of DNA extracts from each species was selected for an initial survey of associated mycorrhizal lineages. This was accomplished by selectively amplifying the fungal nuclear ribosomal internal transcribed spacer region (ITS; ITS1, 5.8S, ITS2) with PCR using the fungal specific primer pair ITS1-F/ITS4 (White *et al.* 1990; Gardes & Bruns 1993). This group was also amplified with two additional pairs, ITS1-OF/ITS4-OF (Taylor & McCormick 2008) and

**Table 1** A county level list of the locations from where root samples were collected from each *Hexalectris* species. The number of populations and individuals sampled from each county is presented with corresponding population-level voucher specimen references. All vouchers are located at MU unless otherwise noted. Duplicates of Mexican collections were deposited at IBUG

| *Hexalectris* species | Collection location (country, state, county/parish/municipio) | Number of populations | Number of individuals | Voucher specimen |
|---|---|---|---|---|
| *arizonica* | USA, Arizona, Cochise | 3 | 8 | AHK *et al*. 347, AHK *et al*. 344, RAC 1136 |
| *arizonica* | USA, Arizona, Pima | 1 | 8 | AHK and FTF 341 |
| *arizonica* | USA, Arizona, Santa Cruz | 1 | 5 | AHK and FTF 343 |
| *arizonica* | USA, Texas, Brewster | 1 | 1 | AHK and AF 30 |
| *arizonica* | USA, Texas, Dallas | 2 | 8 | AHK 76, N A. |
| *brevicaulis* | Mexico, Jalisco, Tecolotlan | 1 | 2 | PCR *et al*. 4403 |
| *brevicaulis* | Mexico, Jalisco, San Cristobal de la Barranca | 1 | 1 | PCR *et al*. 4399 |
| *brevicaulis* | Mexico, Jalisco, Zapopan | 1 | 2 | PCR *et al*. 4400 |
| *brevicaulis* | Mexico, Jalisco, Ejutla | 1 | 2 | PCR *et al*. 4408 |
| *colemanii* | USA, Arizona, Pima | 2 | 9 | AHK *et al*. 65, AHK *et al*. 63 |
| *grandiflora* | USA, Texas, Dallas | 2 | 2 | MBM *s.n.* (BRIT), MBM *s.n.* (BRIT) |
| *grandiflora* | USA, Texas, Jeff Davis | 2 | 8 | AHK 22, AHK and JK 24 |
| *nitida* | USA, Texas, Brewster | 1 | 1 | AHK and AF 31 |
| *nitida* | USA, Texas, Dallas | 3 | 8 | AHK 78, AHK *et al*. 80, AHK *et al*. 83 |
| *parviflora* | Mexico, Zacatecas, Trinidad Garcia de la Cadena | 1 | 1 | PCR and AHK 4526 |
| *parviflora* | Mexico, Jalisco, Cuquio | 1 | 2 | PCR *et al*. 4533 |
| *revoluta* | USA, Texas, Brewster | 2 | 3 | AHK 260, AHK 263 |
| *spicata* | USA, Alabama, Sumter | 1 | 1 | AHK 67 |
| *spicata* | USA, Florida, Citrus | 1 | 6 | AHK 54 |
| *spicata* | USA, Florida, Hernando | 1 | 5 | AHK 58 |
| *spicata* | USA, Indiana, Harrison | 1 | 5 | AHK and KJK 83 |
| *spicata* | USA, Louisiana, Natchitoches | 1 | 1 | AHK 68 |
| *spicata* | USA, North Carolina, Alleghany | 1 | 12 | AHK 15 |
| *spicata* | USA, North Carolina, Hoke | 1 | 5 | AHK 460 |
| *spicata* | USA, Ohio, Adams | 1 | 7 | AHK 17 |
| *spicata* | USA, Oklahoma, Caddo | 1 | 5 | AHK and LKM 81 |
| *spicata* | USA, Texas, Brewster | 1 | 3 | AHK and AF 33 |
| *spicata* | USA, Texas, Dallas | 2 | 4 | AHK 207, NA |
| *spicata* | USA, Texas, Hayes | 1 | 2 | AHK *et al*. 86 |
| *warnockii* | USA, Texas, Brewster | 2 | 3 | AHK and AF 32, AHK 259 |
| *warnockii* | USA, Texas, Dallas | 1 | 4 | AHK 70 |
| Total | | 42 | 134 | |

A key to collector abbreviations is as follows: MBM, Marcy Brown-Marsden; PCR, Pablo Carrillo-Reyes; RAC, Ronald A. Coleman; FTF, Frank T. Farruggia; AF, Allison Freeman; JK, John Karges; AHK, A. H. Kennedy; KJK, K. J. Kennedy; LKM, Lawrence K. Magrath; N.A., a voucher collection was not made.

ITS1/ITS4-Tul (White *et al*. 1990; Taylor & McCormick 2008), because the ITS of *Tulasnella*, a major lineage of known orchid mycorrhizal forming fungi, has experienced accelerated rates of evolution resulting in ineffective amplification with ITS1-F for some of its core members (Suárez *et al*. 2006; Taylor & McCormick 2008). Results from this survey revealed that the ITS1-OF/ITS4-OF pair was effective at amplifying the ITS from the full range of basidiomycete fungi that were detected by all three pairs, therefore, PCR was conducted on the remaining DNA extracts with only ITS1-OF/ITS4-OF.

The PCRs were conducted with QIAGEN Standard PCR reagents in an MJ Research DNA Engine (Waltham, MA, USA) following the conditions of Gardes & Bruns (1993) with the following final concentrations per 25 μL: 200 μM of each dNTP, 1.5 mM MgCl2 and 2.5 units of QIAGEN *Taq* DNA Polymerase. When multiple and discrete bands were detected by gel electrophoresis, each was individually excised for sequencing. PCR products were purified with the Wizard SV Genomic DNA Purification System (Promega, Madison, WI, USA) and labelled with BigDye v3.1 (Applied Biosystems, Foster City, CA, USA). Sequencing was conducted on either an ABI 3130xl or a 3730 DNA Analyzer (Applied Biosystems) at the Miami University, Center for Bioinformatics and Functional Genomics. Electropherograms were assembled and edited in Sequencher 3.1 (Genecodes Inc., Ann Arbor, MI, USA). When multiple bands were present and too similar in size to excise individually, and

whenever electropherograms indicated the presence of a heterogeneous pool of PCR products, original PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Six to ten resulting colonies were selected from each plated reaction and re-sequenced directly. All sequences were submitted to NCBI's GenBank (http://www.ncbi.nlm.nih.gov), accessions HQ667792–HQ667931.

### *Fungal identifications*

A combination of BLAST searches and phylogenetic analyses were used to identify mycorrhizal fungi (Taylor & Bruns 1997; Taylor *et al.* 2003; McCormick *et al.* 2004; Shefferson *et al.* 2005, 2007; Girlanda *et al.* 2006; Suárez *et al.* 2006, 2008; Otero *et al.* 2007; Taylor & McCormick 2008; Barrett *et al.* 2010). Sequences that differed by less than 1% of their nucleotide positions and were generated from the same individual were represented only once in the final matrix (Suárez *et al.* 2006).

*Hexalectris*-associated fungal ITS sequences were highly divergent and not alignable as a single data matrix. Thus, they were grouped by similarity with those in GenBank, which were identified with Discontinuous MegaBLAST (Taylor *et al.* 2003; Shefferson *et al.* 2005; Taylor & McCormick 2008). This sorted most sequences to one of four agaricomycete families: Sebacinaceae, Ceratobasidiaceae, Russulaceae and Thelephoraceae. Sequences were initially considered members of a particular family when pairwise similarity values to several vouchered specimens from that family were greater than 85%. A selection of sequences from these searches was added to the appropriate family-level matrix along with additional sequences from previously published phylogenetic analyses, with vouchered teleomorphs preferentially selected when possible. Additional sequences were added to the Sebacinaceae matrix based on Taylor *et al.* (2003), Weiss *et al.* (2004), and Suárez *et al.* (2008), and from the UNITE fungal rDNA sequence database (Kõljalg *et al.* 2005; http://unite.ut.ee). Finally, sequences were also added to the Ceratobasidiaceae matrix based on Taylor & McCormick (2008); to the Russulaceae matrix based on Miller *et al.* (2001), Miller & Buyck (2002), and Larsson & Larsson (2003); and to the Thelephoraceae matrix based on Taylor & Bruns (1997).

Each family level matrix was aligned with MUSCLE v3.6 (Edgar 2004) and adjusted manually in SE-AL v2.0a11 (Rambaut 2002). The Russulaceae alignment was constructed by adding the selected GenBank sequences to the alignment of Miller & Buyck (2002). Errors in positional homology were reduced in each alignment using the G blocks web server (Castresana 2000) with 'less stringent' alignment settings (Taylor & McCormick 2008).

Phylogenetic analyses were conducted with MRBAYES v3.1.2 (Huelsenbeck & Ronquuist 2001; Ronquist & Huelsenbeck 2003). Outgroups for the Sebacinaceae, Ceratobasidiaceae, Russulaceae and Theleporaceae were based on previous studies and included *Sebacina vermifera* (Weiss *et al.* 2004), *Botryobasidium subcoronatum* (Moncalvo *et al.* 2006), *Gloeocystidiellum aculeatum* (Miller *et al.* 2001; Miller & Buyck 2002; Larsson & Larsson 2003), and *Sarcodon imbricatus* (Taylor & Bruns 1997), respectively. The model of DNA sequence evolution was determined for each matrix using the Akaike Information Criterion (AIC; Posada & Buckley 2004) in MRMODELTEST v2.2 (Nylander 2004). The 'temperature' parameter was set to 0.05 for the analyses of the Sebacinaceae, Russulaceae, and Thelephoraceae matrices due to inefficient Metropolis coupling during initial analyses. All other parameter values were left at default. The posterior probability (pp) distribution of trees was estimated with two independent Markov Chain Monte Carlo (MCMC) simulations, sampling trees every 100 generations. Each analysis was terminated after convergence, which was considered once the average standard deviation of split frequencies was <0.01 (Pelser *et al.* 2007). Stationarity was estimated by plotting the likelihood scores of all sampled trees and locating the stable likelihood plateau. All trees prior to this plateau were discarded as the 'burnin', while the remaining was used to build 50% majority rule consensus trees.

Relative mycorrhizal specificity and phylogenetic breadth of association within each agaricomycete family was estimated for each *Hexalectris* species. Relative mycorrhizal specificity was estimated by calculating the average uncorrected genetic distance in PAUP* version 4b10 (Swofford 2002) for all pairwise comparisons of ITS sequences identified to the same fungal family and isolated from a single *Hexalectris* species (i.e., $p_i$, Nei & Tajima 1981; Taylor *et al.* 2004; Shefferson *et al.* 2007). Relative breadth was estimated using maximum genetic distance between any two ITS sequences for the same fungal family and isolated from a single *Hexalectris* species. Pairwise genetic distances were calculated based on the final alignments produced after the removal of unalignable sites by Gblocks.

## Results

### *Similarity searches*

Results from MegaBLAST searches indicated that 98% of sampled plants contained root-associated ITS sequences which were highly similar to those from four different agaricomycete families: Sebacinaceae, Ceratobasidiaceae, Russulaceae and Thelephoraceae (Table 2). Eighty one percentage of all surveyed *Hexalectris*

**Table 2** A list of fungal taxa that were identified within the roots of each *Hexalectris* species based on MegaBLAST searches of GenBank. The final row indicates the number of individual plants that were sampled for each *Hexalectris* species. Each column indicates the number of individual plants of each species that associated with a fungus from a particular taxon. Numbers in parentheses represent the number of individual plants that were associated with fungi from multiple taxa. The final column represents the total number of plants, regardless of species, that were in association with a particular fungal taxon. Notice that the sum of several columns exceeds the total number of sampled plants. This is because some plants are associated with fungi from more than one taxon. Multiple sequences from an individual plant were considered identical when they differed by less than 1% (Suárez *et al*. 2006)

| | | | | *H. spicata* species complex | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fungal group | *warnockii* | *grandiflora* | *brevicaulis* | *parviflora* | *revoluta* | *colemanii* | *arizonica* | *nitida* | *spicata* | Total |
| Sebacinaceae | — | 2 (1) | 2 (0) | 3 (1) | 3 (1) | 9 (0) | 28 (5) | 8 (0) | 53 (6) | 108 |
| Ceratobasidiaceae | — | 5 (3) | 1 (1) | 1 (1) | — | — | — | — | 3 (1) | 10 |
| Russulaceae | — | 5 (5) | 5 (1) | — | — | — | — | — | — | 10 |
| Thelephoraceae | 7 (0) | — | — | — | — | — | 3 (3) | — | — | 10 |
| Cortinariaceae | — | — | — | — | — | — | — | — | 1 (1) | 1 |
| Ascomycota | — | 2 (1) | — | — | 1 (1) | — | 3 (3) | 1 (1) | 4 (3) | 11 |
| Total plants sampled | 7 | 10 | 7 | 3 | 3 | 9 | 30 | 9 | 56 | |

individuals, and eight of nine species, were associated with members of Sebacinaceae. These associations were especially common within the *H. spicata* species complex, where 94% of individuals were associated with a sebacinaceous fungus. We identified members of the Sebacinaceae as the sole basidiomycete associates of *H. nitida*, *H. colemanii*, and *H. revoluta*. *Hexalectris parviflora* and *H. spicata* also rarely associated with members of the Ceratobasidiaceae, and a single individual of *H. spicata* from Adams County, Ohio associated with a member of Cortinariaceae. Also, *H. arizonica* occasionally associated with members of Thelephoraceae.

Sebacinaceous associates were not as frequently identified in the remaining *Hexalectris* species. In *H. grandiflora*, they were identified in 20% of individuals surveyed, while Ceratobasidiaceae and Russulaceae were more common and were identified in 50% of individuals sampled. However, ceratobasidiaceous fungi were generally only detected by cloning, were rare among clones, and commonly co-occurred with russulaceous fungi. Similarly, 29% of *H. brevicaulis* individuals was associated with Sebacinaceae and 71% was associated with members of Russulaceae. A ceratobasidiaceous fungus was identified in one individual (14%) and was detected by cloning and co-occurred with a russulaceous fungus. All *H. warnockii* individuals associated strictly with members of the Thelephoraceae.

Some ascomycete fungi were also detected within the roots of some species (Table S1, Supporting information). We suggest that these ascomycetous fungi are not mycorrhizal with *Hexalectris* species, but instead are pathogenic or necrotrophic on *Hexalectris* roots or their mycorrhizal fungi because all are likely to be members of groups which are not known to form mycorrhizae, but are known to contain plant and fungal pathogens. These fungi occurred in <9% of individuals, and when they did occur, were often detected only by cloning, were rare among clones, and co-occurred with a known orchid mycorrhiza.

### *Phylogenetic analyses*

The Sebacinaceae matrix contained 149 taxa and 536 nucleotide positions after the removal of 345 nucleotide positions (39%) by Gblocks (Castresana 2000). The GTR+I+G model of sequence evolution was selected. The resulting phylogeny (Fig. 1) reveals that all sebacinaceous fungi associated with *Hexalectris* are members of only one of two major clades within Sebacinaceae subgroup A (Weiss *et al*. 2004). This 'HSF clade' (99% pp) is a large polytomy composed of several subclades (HSF-a–HSF-h). Remarkable fidelity was detected among *Hexalectris* species to HSF subclades. For example, three out of eight species (*H. grandiflora*, *H. brevicaulis*, *H. revoluta*) were restricted to associations with a single HSF subclade. *Hexalectris nitida* and *H. arizonica* were each also restricted to a single HSF subclade, with a single exception for *H. nitida* and two exceptions for *H. arizonica*. The fungal ITS sequences from *H. spicata* are represented in all subclades throughout the HSF polytomy, two of which, HSF-d and HSF-e, contain only *H. spicata*-associated fungi. The breadth of associations for each species was indicated by the average and maximum genetic distances for pairwise ITS sequence comparisons (Table 3).

The Ceratobasidiaceae matrix contained 58 taxa and 573 nucleotide positions after the removal of 234 (27%) by Gblocks. The SYM+I+G model of sequence evolution was selected. All fungi associated with *Hexalectris* with one exception were members of a single narrow clade (HCF-a; Fig. S1, Supporting information). The average

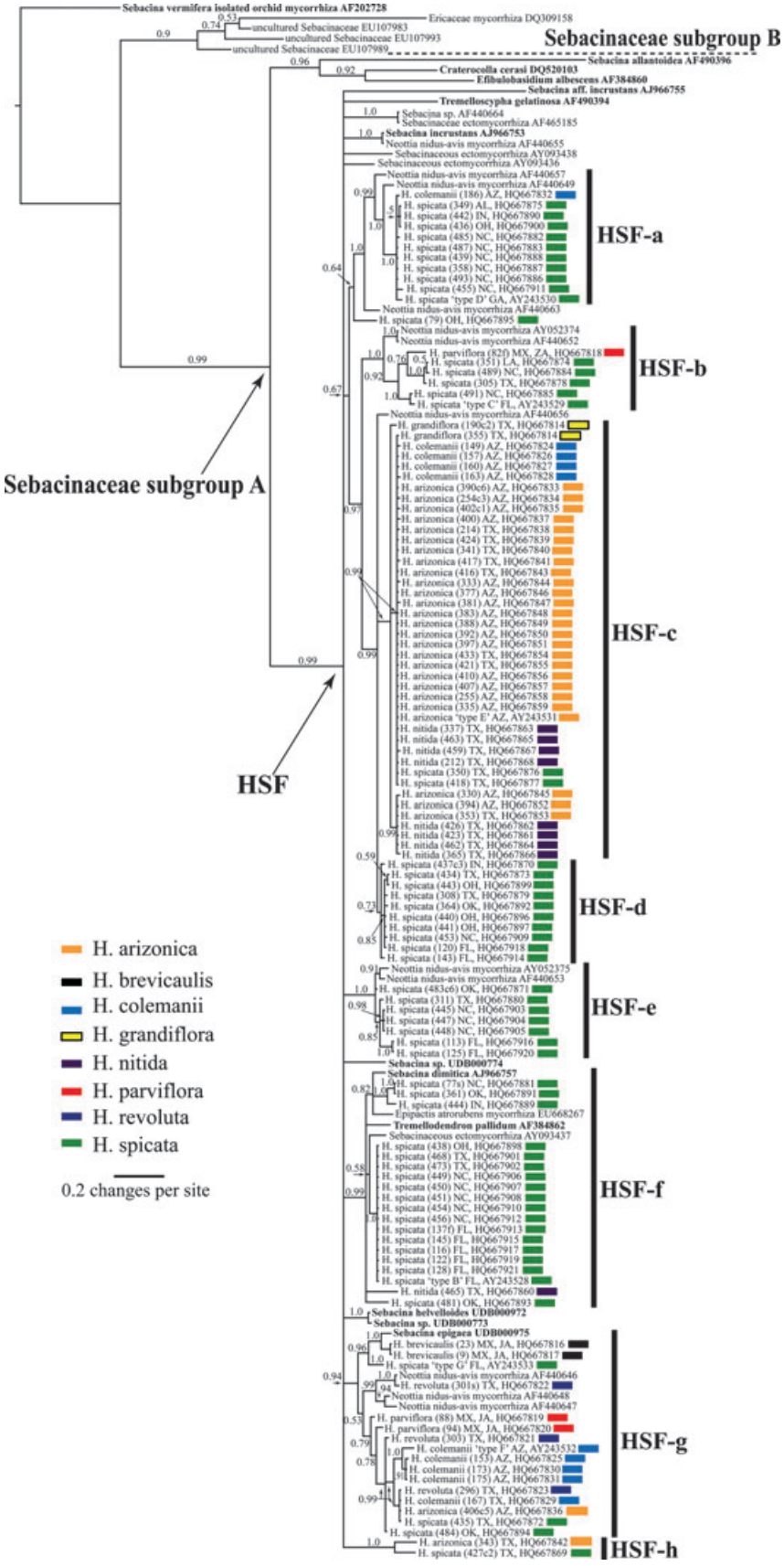



**Fig. 1** A 50% majority rule Bayesian Inference internal transcribed spacer regions phylogeny of Sebacinaceae revealing that the HSF clade within subgroup A contains all the primary fungal associates of the *H. spicata* complex and occasional associates of *H. grandiflora* and *H. brevicaulis*. Values on branches represent posterior probabilities. Accessions in bold represent vouchered herbarium specimens. Taxon labels of *Hexalectris*-associated fungi each include a US or Mexican (MX) state abbreviation indicating collection location. All taxon labels include GenBank or UNITE ('UDB') accession numbers.

**Table 3** Report of $p_i$ within the Sebacinaceae, and the maximum genetic distance among all pairwise comparisons within each species as an estimate of the relative breadth of mycorrhizal associations within this family

| *Hexalectris* species | Average genetic distance ($p_i$) | Maximum genetic distance |
|---|---|---|
| *brevicaulis* | 0.00000 | 0.00000 |
| *grandiflora* | 0.00846 | 0.00846 |
| *arizonica* | 0.01174 | 0.07541 |
| *nitida* | 0.02146 | 0.07784 |
| *revoluta* | 0.03232 | 0.05610 |
| *parviflora* | 0.04043 | 0.05651 |
| *colemanii* | 0.04091 | 0.06586 |
| *spicata* | 0.04746 | 0.09227 |

and maximum genetic distances of pairwise comparisons of ITS sequences for *H. spicata* (0.00053 and 0.00159) and *H. grandiflora* (0.02184 and 0.05696) were low because the ITS sequences of HCF-a were nearly identical.

The Russulaceae matrix contained 97 taxa and 558 nucleotide positions after the removal of 325 base pairs (37%) by Gblocks. The GTR+I+G model of sequence evolution was selected. The average and maximum genetic distances of pairwise comparisons of ITS sequences for *H. brevicaulis* and *H. grandiflora* were 0.10317 and 0.14425; and 0.09211 and 0.11626, respectively. The topology of the resulting tree shows that the russulaceous fungi associated with *H. grandiflora* and *H. brevicaulis* span much of the known species diversity of *Russula* (Fig. 2).

The Thelephoraceae matrix contained 117 taxa and 530 nucleotide positions after the removal of 286 positions (35%) by G blocks. The SYM+I+G model of sequence evolution was selected. The average and maximum genetic distances of pairwise comparisons of ITS sequences for *H. arizonica* and *H. warnockii* were 0.04044 and 0.05979 and 0.04701 and 0.06800, respectively. The resulting 50% majority rule tree reveals that, although all *H. warnockii* and associates are restricted to a single clade (HTF; Fig. 3) which contains most of the sampled ITS sequences, they are members of several HTF subclades. The occasional associates of this family and *H. arizonica* were also all members of HSF and are similarly distributed throughout this clade.

## Discussion

### *Function and identity of fungal root associates in* Hexalectris

A persistent problem concerning studies of mycorrhizal specificity is whether a fungus that is detected in the mycorrhizal tissue is functioning as a mycorrhiza. This is problematic even for tissues that are heavily colonized by pelotons because other non-mycorrhizal fungi may be present (Taylor *et al.* 2003; Shefferson *et al.* 2005). Although the function of each fungus identified within *Hexalectris* mycorrhizal tissue was not demonstrated directly, we assume mycorrhizal function for the detected agaricomycete fungi based on previous empirical evidence and our phylogenetic results. It has been hypothesized that MHPs form mycorrhizae strictly with fungi that have access to a large and persistent carbon source, such as ectomycorrhizae (Taylor & Bruns 1997; Taylor *et al.* 2002; Leake 2005). Among the root-associated fungi identified in the present study, it is only the ectomycorrhizal agaricomycete fungi that are likely to have access to such a carbon supply. Our results suggest that *Hexalectris* agaricomycete associates are ectomycorrhizal because each is intermixed in subclades with known ectomycorrhizal fungi that have been previously reported as mycorrhizae of other orchid species (Taylor & Bruns 1997, 1999; McKendrick *et al.* 2002; Selosse *et al.* 2002a,b; Taylor *et al.* 2003, 2004; Bidartondo & Read 2008; Suárez *et al.* 2008; Barrett *et al.* 2010). In fact, several studies have directly demonstrated that such ectomycorrhizal fungi syphon photosynthetically derived carbon to mycoheterotrophic orchids (Björkman 1960; Furman & Trappe 1971; Taylor & Bruns 1997; McKendrick *et al.* 2000; Selosse *et al.* 2002b; Gebauer & Meyer 2003; Trudell *et al.* 2003; Bidartondo *et al.* 2004; Girlanda *et al.* 2006; Zimmer *et al.* 2008; Roy *et al.* 2009).

Previous identifications of root associated fungi in *H. spicata*, *H. arizonica*, and *H. colemanii* suggested that the primary mycorrhizal symbionts of *Hexalectris* are sebacinaceous fungi (Taylor *et al.* 2003). Our increased sampling revealed that sebacinaceous fungi are indeed dominant, especially within the *H. spicata* complex, but that *H. brevicaulis* and *H. grnadiflora* display mixed associations with Russulaceae and Sebacinaceae, and *H. warnockii* associates strictly with Thelephoraceae.

Sebacinaceae is comprised of two major and well-supported ectomycorrhizal clades that are divergent in terms of their mycorrhizal associations (Sellose *et al.* 2007; Selosse *et al.* 2009). Members of clade B form endomycorrhizae in several epiphytic and terrestrial autotrophic orchids (Bidartondo & Read 2008; Suárez *et al.* 2008), several Ericaceae (Sellose *et al.* 2007), and are endosymbionts of some liverworts (Kottke *et al.* 2003). However, members of clade A are only known to form ectomycorrhizae and endomycorrhizae with orchid MHPs (Selosse *et al.* 2002a,b; Selosse *et al.* 2009).

Sebacinaceous fungi were only occasionally identified as associates of *H. brevicaulis* and *H. grandiflora* (Table 2). However, members of the Russulaceae, like

the Thelephoraceae, are comprised of some of the most abundant ectomycorrhizal fungi on Earth (Horton & Bruns 2001) and were the dominant associates of *H. brevicaulis*. Although no single dominant fungal family was identified in *H. grandiflora*, sebacinaceous and russulaceous fungi were identified from five of seven individuals, showing that members of the Russulaceae, and to a lesser extent, members of Sebacinaceae, are the fungal conduits of photosynthetic carbon to these species.

Members of Ceratobasidiaceae were also identified from *H. grandiflora* and *H. brevicaulis*, and were occasionally associated with *H. parviflora* and *H. spicata* (Table 2). Ceratobasidiaceous fungi are commonly isolated from orchid mycorrhizal tissues (Taylor *et al.* 2002) and have been demonstrated as mycorrhizal (Cameron *et al.* 2006; Paduano *et al.* 2010). However, many ceratobasidiaceous fungi also function ecologically as saprotrophs and plant parasites (Roberts 1999). Taylor *et al.* (2003) identified *Thanatephorus ochraceus* from the roots of several *H. spicata* individuals from Florida, the same ceratobasidiaceous fungus that was identified in several *Hexalectris* species in the present study (Fig. S1, Supporting information). They considered the mycorrhizal status of this fungus as unlikely because, although the hyphae of these fungi formed partially coiled pelotons in roots, they also formed uncoiled hyphae in nonmycorrhizal rhizome tissue, suggesting a potentially pathogenic interaction. In the present study, as in Taylor *et al.* (2003), *T. ochraceus* occurred sporadically throughout our sampling and at a low abundance suggesting that this fungus is at least unlikely to be a primary or common symbiont of any *Hexalectris* species.

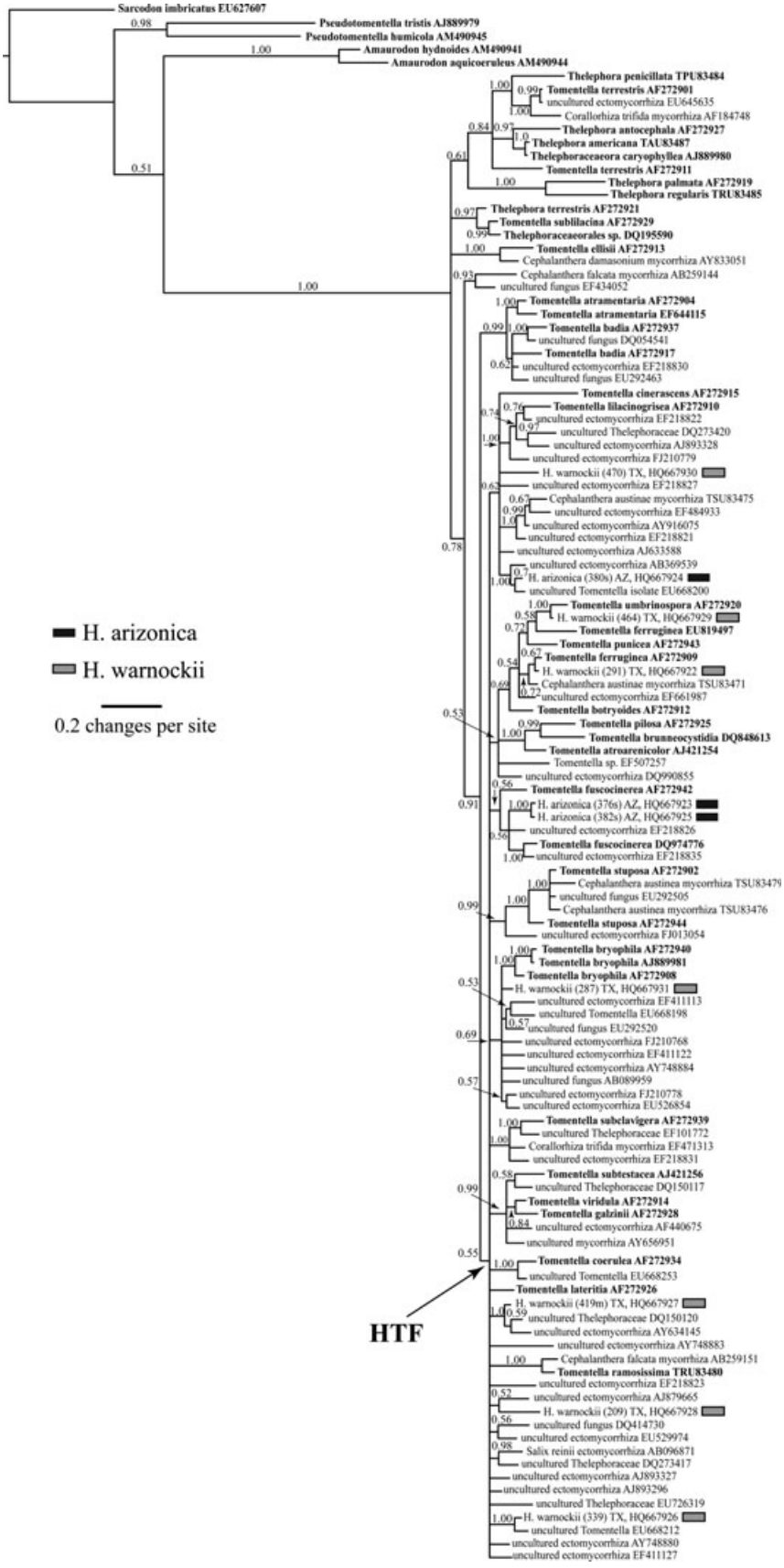
### *Mycorrhizal specificity in* Hexalectris *and its species*

Remarkable levels of mycorrhizal specificity have been detected in MHPs from a broad range of plants including lycopod gametophytes (Winther & Friedman 2007), an epiparasitic liverwort (Bidartondo *et al.* 2003), nearly all species of the ericaceous Monotropoideae (Bidartondo & Bruns 2002), Gentianaceae (Bidartondo *et al.* 2002), Burmaniaceae (Merckx & Bidartondo 2008), and the many orchids discussed herein. It is therefore not surprising that high levels of mycorrhizal specificity were identified in *Hexalectris* species. Nevertheless, the levels of mycorrhizal specificity detected in *Hexalectris* are striking considering that it is common for a typical autotrophic ectomycorrhizal plant to associate with dozens of phylogenetically distant fungal species at any one time, and with thousands more across a plant species' entire geographic distribution (Bruns *et al.* 2002).

Extreme specificity for sebacinaceous fungi in the *H. spicata* species complex is evidenced by the phylogenetic placement of nearly all of their mycorrhizal fungi in the HSF clade of Sebacinaceae subgroup A (Fig. 1). Some exceptions to this fidelity were detected in a few *Hexalectris* species of this complex. In these cases, one or a few individuals of each species were associated with fungi from other agaricomycete fungal families (Table 2; Fig. 1). Exceptional associations like these are a common feature of orchids that otherwise display high levels of specificity towards a narrow clade of mycorrhizal partners (Shefferson *et al.* 2005).

Although many of the interior nodes in the HSF clade are unresolved, several nodes near the tips are sufficiently resolved to reveal that fungal associates of each species of the *H. spicata* complex are restricted to one or more subclades (Fig. 1). Striking examples of specificity include *H. arizonica*, *H. nitida*, and *H. revoluta*, whose fungal ITS sequences have the lowest $p_i$ values among members of the complex (Table 3). These low values reflect the fact that these species associate strictly with a single HSF subclade. The only exceptions to this are a single fungal ITS sequence from *H. nitida* nested within the HSF-f clade, and a single *H. arizonica* associated fungal ITS sequence nested within each of clades HSF-g and -h. It is fascinating that *H. nitida* and *H. arizonica* have the highest fidelity towards their mycorrhizal partners because most members of these species are cleistogamous and have experienced several, apparently convergent, morphological adaptations that accommodate self-pollination (Kennedy & Watson 2010). We hypothesize that self-pollination increases the likelihood that a highly successful mycorrhizal partnership will be formed by future generations due to an accompanying loss of heterozygosity and fixation of alleles (Hamrick & Godt 1989), which results in an inherited predisposition for successful mycorrhizal associations within a species. Evidence that targeting of particular fungal clades by MHPs may be a heritable characteristic has been demonstrated by several studies (Taylor & Bruns 1999; Taylor *et al.* 2004). For example, some monotropoid species (Ericaceae) were shown to be more likely to live to reproductive age when they were mycorrhizal with the same *Russula* species as their maternal parent (Bidartondo & Bruns 2005). In the pres-

**Fig. 2** A 50% majority rule Bayesian Inference internal transcribed spacer phylogeny revealing that the russulaceaous fungi associated with *H. grandiflora* and *H. brevicaulis* are widely distributed throughout this family. Values on branches represent posterior probabilities. Accessions in bold represent vouchered herbarium specimens. Taxon labels of *Hexalectris*-associated fungi each include a US or Mexican (MX) state abbreviation indicating collection location. All taxon labels include GenBank accession numbers.



ent study, intermixed individuals of *H. warnockii*, *H. grandiflora*, *H. spicata*, *H. nitida*, and *H. arizonica* were sampled from a single site in Dallas County, TX, and in each case their fungal affinities were retained.

It may be hypothesized that a species with a relatively large geographic distribution has a commensurate breadth of associations because of its access to a presumably greater number of fungal taxa throughout its range. However, this is not supported by our data and has been rejected in *Cypripedium* (Shefferson *et al*. 2007). For example, although *H. spicata* associates with each HSF subclade, has the broadest host sebacinaceous specificity, and the largest geographic distribution in the *H. spicata* complex, its North Carolina fungal associates alone span nearly the entire breadth of associations identified from throughout its entire distribution. Also, in *H. colemanii*, two populations growing in similar habitats only a few dozen kilometres apart associated with members of the widely distant subclades HSF-a, -c, and -g, members of which were identified from other *Hexalectris* species from western Mexico to the eastern United States. It is therefore reasonable to infer wide distributions for many of the sebacinaceous fungi that host *Hexalectris* species, and geographic distributions for *Hexalectris* species that are not solely restricted by those of ectomycorrhizal fungi.

*Hexalectris grandiflora* and *H. brevicaulis* displayed similar levels of fidelity to HSF clades as did several species of the *H. spicata* complex. However, these species' associations with Russulaceae were comparatively broad (Fig. 2). The identification of fungal mycorrhizae from two distantly related families co-occurring within a single individual or species was also reported by Suárez *et al.* (2008) who identified co-occurring Sebacinaceae and Tulasnellaceae in *Stelis* and *Pleurothallis*. The pattern of specificity identified within *H. warnockii* was unique among *Hexalectris* species in that its members associated strictly with the Thelephoraceae and with a broad range of fungi within this family.

## Conclusion

Our results in context with the phylogeny of *Hexalectris* (Kennedy & Watson 2010) suggest that associations in this genus are rapidly evolving heritable characteristics. For example, the *H. spicata* species complex appears to be recent and rapidly diverging yet each of its species associates with a unique group of closely related Sebacinaceae subgroup A clades. Also, this context reveals a specific pattern of phylogenetically large host shifts that have occurred in the evolutionary history of this genus. For instance, the four major lineages of *Hexalectris* (*H. warnockii*, *H. brevicaulis*, *H. grandiflora*, and the *H. spicata* species complex) associated primarily with members of different ectomycorrhizal fungal families. Lastly, patterns of associations among these lineages help provide insight into a potential mechanism for the evolution of specificity. For example, the shift from mixed associations with Russulaceae and Sebacinaceae subgroup A in *H. grandiflora* to fixation of associations with Sebacinaceae subgroup A in the *H. spicata* species complex suggests that host shifts may be facilitated through an intermediate taxon having mixed associations.

Finally, an understanding of phylogenetic species delimitations in *Hexalectris* enabled a leap in our understanding of mycorrhizal specificity in *Hexalectris*, particularly within the *H. spicata* species complex. For example, Kennedy & Watson (2010) revealed two additional species within this complex, *H. arizonica* and *H. colemanii,* which were previously grouped with *H. spicata* and *H. revoluta*, respectively. Taylor *et al.* (2003) reported divergence in mycorrhizal associations between *H. spicata* var. *spicata* and var. *arizonica*. However, phylogenic studies revealed two distinct lineages within *H. spicata*, each of which was given species status (*H. spicata s.s.* and *H. arizonica*) with circumscriptions that only roughly correspond to those of *H. spicata* var. *spicata* and var. *arizonica*. Knowledge of these discrete lineages enabled our finding that *H. spicata* s.s. and *H. arizonica* have very different breadths of mycorrhizal associations and that although each targets a different set of HSF clades, some are targeted by both species. Similarly, differences in the level of specificity and targeted clades were identified between the phylogenetic species *H. revoluta* and *H. colemanii*. These patterns of mycorrhizal associations support the conclusion that *H. revoluta* and *H. arizonica* be recognized at species rank and demonstrate the importance of working with well-delimited phylogenetic units when studying mycorrhizal specificity.

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**Fig. 3** A 50% majority rule Bayesian Inference internal transcribed spacer phylogeny revealing that all fungal associates of *H. warnockii* are members of Thelephoraceae and widely distributed throughout much of its diversity. Values on branches represent posterior probabilities. Accessions in bold represent vouchered herbarium specimens. Taxon labels of *Hexalectris*-associated fungi each include a US or Mexican (MX) state abbreviation indicating collection location. All taxon labels include GenBank accession numbers.

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## References

Barrett CF, Freudenstein JV (2008) Molecular evolution of *rbc*L in the mycoheterotrophic coralroot orchids (*Corallorhiza* Gagnebin, Orchidaceae). *Molecular Phylogenetics and Evolution*, **47**, 665–679.

Barrett CF, Freudenstein JV, Taylor DL, Kõljalg U (2010) Rangewide analysis of fungal associations in the fully mycoheterotrophic *Corallorhiza striata* complex (Orchidaceae) reveals extreme specificity on ectomycorrhizal *Tomentella* (Thelephoraceae) across North America. *American Journal of Botany*, **97**, 628–643.

Bernard N (1904) Le champignon endophyte des orchidées. *Comptes Rendus de l' Academie des Sciences*, **138**, 828–830.

Bidartondo MI, Bruns TD (2002) Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups. *Molecular Ecology*, **11**, 557–569.

Bidartondo MI, Bruns TD (2005) On the origins of extreme mycorrhizal specificity in the Monotropoideae (Ericaceae): performance trade-offs during seed germination and seedling development. *Molecular Ecology*, **14**, 1549–1560.

Bidartondo MI, Read DJ (2008) Fungal specificity bottlenecks during orchid germination and development. *Molecular Ecology*, **17**, 3707–3716.

Bidartondo MI, Redecker D, Hijri I *et al.* (2002) Epiparasitic plants specialized on arbuscular mycorrhizal fungi. *Nature*, **419**, 389–392.

Bidartondo MI, Bruns TD, Weiβ M *et al.* (2003) Specialized cheating of the ectomycorrhizal symbiosis by an epiparasitic liverwort. *Proceedings of the Royal Society of London Series B- Biological Sciences*, **270**, 835–842.

Bidartondo MI, Burghardt B, Gebauer G *et al.* (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **271**, 1799–1806.

Björkman E (1960) *Moiwtropa hvpopitys* L.-an epiparasite on tree roots. *Physiologia Plantarum*, **13**, 308–327.

Bruns TD, Bidartondo MI, Taylor DL (2002) Host specificity in ectomycorrhizal communities: what do the exceptions tell us? *Integrative and Comparative Biology*, **42**, 352–359.

Cameron K, Molina M (2006) Photosystem II gene sequences of *psb*B and *psb*C clarify the phylogenetic position of *Vanilla* (Vanilloideae, Orchidaceae). *Cladistics*, **22**, 239–248.

Cameron DD, Leake JR, Read DJ (2006) Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. *New Phytologist*, **171**, 405–416.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*, **17**, 540–552.

Currah RS (1997) Fungi from orchid mycorrhizas. In: *Orchidbiology: Reviews and Perspectives, VII* (eds Arditti J, Pridgeon AM), pp. 117–153. Kluwer, Boston, MA.

Dressler R (1981) *The Orchids: Natural History and Classification*. Harvard University Press, Cambridge.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, **32**, 1792–1797.

Freudenstein JV, Barrett CF (2009) Mycoheterotrophy and diversity in Orchidaceae with a focus on *Corallorhiza*. Proceedings of the Fourth International Conference on the Biology of the Monocotyledons. University of Copenhagen, Copenhagen.

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

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.

Gebauer G, Meyer M (2003) $^{15}N$ and $^{13}C$ natural abundance of autotrophic and mycoheterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist*, **160**, 209–223.

Girlanda M, Selosse MA, Cafasso D *et al.* (2006) Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. *Molecular Ecology*, **15**, 491–504.

Goldman DH (2005) *Hexalectris*. In: *Genera orchidacearum* (eds Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN), Vol. 4, pp. 170–173. Oxford University Press, Oxford.

Goldman DH, Coleman RA, Magrath LK, Catling PM (2002) *Hexalectris*. In: *The Flora of North America* (eds Flora of North America Editorial Committee), Vol. 26, pp. 603–607. Oxford University Press, Oxford.

Hamrick JL, Godt MJ (1989) Allozyme diversity in plant species. In: *Plant Population Genetics, Breeding, and Genetic Resources* (eds Brown MD, Clegg MT, Kahler AL, Weir BS). pp. 43–63, Sinauer Associates Inc, Sunderland, MA.

Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology*, **10**, 1855–1871.

Huelsenbeck JP, Ronquuist F (2001) MrBayes: Bayesian inference of phylogeny. *Bioinformatics*, **17**, 754–755.

Hynson NA, Bruns TD (2010) Fungal hosts for mycoheterotrophic plants: a nonexclusive, but highly selective club. *New Phytologist*, **185**, 598–601.

Kennedy AH, Watson LE (2010) Species delimitations and phylogenetic relationships within the fully myco-heterotrophic *Hexalectris* (Orchidaceae). *Systematic Botany*, **35**, 64–76.

Kõljalg U, Larsson K, Abarenkov K *et al.* (2005) UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. *New Phytologist*, **166**, 1063–1068.

Kottke I, Beiter A, Weiβ M *et al.* (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while *Aneura pinguis* (Metzgeriales) is associated with a *Tulasnella* species. *Mycological Research*, **107**, 957–968.

Larsson E, Larsson KH (2003) Phylogenetic relationships of russuloid basdiomycetes with emphasis on aphyllophoralean taxa. *Mycologia*, **95**, 1037–1065.

Leake JR (1994) Tansley Review No. 69. The biology of mycoheterotrophic ('Saprophytic') plants. *New Phytologist*, **127**, 171–216.

Leake JR (2005) Plants parasitic on fungi: unearthing the fungi in myco-heterotrophs and debunking the 'saprophytic' plant myth. *Mycologist*, **19**, 113–122.

McCormick MK, Whigham DF, O'Neill J (2004) Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist*, **163**, 425–438.

McKendrick SL, Leake JR, Taylor DL, Read DJ (2000) Symbiotic germination and development of mycoheterotrophic plants in nature: ontogeny of *Corallorhizatrifida* and characterization of its mycorrhizal fungi. *New Phytologist*, **145**, 523–537.

McKendrick SL, Leake JR, Taylor DL, Read DJ (2002) Symbiotic germination and development of the mycoheterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. *New Phytologist*, **154**, 233–247.

Merckx V, Bidartondo MI (2008) Breakdown and delayed cospeciation in the arbuscular mycorrhizal mutualism. *Proceedings of the Royal Society of London Series B- Biological Sciences*, **275**, 1029–1035.

Miller SL, Buyck B (2002) Molecular phylogeny of the genus *Russula* in Europe with a comparison of modern infrageneric classifications. *Mycological Research*, **106**, 259–276.

Miller SL, McClean TM, Walker JF, Buyck B (2001) A molecular phylogeny of the Russulales including agaricoid, gasteroid and pleurotoid taxa. *Mycologia*, **93**, 344–354.

Moncalvo JM, Nilsson RH, Koster B *et al.* (2006) The cantharelloid clade: dealing with incongruent gene trees and phylogenetic reconstruction methods. *Mycologia*, **98**, 937–948.

Nei M, Tajima F (1981) DNA polymorphisms detectable by restriction endonucleases. *Genetics*, **97**, 145–163.

Nylander JAA (2004) *MrModeltest Version 2.2*. Program distributed by the author, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

Otero JT, Flanagan NS, Herre EA *et al.* (2007) Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides* (Orchidaceae). *American Journal of Botany*, **94**, 1944–1950.

Paduano C, Rodda M, Ercole E *et al.* (2010) Pectin localization in the Mediterranean orchid *Limodorum abortivum* reveals modulation of the plant interface in response to different mycorrhizal fungi. *Mycorrhiza*, DOI: 10.1007/s00572-010-0315-5.

Pelser PB, Nordenstam B, Kadereit JW, Watson LE (2007) An ITS phylogeny of tribe Senecioneae (Asteraceae) and a new delimitation of *Senecio* L. *Taxon*, **56**, 1077–1104.

Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of akaike information criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology*, **53**, 793–808.

Rambaut A (2002) *Se-Al Sequence Alignment Editor*, Version 2.0a11. http://tree.bio.ed.ac.uk/software/seal/ (Accessed February 2010).

Rasmussen HN (1995) *Terrestrialorchids: From Seed to Mycotrophic Plant*. Cambridge University Press, Cambridge.

Roberts P (1999) *Rhizoctonia-Forming Fungi. A Taxonomic Guide*. The Herbarium, Royal Botanic Gardens, Kew, London.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Roy M, Whatthana S, Richard F *et al.* (2009) Mycoheterotrophic orchids from Thailand tropical dipterocarpacean forests associate with a broad diversity of ectomycorrhizal fungi. *BMC Biology*, **7**, 51.

Sachs JL, Simms EL (2006) Pathways to mutualism breakdown. *Trends in Ecology and Evolution*, **21**, 585–592.

Sellose MA, Setaro S, Glatard F *et al.* (2007) Sebacinales are common mycorrhizal associates of Ericaceae. *New Phytologist*, **174**, 864–878.

Selosse MA, Bauer R, Moyersoen B (2002a) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. *New Phytologist*, **155**, 183–195.

Selosse MA, Weiβ M, Jany JL, Tillier A (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.

Selosse MA, Dubois M-P, Alvarez N (2009) Do Sebacinales commonly associate with plant roots as endophytes? *Mycological Research*, **113**, 1062–1069.

Shefferson RP, Weiβ M, Kull T, Taylor DL (2005) High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Molecular Ecology*, **14**, 613–626.

Shefferson RP, Taylor DL, Weiβ M *et al.* (2007) The evolutionary history of mycorrhizal specificity among lady's slipper orchids. *Evolution*, **61**, 1380–1390.

Smith SE, Read DJ (2008) *Mycorrhizal Symbiosis*, 3rd edn. Academic Press, San Diego, CA.

Sosa V (2007) A molecular and morphological phylogenetic study of subtribe Bletiinae (Epidendreae, Orchidaceae). *Systematic Botany*, **32**, 34–42.

Suárez JP, Weiβ M, Abele A *et al.* (2006) Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. *Mycological Research*, **110**, 1257–1270.

Suárez JP, Weiβ M, Abele A *et al.* (2008) Members of the Sebacinales subgroup B form mycorrhizae with epiphytic orchids in a neotropical mountain rain forest. *Mycological Progress*, **7**, 75–85.

Swofford DL (2002) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4. Sinauer Associates, Sunderland, MA.

Taylor DL, Bruns TD (1997) Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 4510–4515.

Taylor DL, Bruns TD (1999) Population, habitat and genetic correlates of mycorrhizal specialization in the 'cheating'

orchids *Corallorhiza maculata* and *C.mertensiana*. *Molecular Ecology*, **8**, 1719–1732.

Taylor DL, McCormick MK (2008) Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. *New Phytologist*, **177**, 1020–1033.

Taylor DL, Bruns TD, Leake JR, Read DJ (2002) Mycorrhizal specificity and function in mycoheterotrophic plants. In: *The Ecology of Mycorrhizas* (eds van der Heijden MGA, Sanders RI), pp. 375–414. Springer-Verlag, Berlin.

Taylor DL, Bruns TD, Szaro TM, Hodges SA (2003) Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. *American Journal of Botany*, **90**, 1168–1179.

Taylor DL, Bruns TD, Hodges SA (2004) Evidence for mycorrhizal races in a cheating orchid. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **271**, 35–43.

Thompson JN (1994) *The Coevolutionary Process*. The University of Chicago Press, Chicago.

Trudell SA, Rygiewicz PT, Edmonds RL (2003) Nitrogen and carbon stable isotope abundances support the myco-heterotrophic nature and host-specificity of certain achlorophyllous plants. *New Phytologist*, **160**, 391–401.

Weiss M, Selosse MA, Rexer KH *et al.* (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycological Research*, **108**, 1003–1010.

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. 315–324. Academic Press, San Diego, CA.

Winther JL, Friedman WE (2007) Arbuscular mycorrhizal associations in Lycopodiaceae. *New Phytologist*, **177**, 790–801.

Zimmer K, Meyer C, Gebauer G (2008) The ectomycorrhizal specialist orchid *Corallorhizatrifida* is a partial mycoheterotroph. *New Phytologist*, **178**, 395–400.

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## Supporting information

Additional supporting information may be found in the online version of this article.

**Table S1** Ascomycete fungi from roots of *Hexalectris* species, identified based on MegaBLAST searches of GenBank.

**Fig. S1** A 50% majority rule Bayesian Inference ITS phylogeny revealing that the positions of *Hexalectris* associated ceratobasidiaceous fungi are members of a narrow subclade (HCF-a) within this family.

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