**Methods**

Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas

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**Summary**

- Despite advances owing to molecular approaches, several hurdles still obstruct the identification of fungi forming orchid mycorrhizas. The Tulasnellaceae exhibit accelerated evolution of the nuclear ribosomal operon, causing most standard primers to fail in polymerase chain reaction (PCR) trials. Insufficient sequences are available from well characterized isolates and fruitbodies. Lastly, taxon-specific PCR primers are needed in order to explore the ecology of the fungi outside of the orchid root. Here, progress in overcoming these hurdles is reported.

- Broad-spectrum basidiomycete internal transcribed spacer (ITS) primers that do not exclude most known Tulasnellaceae are presented. BLAST searches and empirical PCR tests support their wide utility within the Basidiomycota.

- Taxon-specific ITS primers are presented targeted to orchid-associated *Tulasnella*, and a core component of the *Thelephora–Tomentella* complex. The efficiency and selectivity of these primer sets are again supported by BLAST searches and empirical tests.

- Lastly, ITS DNA sequences are presented from several strains of *Eпуlorhiza*, *Ceratorhiza*, *Ceratobasidium*, *Sistotrema*, *Thanatephorus* and *Tulasnella* that were originally described in the landmark mycorrhizal studies of Currah and Warcup. Detailed phylogenetic analyses reveal some inconsistencies in species concepts in these taxonomically challenging resupinate basidiomycetes, but also help to place several sequences from environmental samples.

**Key words:** *Ceratobasidium*, mycorrhiza, Orchidaceae, *Rhizoctonia*, ribosomal ITS sequence, *Thanatephorus*, *Tomentella*, *Tulasnella*.

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**Introduction**

The Orchidaceae is the most species-rich family of flowering plants. Along with other unique features, the Orchidaceae is characterized by a novel form of mycorrhizal interaction. The diagnostic feature of these mycorrhizas is the intracellular coils of hyphae, which have a superficial resemblance to the Paris form of arbuscular mycorrhizas (Smith & Read, 1997). However, rather than the Glomeromyctan fungi which engage in all arbuscular mycorrhizal associations, nearly all known orchid mycorrhizas are formed with fungi of the Basidiomycota (Rasmussen, 1995; Taylor et al., 2002). The identification of orchid mycorrhizal fungi is a critical first step in exploring the biology of this symbiosis, on which all orchids so far studied
depend to complete their life cycles in nature (Arditti et al., 1990). This first step is difficult for a number of reasons. The majority of fungi that have been recorded as orchid mycorrhizal symbionts belong to the anamorphic form-genus Rhizoctonia (Burgeff, 1959; Hadley, 1982; Rasmussen, 1995). This genus includes fungi with perfect states belonging to the Ascomycota and the Pucciniomycotina and Agaricomycotina of the Basidiomycota (Roberts, 1999). However, all recorded orchid-associated Rhizoctonia species belong to the Ceratobasidiaceae, Sebacinaeae and Tulasnellaceae of the Agaricomycotina (Wells, 1994). The best-known Rhizoctonia species among these three families is the damping-off root pathogen R. solani (teleomorph Thanatephorus cucumeris) of the Ceratobasidiaceae. All three families lie in a gray area occupied by diverse basal hymenium-forming fungi, most having septate basidia, leading to perpetual disagreements about the relationships among Rhizoctonia species and the placements of these families within the Agaricomycotina (see Wells, 1994; Weiss & Oberwinkler, 2001).

Morphology is naturally the first choice for species discrimination in eukaryotes, including fungi. However, in most fungi where complex fruit bodies are absent, such as the three families containing orchid-associated Rhizoctonia species, morphological species delimitation is difficult. This difficulty is further multiplied when even the cryptic, resupinate fruiting structures are rarely seen. In the Ceratobasidiaceae, vegetative hyphal morphologies are mostly homogeneous within genera, while many characters overlap between species or vary environmentally or developmentally within individuals (Andersen, 1990). Basidial morphology provides reliable identification of orchid-associated Rhizoctonia species at the morpho-species level (Warcup & Talbot, 1966, 1967, 1971, 1980), but orchid isolates have very rarely been induced to fruit in culture (Ramsay et al., 1986; Currah et al., 1987, 1990; Milligan & Williams, 1988). Septal ultrastructure is a concrete character which clearly distinguishes the major clades within Rhizoctonia (Khan & Kimbrough, 1982; Marchisio et al., 1985; Currah & Sherburne, 1992), although the Sebacinaeae and Tulasnellaceae require detailed observation to separate (Andersen, 1996). However, the methods involved are laborious and ultrastructure does not separate species within a genus.

The fungal isolation step is another major stumbling block in orchid mycorrhizal research. The symbionts of some orchid species can be routinely isolated (Rasmussen, 1995). However, isolation success in many orchids varies with season and prior disturbance (Ramsay et al., 1986) and has been shown to decline within hours of collection in some epiphytic Andean orchids (Suarez et al., 2006). Furthermore, the symbionts of a number of orchids, especially nonphotosynthetic ones, are difficult or impossible to isolate (Downie, 1943; Burgeff, 1959; Warcup, 1981, 1985; Taylor & Bruns, 1997; Taylor et al., 2003). Finally, nonsymbiotic fungi can be isolated (Warcup & Talbot, 1967; Suarez et al., 2006), leading to suspect conclusions concerning the biology of the symbiosis (see Taylor et al., 2002).

Molecular methods based on fungal-specific PCR amplification of the nuclear ribosomal internal transcribed spacer (ITS) have revolutionized characterization of ecto-, ericoid and arbuscular mycorrhizas (Gardes et al., 1991; Gardes & Bruns, 1993; Redecker, 2000; Horton & Bruns, 2001; Vralstad et al., 2002). While the ITS region has certain limitations, it is unlikely to be displaced as the most effective single locus for identification of environmental fungi at the species to genus level (Bruns, 2001 contra Seifert et al., 2007). PCR-based approaches are helping to overcome the problems associated with limited morphological variation and culture biases in orchid mycorrhizal research (Taylor & Bruns, 1997; Bidartondo et al., 2004; McCormick et al., 2004; Selosse et al., 2004; Taylor et al., 2004; Suarez et al., 2006). However, three major hurdles still stand in the way of comprehensive and unbiased molecular identification of orchid mycorrhizal symbionts. First, the most commonly encountered fungal symbionts of orchids belong to the Tulasnellaceae, yet these fungi have proven difficult to characterize using standard PCR primer sets, apparently because of accelerated evolution of the nuclear ribosomal operon (Binder et al., 2005; Moncalvo et al., 2006) and consequent mutation of bases in conserved regions to which primers hybridize (Taylor et al., 2002). A compelling example of this problem is seen in the recent study of mycorrhizal associations in several epiphytic species of the Pleurothallinae growing in the Andes (Suarez et al., 2006). Electron microscopic examination of mycorrhizal tissues with pelotons revealed a predominance of fungi with dolipore septa and imperforate, slightly curved parenthesomes that are diagnostic of the Tulasnellaceae. However, using an array of standard primers, few of these fungi were amplified. Instead, a variety of low-level contaminants, particularly ascomycetes, were amplified (the septa of which were not seen in mycorrhizal structures). Only when nested PCR and several Tulasnella-specific primers were used did the true mycorrhizal fungi appear in the molecular surveys. Secondly, owing to the extremely high diversity of fungi in environmental samples such as ectomycorrhizal roots or soil, it has been difficult to track particular fungal species outside of the orchids with which they associate. Third, there is a paucity of ITS sequences from well characterized isolates or fruitbodies in several of the most important orchid-associated clades, particularly within the Tulasnellaceae, Sebacinaeae and Ceratobasidiaceae. The result is that many fungal clades are known only from sequence data, without connection to a whole organism whose physiology, morphology, anatomy, etc. can be studied.

To help combat these issues, we have developed new fungal-selective primers which minimize amplification of plant sequences while allowing robust amplification of all tested Basidiomycota, including Tulasnella. The purpose of this primer pair is to characterize fungal diversity in mycorrhizas of unstudied orchids. In addition, we have developed more selective primer sets to amplify the ITS from the orchid-associated core of the genus Tulasnella, and the Thelephora–Tomentella complex.
Methods

isolated orchid strains. These studies are widely used for comparison of newly
& Sherburne, 1992; Currah & Zelmer, 1992; Zelmer and colleagues obtained numerous isolates from North American
terrestrial orchids, characterized their anamorphic states
The optimal primers we developed are listed in Table 1.
alignments. Previously described primers commonly used to
amplicons were located on the SSU and LSU alignments. Prospective new primer regions were then
imported to NetPrimer (Premier Biosoft, Palo Alto, CA, USA)
and checked for unwanted secondary structure and cross-
hybridization and also modified to achieve desirable annealing
temperatures (between 50 and 65°C and < 3°C difference
between paired primers).
Prospective primers obtaining relatively high scores in Net
Primer (above 87) were then tested for specificity to the target
clad and breadth of amplification within the target clade both
in silico and empirically. In silico testing was carried out using
the ‘find short nearly exact matches’ version of nucleotide
BLAST for searching GenBank on the NCBI website (http://
top 1000–5000 matches from each search were assessed using
the Taxonomy Reports and Lineage Reports output options.
The optimal primers we developed are listed in Table 1.
Empirical tests of primer performance were carried out
using 56 DNA extracts, representing the following: most
major clades of the Agaricomycotina (= hymenomycetes)
(Hibbett et al., 2007), including Tremellomycetes, Dacrymycetes,
Auriculariales, Gomphales, Cantharellales, Hymenochaetales,
Polyporales, Russulales, Sebacinales, Thelephorales, and
Agaricales, but missing the Geastrales, Hysterangiales, Phallales,
Gloeophorales, Wallemiomycetes and Entorrhizomycetes;
the major orchid-associated Rhizoctonia clades Tulasnellaceae,
Ceratobasidiaceae, and Sebacinaeaceae; diverse members of the
Thelephorales; several vascular plants, including three orchid
species (Table 2; additional details of DNA sources are given in
Supplementary Material, Table S1).

DNA extraction
In general, fungal genomic DNAs for empirical primer tests
and sequencing were extracted from either dried fruitbodies
or mycelium grown from pure cultures in broth. Because
the DNAs were obtained over a 15-yr period, a variety of
extraction methods were utilized, including the CTAB

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target clade</th>
<th>Sequence</th>
<th>Paired primer</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1-OF</td>
<td>All Basidiomycota</td>
<td>AAACGTCCATTAGAGGAAGT</td>
<td>ITS4-OF</td>
<td>60</td>
</tr>
<tr>
<td>ITS4-OF</td>
<td>All Basidiomycota</td>
<td>AAACGTCCATTAGAGGAAGT</td>
<td>ITS1-OF</td>
<td>54</td>
</tr>
<tr>
<td>ITS4-Tul</td>
<td><em>Tulasnella</em></td>
<td>CGATACGGGAACCCGTTT</td>
<td>ITS1 or ITS5</td>
<td>62</td>
</tr>
<tr>
<td>SSU1318-Tom</td>
<td><em>Thelephoraceae</em></td>
<td>CGGAGGGAACCCGTTT</td>
<td>LSU-Tom4</td>
<td>62</td>
</tr>
<tr>
<td>LSU-Tom4</td>
<td><em>Tomentella/Thelephora</em></td>
<td>GCCCATTTCAAGAGACCTTA</td>
<td>SSU1318-Tom</td>
<td>62</td>
</tr>
</tbody>
</table>

Sequences of new primers designed in this study are given, along with recommendations for primers with which to pair the new primers and annealing temperatures for the PCR. In one case, one of the two primers in the recommended pair has been previously published: ITS1; ITS5 is also a good option (White et al., 1990).

These primers sets should help to elucidate the distribution
and natural histories of particular orchid-associated fungi in
natural environments. Lastly, we have sequenced the ITS region
from several fungi isolated from orchids in the landmark studies of Jack Warcup and Randolf Currah in order to
improve phylogenetic resolution of orchid-associated fungi
and in the hope that additional clades of environmental
sequences can be connected to whole organisms. Warcup and
Talbot isolated mycorrhizal fungi from a wide spectrum of
Australian terrestrial orchids and were one of the few teams who succeeded in inducing teleomorph formation from a large percentage of their isolates. The sexual structures allowed
detailed taxonomic work as well as analyses of patterns of
specificity in these orchids (Warcup & Talbot, 1966, 1967,
1971, 1980; Warcup, 1971, 1981, 1985). In turn, Currah and
doctorates obtained numerous isolates from North American
terrestrial orchids, characterized their anamorphic states
(rarely, teleomorphs) and conducted a study of septal
ultrastructure in representative strains (Currah, 1987; Currah
et al., 1987, 1988, 1990, 1997; Mordue et al., 1989; Currah
& Sherburne, 1992; Currah & Zelmer, 1992; Zelmer et al.,
1996). These studies are widely used for comparison of newly
isolated orchid strains.

Materials and Methods

Primer design and testing
An alignment of the 3′ region of the nuclear small subunit
ribosomal gene with representatives of the major fungal
phyla (Chytridiomycota, Blastocladiomycota, Zygomycota,
Glomeromycota, Ascomycota, Basidiomycota), diverse
basidiomycetes, the major Rhizoctonia groups and other
orchid-associated lineages was initiated in ClustalW and
modified by eye in *nup*60 (Swofford, 1990) and Se-Al
(Rambaut, 1996). A similar alignment of the 5′ end of the
nuclear large subunit ribosomal gene was also constructed.
GenBank sequences from diverse vascular plants, including
several members of the Orchidaceae, were added to both
alignments. Previously described primers commonly used to
amply the ITS region were located on the SSU and LSU
alignments. Prospective new primer regions were then
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Table 2  Results of empirical polymerase chain reaction (PCR) trials to test primer breadth and selectivity

<table>
<thead>
<tr>
<th>Family/lineage</th>
<th>ITS1/ITS4</th>
<th>ITS1F/ITS4</th>
<th>ITS1OF/ITS4OF</th>
<th>ITS1/ITS4-Tul SSU1318 Tom/LSU-Tom4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortinarius traganus</strong></td>
<td>Agaricales</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Galera patagonica</strong></td>
<td>Agaricales</td>
<td>+++</td>
<td>+++</td>
<td>(+)</td>
</tr>
<tr>
<td><strong>Fomitopsis pinicola</strong></td>
<td>Aphyllophorales</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Auricularia cornea</strong></td>
<td>Auriculariales</td>
<td>+++</td>
<td>+++</td>
<td>(+)</td>
</tr>
<tr>
<td><strong>Exidia crenata</strong></td>
<td>Auriculariales</td>
<td>+++</td>
<td>+++</td>
<td>(+)</td>
</tr>
<tr>
<td><strong>Exidia sp.</strong></td>
<td>Auriculariales</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Exidiopsis punicea</strong></td>
<td>Auriculariales</td>
<td>+++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>Heterochaete sp.</strong></td>
<td>Auriculariales</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Tipularia protocorm mycorrhiza</strong></td>
<td>Auriculariales</td>
<td>MB</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Tipularia protocorm mycorrhiza</strong></td>
<td>Auriculariales</td>
<td>MB</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>Alpova sp.</strong></td>
<td>Boletales</td>
<td>+++</td>
<td>+++</td>
<td>(+)</td>
</tr>
<tr>
<td><strong>Boletus edulis</strong></td>
<td>Boletales</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Dacrymyces capitatus</strong></td>
<td>Dacrymycetales</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Dacrymyces cerasi</strong></td>
<td>Dacrymycetales</td>
<td>+</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Geastrum mammosum</strong></td>
<td>Geastrales</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Gomphus floccosus</strong></td>
<td>Gomphoid-Phalloid</td>
<td>++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>Polyporus brumalis</strong></td>
<td>Polyporoid</td>
<td>+++</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Trametes versicolor</strong></td>
<td>Polyporoid</td>
<td>+++</td>
<td>+++</td>
<td>(+)</td>
</tr>
<tr>
<td><strong>Trichaptum abietinum</strong></td>
<td>Polyporoid</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Ceratobasidium sp.</strong></td>
<td>Rhizoctonia, Ceratobasiidae</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Ceratobasidium sphaerosporum</strong></td>
<td>Rhizoctonia, Ceratobasiidae</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Moniliopsis anomala</strong></td>
<td>Rhizoctonia, Ceratobasiidae</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Rhizoctonia versicolor</strong></td>
<td>Rhizoctonia, Ceratobasiidae</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Sistotrema sp.</strong></td>
<td>Rhizoctonia, Ceratobasiidae</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Thanatephorus ochraceus</strong></td>
<td>Rhizoctonia, Ceratobasiidae</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Fungus isolated from Hexaeclites spicata</strong></td>
<td>Rhizoctonia, Sebacinaceae</td>
<td>+++</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Sebacina vermintera</strong></td>
<td>Rhizoctonia, Sebacinaceae</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td><strong>Epulorhiza anaticula</strong></td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>(+)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Tulasnella cystidiophora</strong></td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>(+)</td>
<td>++</td>
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<tr>
<td><strong>Tulasnella calospora</strong></td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Tulasnella irregularis</strong></td>
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<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Tulasnella sp.</strong></td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>(+)</td>
<td>+++</td>
</tr>
<tr>
<td>from Goodyera</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>(+)</td>
<td>+++</td>
</tr>
<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>(+)</td>
<td>+++</td>
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<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>from Tipularia</td>
<td>Rhizoctonia, Tulasnellaceae</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
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<tr>
<td><strong>Lactarius resimus</strong></td>
<td>Russulaceae</td>
<td>+++</td>
<td>++</td>
<td>–</td>
</tr>
</tbody>
</table>

The intensity of PCR products produced from each taxon with the various primer pairs are indicated from barely visible, (+), to very bright, ++.
### Table 2 continued

<table>
<thead>
<tr>
<th>Family/lineage</th>
<th>ITS1/ITS4</th>
<th>ITS1F/ITS4</th>
<th>ITS1OF/ITS4OF</th>
<th>ITS1/ITS4-Tul</th>
<th>SSU1318 Tom/LSU-Tom4</th>
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</thead>
<tbody>
<tr>
<td>Lactarius torminosus</td>
<td>Russulaceae</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Russula brevipes</td>
<td>Russulaceae</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Hydnellum peckii</td>
<td>Thelephoraceae</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Fungus isolated from Cephalanthera austinae</td>
<td>Thelephoraceae</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Cephalanthera austinae</td>
<td>Thelephoraceae</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Fungus isolated from Hydnellum peckii</td>
<td>Thelephoraceae</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Corallorhiza odontorhiza</td>
<td>Thelephoraceae</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Fungus isolated from Corallorhiza odontorhiza</td>
<td>Thelephoraceae</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Tomentella sp.</td>
<td>Thelephoraceae</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Tomentella sp.</td>
<td>Thelephoraceae</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Sirobasidium magnum</td>
<td>Tremellales</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>Tremella mesenterica</td>
<td>Tremellales</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Cuphea miniata stem</td>
<td>Eudicots; Myrtales</td>
<td>+++</td>
<td>(+)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Phacelia viscidia stem</td>
<td>Eudicots; Solanales</td>
<td>+++</td>
<td>(+)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Verbena species stem</td>
<td>Eudicots; Lamiales</td>
<td>+++</td>
<td>MB</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Silene vulgaris stem</td>
<td>Eudicots; Caryophyllales</td>
<td>+++</td>
<td>(+)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dalechampia volubilis stem</td>
<td>Eudicots; Malpighiales</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Corallorhiza maculata stem</td>
<td>Monocots; Orchidaceae</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Corallorhiza mertensiana stem</td>
<td>Monocots; Orchidaceae</td>
<td>+++</td>
<td>MB</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cypripedium guttatum stem</td>
<td>Monocots; Orchidaceae</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

The intensity of PCR products produced from each taxon with the various primer pairs are indicated from barely visible, (+), to very bright, +++.

Unless otherwise indicated, the band is of the expected size for a given primer pair. MB stands for multiple bands of incorrect sizes. Note that the amplicons from Corallorhiza stems when using ITS1-OF and ITS4-OF were found to derive from basidiomycetous yeasts.

The method of Gardes & Bruns (1996a), the SDS/Gene Clean method of O’Donnell (see Taylor & Bruns, 1997), the Qiagen Plant DNeasy and Genomic Tip kits (Qiagen, Valencia, CA, USA) and the Omega Fungal EZNA kit (Omega Biotech, Doraville, GA, USA). In selecting strains for DNA sequencing, we acquired representative strains from the landmark studies of Warcup & Talbot (1966, 1967, 1971, 1980) and Currah et al. (1987, 1990) (JHW 062; JHW 0632 – type strain; JHW 0750; UAMH 5404; UAMH 5428; UAMH 5430; UAMH 5443; UAHM 6440).

**PCR amplification**

Amplification reactions of 25 µl were carried out with final concentrations of 200 µm each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 25 mM MgCl, 0.1 mg ml⁻¹ gelatin, and 0.5 units of Sigma RedTaq DNA polymerase (Sigma-Aldrich, Saint Louis, MO, USA). Routine amplifications consisted of 35 cycles in a MJ PTC-200 thermocycler and employed a 2 min initial denaturation at 96°C before thermocycling, with 30 s denaturation at 94°C followed by a 40 s annealing at various temperatures (Table 1) and 72°C elongation for 1 min. The last cycle was followed by extension at 72°C for 10 min.

**DNA sequencing and cloning**

Primer pairs ITS1-OF plus ITS4-OF; ITS1 (White et al., 1990) plus ITS4-Tul; and ITS1-F (Gardes & Bruns, 1993) plus TW13, (GGTCCGTGTTCACAGACG http://plantbio.berkeley.edu/~bruns/) were used for initial amplification, followed by Qiagen Qiaquick cleanup and cycle sequencing with BigDye Terminator 3.1 (Applied Biosystems, Foster City, CA, USA) using ITS1 and ITS4 (White et al., 1990). Products
were cleaned over Sephadex G50 and separated on an ABI 3130XL capillary system. Mixed fragments were obtained from ITS1-OF/ITS4-OF amplifications from stems of two Corallorrhiza species. We therefore cloned and sequenced these amplicons. PCR products were purified with Zymo 5 Clean & Concentrator columns (Zymo Research, Orange, CA, USA) then cloned using the TOPO TA for sequencing kit with vector PCR4.0 (Invitrogen, Carlsbad, CA, USA) following manufacturers’ instructions. Discrete colonies were directly amplified using M13 primers and sequenced, as described earlier. Sequences have been submitted to GenBank under accessions EU218878-EU218895.

Phylogenetic analyses

Close relatives of our sequenced specimens were identified through Discontinuous MegaBLAST searches of GenBank and masked. FASTA searches of our website (http://biotech.inbre.alaska.edu/fungal-metagenomics/). Sets of closely related sequences were then aligned using Muscle (Edgar, 2004) followed by manual optimization in Se-Al (Rambaut, 1996). The ITS sequences within the Tulasnellaceae were extremely diverse, and positional homology when we attempted a global alignment of all sequences was highly suspect. We therefore created an alignment including only the 5.8S portion of the ITS region, then a maximum parsimony tree for all sequences was estimated in PAUP*4.0b10, which was used to identify clades that could be used to create three separate alignments spanning the entire ITS1–5.8S–ITS2 region. Similar approaches were used by Suarez et al. (2006) and Shefferson et al. (2007). We started with 154 taxa in the Tulasnellaceae, but pruned numerous highly similar sequences for ease of visualization of the resulting trees. To further evaluate the effects of uncertain positional homology in the alignments, all alignments were also pruned to leave only conserved positions using the lenient Gblocks web server (Castresana, 2000). Trees produced from complete alignments versus pruned ‘Gblock’ alignments were compared. Best-fitting models of molecular evolution were determined for each alignment using ModelTest 2.0 (Posada & Crandall, 1998) and Aikake Information Criteria. Maximum-likelihood trees were inferred using the genetic algorithm-driven program GARLI (Zwickl, 2006) using default search settings; the same settings were used to carry out 100 bootstrap replicates for each dataset, except that the search termination criterion for consecutive generations without an improvement in likelihood was dropped from 10 000 to 5000. For the three Tulasnellaceae alignments, the GTR + I + G model was used, since it was the closest available model to the one specified by ModelTest. For the Ceratobasidiales, the HKY + I + G model was used. Likelihood trees were compared to parsimony trees estimated in PAUP*4.0b10 using heuristic searches with 10 random addition replicates, equal weights and maximum trees set to 100 000. The three Tulasnellaceae trees are shown with midpoint rooting (Farris, 1972), because of a lack of an alignable, a priori, outgroup. Botryobasidium plus Hypalorhiza were designated as outgroups in the Ceratobasidiales analyses based upon Moncalvo et al. (2006). Alignments and additional information are available on our website (http://mercury.bio.uaf.edu/~lee_taylor/orchid_primers.html).

Results and discussion

New basidiomycete ITS primers: ITS1-OF/ITS4-OF

A very effective primer for the amplification of the ITS region from essentially all Eumycota, ITS1-F, and which minimizes the amplification of plant sequences, was developed by Gardes & Bruns (1993). However, the nuclear ribosomal operon of the Tulasnellaceae is evolving exceedingly rapidly (Taylor et al., 2002; Binder et al., 2005; Moncalvo et al., 2006), and hence many primer sites which are generally conserved across the Eumycota are not conserved in the Tulasnellaceae (Figs 1, 2). The primer ITS-1F does not effectively amplify some core species within the Tulasnellaceae (e.g. Tulasella irregularis and Epulorhiza anaticula, Table 2; also see Suarez et al., 2006). Hence, we sought to design a pair of ITS primers that would amplify Tulasella species and as many other Basidiomycota as possible, while selecting against amplification of orchid genomic regions. The forward primer ITS1-OF overlaps with ITS1-F but is positioned two bases 5′ in the small subunit. Note that ITS1-OF is really two primers of nearly identical sequence that must be ordered separately and then combined before use; synthesis of a single degenerate primer is not recommended. The altered placement and two positions that differ among the primer forms provide an improved fit to the few available Tulasella sequences, and the 10 3′ bases perfectly match all other Basidiomycota inspected (see alignment, Fig. 1). The primer has one fewer mismatch with conserved vascular plant sequences than does ITS1-F, but still has a mismatch at the critical 3′-most base and at three other positions. The reverse primer ITS4-OF is slightly 3′ of ITS4 and binds in a highly conserved region of the large subunit (see alignment, Fig. 2). Again, however, the primer has a mismatch with all inspected orchid sequences at the 3′-most base. The primer is a perfect match to all inspected Basidiomycota with the exception of a few noncritical bases at the 5′ end in various Tulasnellaceae (owing to the rapid evolution in this lineage, no entirely conserved regions were found). The primer has a few mismatches with some inspected members of the Ascomycota, Zygomycota and Chytridiomycota, although it is not safe to assume that amplification of species in these taxa will be prevented.

Broader in silico analyses of primer specificity were performed using the short-exact match option in BLASTn searches of the complete nr database on GenBank. These analyses were largely congruent with the patterns seen in the alignments of a few selected taxa. BLASTn lineage reports utilize the hierarchical
Fig. 1 Alignment of a region of the ribosomal small subunit from diverse fungi used for primer design. The small subunit (SSU) alignment shows locations of previously published and new primers for amplification of the internal transcribed spacer (ITS) region. The alignment is in pretty format with all sequences compared to *Thanatephorus cucumeris* as the reference sequence, shown at both the top and bottom. Bases in other taxa which are identical to the reference sequence are indicated with a ‘.’ while alternative bases are spelled out. To maximize representation in several clades of orchid fungi, sequences that do not span the entire aligned region were included, with missing bases coded as ‘?’ while gaps resulting from indels are represented by the ‘–’ symbol. Boxes highlight bases within particular taxa that contribute to the specificity of particular primers. Two portions at the 3′ end of the SSU have been concatenated, with the join indicated by ‘+++’. The portions span positions 1307–1341 and 1713–1821 of the *Saccharomyces cerevisiae* GenBank V01335 nuclear SSU gene.
Fig. 2. Alignment of a region of the ribosomal large subunit from diverse fungi used for primer design. The large subunit (LSU) alignment shows the locations of previously published and new primers for amplification of the internal transcribed spacer (ITS) region.
NCBI taxonomy and sort taxa in order of the proportion of best matches to the query within a taxon. The top taxon reported for ITS1-OF was *Entoloma* (Basidiomycota), but equally high matches were distributed throughout the Basidiomycota and occurred in many other Eumycota. Taxonomy reports show all significant matches to a query, organized according to the NCBI taxonomic hierarchy. For ITS1-OF there were 5007 hits in the following groups: Fungi, 4694; Ascomycota, 2611; Basidiomycota, 1214; Glomeromycota, 615; Zygomycota, 75; Chytridiomycota, 74; Embryophyta and Orchidaceae, both 0.

The top-ranked taxon in the ITS4-OF lineage report was *Tulasnella*. The taxonomy report showed hits to important groups as follows: Fungi, 4661; Basidiomycota, 4253; Glomeromycota, 191; Zygomycota, 53; Chytridiomycota, 3; Embryophyta, 16; Orchidaceae, 0. To some degree, the numbers of hits to particular lineages likely reflect biases in the sequences available on GenBank. For example, the 3′ end of the SSU is sequenced less often than the 5′ end of the LSU in molecular systematic studies of basidiomycetes, which may explain the lower number of basidiomycete hits to ITS1-OF.

It should also be noted that many of the *blast* best hits are not 100% identical to the primer sequence, meaning that primer specificity is likely to be narrower than the spectrum of hits. In addition to primers that do not select against *Tulasnella* species, it is advantageous in some situations to select for *Tulasnella* species while minimizing amplification of other fungal taxa. The primer ITS4-Tul was designed with this objective in mind. For example, the distributes of *Tulasnella* species in soil samples has been investigated using this primer (M. K. McCormick et al., unpublished). ITS4-Tul sits between ITS4 (White et al., 1990) and ITS4B (Gardes & Bruns, 1993) at the 5′ end of the nuclear large subunit; it is best paired with ITS1 or ITS5 (which do not exclude *Tulasnella* species). The primer can also be paired with ITS1-F, but we advise against this combination because ITS1-F excludes many *Tulasnella* species. As can be seen in the LSU alignment (Fig. 2), ITS4-Tul is a close or perfect match to several core species of the genus *Tulasnella*, but mismatches most other fungi, including some *Tulasnella*-like environmental sequences and more divergent members of the genus such as *Tulasnella cystidiophora*.

The *blast* searches support these observations more broadly: the Tulasnellaceae was the top-ranked taxon in the lineage report, while only *Tulasnella* species were returned as perfect matches. In empirical tests, outside of *Tulasnella*, weak bands were produced only with several members of the Thelephoraceae (Table 2). Bands of incorrect sizes were not seen. Within the Tulasnellaceae, *T. irregularis*, *T. violea*, *T. calopora* and all isolates from the orchids *Liparis lilifolia*, *Tipularia discolor* (adults) and *Goodyera repens* amplified strongly. These species and isolates have diverse ITS sequences and thus represent considerable phylogenetic breadth within *Tulasnella* (see McCormick et al., 2004 and Figs S1–S3). No failures were seen with tested species of *Tulasnella* except *Tulasnella cystidiophora*. This is not known to be an orchid mycorrhizal fungus, and is distantly related to the clades of *Tulasnella* containing orchid-associated strains (Shefferson et al., 2007).

We designed ITS4-Tul over 10 yr ago and it has been used widely to study orchid mycorrhizas (Bidartondo et al., 2004; Selosse et al., 2004; Julou et al., 2005; Shefferson et al., 2005, 2007; Abadie et al., 2006; Girlanda et al., 2006; Suarez et al., 2006), and has also been used to screen ectomycorrhizas rapidly for the presence and diversity of *Tulasnella* species (Bidartondo et al., 2003), but it has not been formally published. Here, we tested ITS4-Tul along with the new primers and formally describe its design and testing.
Sporobolomyces, and the following members of the basidiomycetes of screen ectomycorrhizal root tips and soil samples for fungi in the Thelephoraceae. We have designed (McKendrick et al., 2007). Journal compilation ©

Some mycoheterotrophic orchids, including Cephalanthera austiniae (Taylor & Bruns, 1997) and Coralloriza trifida (McKendrick et al., 2000), associate specifically with ectomycorrhizal fungi in the Thelephoraceae. We have designed these Thelephoraceae-selective ITS primers in order to rapidly screen ectomycorrhizal root tips and soil samples for fungi in the Thelephora–Tomentella complex. Once amplified, species-level diagnosis can be attempted through PCR-RFLP analysis or cloning and sequencing. SSU1318-Tom aligns c. 490 bp from the 3′ end of the nuclear small subunit gene. It has one base position at the 3′ end which is conserved in the few available sequences from the Thelephoraceae yet differs from many other basidiomycetes (Fig. 1). As with ITS4-Tul, LSU-Tom4 sits at the 5′ end of the large subunit between ITS4 and ITS4-B. It aligns well with many Tomentella and Thelephora sequences, but mismatches other fungi, including related taxa such as Pseudotomentella and Hydnum (Fig. 2). The spectrum of BLAST hits to SSU1318 was somewhat wider than we had expected. SSU1318-Tom had perfect matches to several ascomycetes, including Saccharomyces unisporus, several species of Candida, Peziza, Chalarra, a wide variety of zygomycetes including Rhizopus and Mucor, and the basidiomycetes Bensingtonia, Donkioporia, Kondoia, and Sporobolomyces, and the following members of the Thelephoraceae: Bankera, Boletopsis, Hydnellum, Phellodon, Polyzellus, Sarcodon, Thelephora, Tomentella. There were no perfect matches to Viridiplantae, and the predominant basidiomycete matches belonged to the Thelephoraceae, as intended. Tomentella and the Thelephoraceae were the top taxa in the lineage report for LSU-Tom4. The only perfect matches outside the Thelephoraceae were to Phaeoclavulina (Basidiomycota) and Buellia and Piedraaria (Ascomycota).

Empirical tests show the SSU1318-Tom/LSU-Tom4 pair to be highly specific: strong amplification was obtained from the array of orchid isolates and Tomentella and Thelephora fruitbodies, but not from any other tested fungi. Bands of incorrect size were not seen with this primer pair. While the in silico specificity of SSU1318-Tom is not as strict as we had hoped, it nevertheless mismatches the majority of Basidiomycota and Ascomycota, and so should act synergistically with LSU-Tom4 in targeting only the Tomentella + Thelephora lineage. In other words, SSU1318-Tom is a better choice as the forward primer than universal primers such as ITS1-F or ITS1, if the goal is to selectively amplify the core Tomentella + Thelephora clade. When used on soil samples from which a wide variety of ectomycorrhizal fungi had previously been amplified, this primer pair selectively amplified only Tomentella spp. (M. K. McCormick et al., unpublished).

Note that these primers can positively identify the presence of members of the core Thelephora–Tomentella clade, and thus help track these fungi amidst the overwhelming spectrum of soil fungi. However, these primers should not be used to infer the absence of members of the Thelephoraceae, since our primers target only a subclade. No family-wide synapomorphies were found which could be used to design family- or orderspecific primers, and there is likely great sequence diversity among currently uncharacterized species of Thelephorales.

Phylogenetic analyses of orchid-associated Rhizoctonia strains

While other regions have been investigated (Bruns et al., 1998; Seifert et al., 2007), the ITS region has several features that make it a strong candidate for a universal ‘barcode’ for fungal identification. As pointed out by Bruns (2001) and many others, it is easy to amplify because of its high copy number, because relatively few primer sets are needed as a result of the highly conserved SSU and LSU flanking regions, and because it varies relatively little within species but dramatically between species, and it is far better represented in GenBank than any other locus in fungi. These observations have motivated our efforts to improve the spectrum of ITS primers available for orchid mycorrhizal research, and to generate sequences from selected orchid fungi in order to broaden the basis for comparison of cultured and uncultured fungi.

The major conclusions from detailed phylogenetic analyses of our new ITS sequences from the strains of Warcup and Currah, along with all related sequences from GenBank, are summarized here. More detailed discussions are included with the phylogenetic trees in the Supplementary Material. First, ITS sequences within the Tulasnellaceae are extremely diverse, and must be aligned separately within narrower subclades of the Tulasnellaceae, as pointed out by several previous authors (Suarez et al., 2006; Jefferson et al., 2007). Here, we have divided the available sequences into three subclades. The first clade contains numerous orchid-associated sequences and two separate groupings attributed to T. calospora, the most commonly encountered orchid Tulasnella. Warcup’s strain CBS 573.83 belongs to the first of these T. calospora groupings (Fig. S1). Our sequence from Warcup’s type strain of T. irregularis belongs to the second major Tulasnella clade, and provides a useful phylogenetic marker because its closest sequenced relatives are all unidentified environmental samples (Fig. S2). The third Tulasnella clade contains Currah’s anamorphic strain UAMH 5428, which is morphologically unique as a result of the isthmus-like connections between the monilioid cells, along with T. danica and several unidentified sequences.

The affiliations of strains belonging to the Ceratobasidiales are also complicated (Fig. S4). For example, neither Thanatephorus nor Ceratobasidium is monophyletic in our analyses; this pattern has been pointed out previously (Gonzalez et al., 2001). Currah’s ‘Ceratobasidium obscurum’ UAMH 5443 does not appear to belong to the C. conigerum complex, as was predicted by Roberts (Roberts, 1998a), but is interesting

because it groups with a number of uncultured fungi including putative ectomycorrhiza-formers. Currah’s isolate of the newly described species Thanatephorus pennatus (Currah, 1987), which was synonymized with T. ochraceus (Roberts, 1998b), falls out on a long branch and thus provides a cultured neighbor for one related environmental sequence. The position of ‘Ceratobasidium goodyera-repentis’ UAMH 6440 is not with C. cornigerum, as was expected from Warcup’s work with anamorph-teleomorph connections, but instead is sister to a GenBank sequence labeled Uhohbasidium fusisporum along with numerous sequences from Puerto Rican orchid isolates. Lastly, Currah’s interesting isolate labeled ‘Sistotrema sp.’ (UAMH 5437) appears to be a divergent member of the Ceratobasidiaceae, which has clamp connections. This unexpected connection between clamped orchid strains and Rhizotonia species within the Ceratobasidium–Thanatephorus complex calls for a re-evaluation of the taxonomic implications when clamp connections are observed in orchid mycorrhizal pelotons.

Conclusions

Several of the ITS sequences from the Warcup and Currah strains reported here fall into clades previously represented entirely by uncultured environmental samples, and thus broaden the basis for physiological and taxonomic comparisons. Our phylogenetic analyses also highlight some of the difficulties encountered when attempting to connect anamorphs to teleomorphs based solely on morphological analysis of vegetative characters. Indeed, even strains that fruit are difficult to identify judging by the polyphyletic positions of vegetative characters. Furthermore, as this study and others reveal, the diversity of the cryptic, resupinate fungi that have traditionally fallen under the Rhizoctonia umbrella appears to be immense, and a great deal of additional taxonomic and molecular systematic work on these fungi is needed.

For initial characterization of the unknown fungal symbionts of an orchid, we recommend the use of ITS1-OF with ITS4-OF, which have been shown to be effective across all tested Basidiomycota and to minimize amplification of plant sequences. This primer pair has the advantage over previously published primers in that it does not exclude Tulasnella species, and thus should give a less biased view of orchid associations within the Basidiomycota. Another primer pair which allows amplification of a partial nuclear ribosomal large subunit region from a broad spectrum of basidiomycetes, including the Cantharellales and Tulasnella species, may prove useful in a wider field of research than orchid mycorrhizas alone. Quickly screening ECM roots for this Tulasnella + Thelephora clade and documenting the distribution of this clade in soil are uses we envision for these primers. However, the Thelephoraceae is a very diverse family, and our new primers are not intended to span the entire breadth of the family. The primer ITS4-Tul is quite effective for screening environmental samples for an array of orchid-associated Tulasnella species, although it does not span the entire Tulasnellaceae species. Given the tremendous phylogenetic diversity encompassed by the Tulasnellaceae and Thelephoraceae, it does not seem feasible to design family-wide primer pairs which also exclude all other fungi. Rather, targeting key subclades within these families is a more efficacious approach. We did not attempt the design of Ceratobasidiaceae selective primers because they are relatively uncommon as true mycorrhizal symbionts of orchids. The third major clade under the Rhizoctonia umbrella is the Sebacinaceae, which does include important orchid symbionts (McKendrick et al., 2002; Taylor et al., 2003; Selosse et al., 2004). A selective primer for this clade, ITS35eb, has been designed by Mary Berbee (Setaro et al., 2006).

Until now, it has not been feasible to explore the distribution and activities of orchid fungi in natural environments and outside mycorrhizal structures. Given the extreme dependence of orchids upon their mycorrhizal fungi and the conservation threats facing many wild orchids, improved understanding of the natural histories of their fungal associates is an urgent goal. We expect that the taxon-specific primers that we and others have recently developed will provide exciting new insights into the ecologies of these enigmatic fungi.

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Methods


**Supplementary Material**

The following supplementary material is available for this article online:

**Fig. S1** Maximum-likelihood tree of the first Tulasnellaceae clade.

**Fig. S2** Maximum-likelihood tree of the second Tulasnellaceae clade.

**Fig. S3** Maximum-likelihood tree of the third Tulasnellaceae clade.

**Fig. S4** Maximum-likelihood tree of the Ceratobasidiaceae.

**Table S1** Collection numbers and sources of DNAs used for empirical primer tests and/or DNA sequencing

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