New Phytologist

Methods

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

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Received: *21 August 2007*
Accepted: *18 October 2007*

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 *Epulorhiza*, *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*.

New Phytologist (2008) **177**: 1020–1033

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doi: 10.1111/j.1469-8137.2007.02320.x

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 Glomeromycotan 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, Sebacinaceae 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 Sebacinaceae 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, Sebacinaceae 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.

Table 1 Primer sequences, recommended primer pairs and annealing temperatures

Primer	Target clade	Sequence	Paired primer	Temperature (°C)
ITS1-OF	All Basidiomycota (mix these two primers)	AACTCGGCCATTTAGAGGAAGT AACTTGGTCATTTAGAGGAAGT	ITS4-OF	60
ITS4-OF	All Basidiomycota	GTTACTAGGGGAATCCTTGTT	ITS1-OF	
ITS4-Tul	*Tulasnella*	CCGCCAGATTCACACATTGA	ITS1 or ITS5	54
SSU1318-Tom	Thelephoraceae	CGATAACGAACGAGACCTTAT	LSU-Tom4	62
LSU-Tom4	*Tomentella/Thelephora*	GCCCTGTTCCAAGAGACTTA	SSU1318-Tom	

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 colleagues 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 PAUP*b10 (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 amplify the ITS region 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 clade 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://www.ncbi.nlm.nih.gov/BLAST/; Altshul *et al.*, 1997). The 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 Entorhizomycetes; the major orchid-associated *Rhizoctonia* clades Tulasnellaceae, Ceratobasidiaceae, and Sebacinaceae; diverse members of the Thelephoraceae; 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

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Table 2 Results of empirical polymerase chain reaction (PCR) trials to test primer breadth and selectivity

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

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Table 2 *continued*

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

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.

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 Biotek, 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 of 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, (GGTCCGTGTTTCAAGACG 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

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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 *Corallorhiza* 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 settings in the 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 ones specified by ModelTest. For the Ceratobasidiaceae, 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 *Hyplotrichum* were designated as outgroups in the Ceratobasidiaceae analyses based upon Moncalvo *et al.* (2006). Alignments and additonal 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. *Tulasnella 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 *Tulasnella* 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 *Tulasnella* 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. BLAST lineage reports utilize the hierarchical

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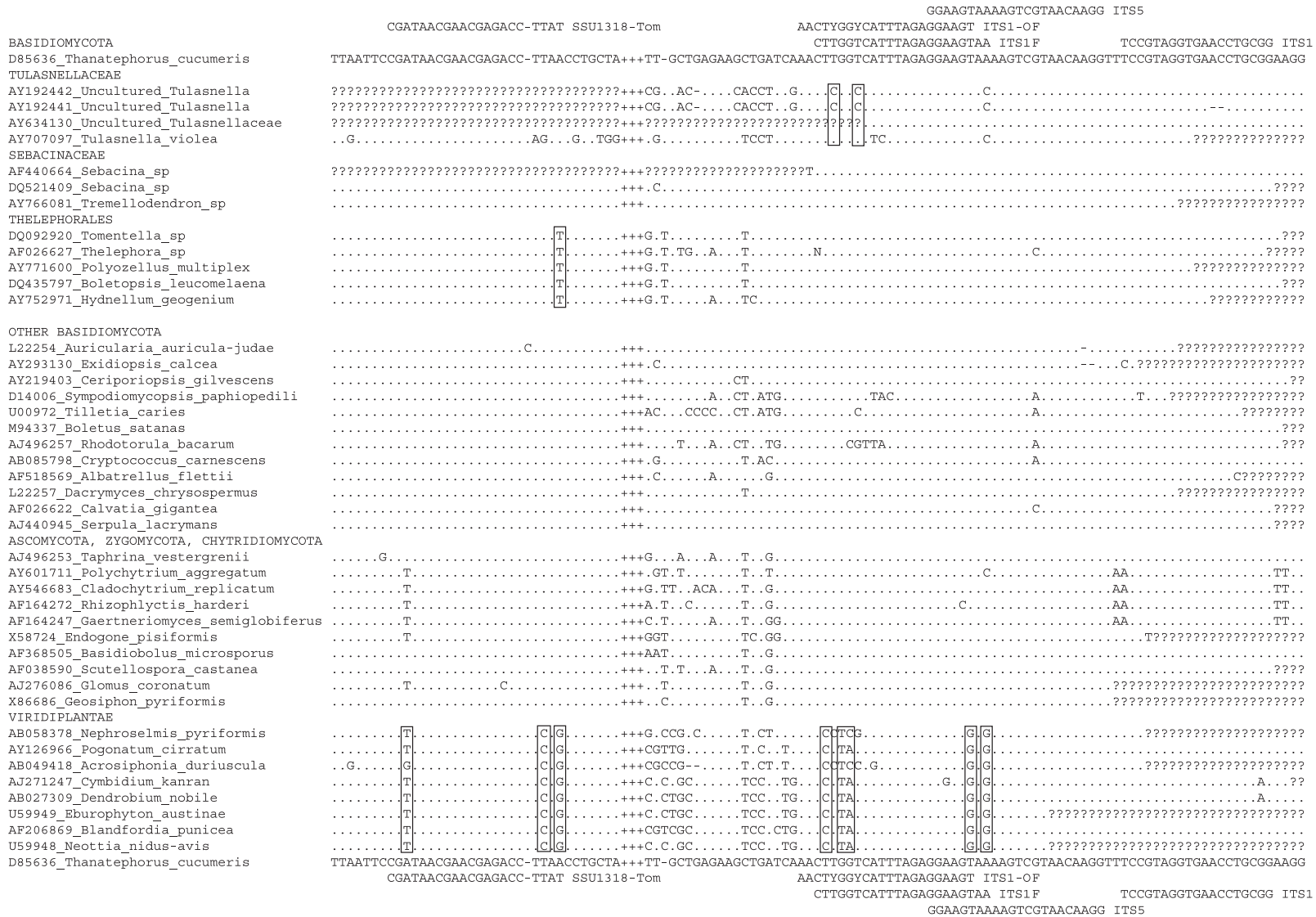

Fig. 1 Alignment of a region of the ribosomal small subunit from diverse fungi used for primer design. The small subunit (SSU) alignment shows locations of previously published and new primers for amplification of the internal transcribed spacer (ITS) region. The alignment is in pretty format with all 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.

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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. Note that primer sequences shown are reverse complements of the actual oligonucleotides that should be synthesized (Table 1). The alignment format follows Fig. 1. Two concatenated portions are again shown, which span positions 37–138 and 179–209 of the *Saccharomyces cerevisiae* GenBank J01355 nuclear LSU gene.

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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 hits are not 100% identical to the primer sequence, meaning that primer specificity is likely to be narrower than the spectrum of hits. Empirical tests agreed well with the predictions from the alignments and BLAST searches. There were no tested Basidiomycota that failed to amplify with this primer pair. By contrast, none of the plant stem DNA extracts produced an amplicon except *Corallorhiza maculata* and *Corallorhiza mertensiana*. These products were cloned and sequenced, and turned out to belong to a spectrum of basidiomycetous yeasts and other fungi, with top BLAST matches to *Cryptococcus* sp. AF444487 (98% identity), *C. huempii* AF444322 (92%), and *C. saitoi* AF444372 (99%), *Udeniomyces pannonicus* AB072232 (99%), *Rhodosporula fujisanensis* AF444490 (96%), *Malessezia restricta* AJ437695 99%), *Fomitopsis* sp. AF509233 (100%) and *Gerronema subclavatum* U66434 (85%). The occurrence of these fungi on orchid floral parts is not surprising, since yeast communities are common on plant leaves and flowers (Glushakova & Chernov, 2004; Maksimova & Chernov, 2004; Wang & Bai, 2004; Inacio *et al.*, 2005) and the tissues were not surface-sterilized. However, the important point is that sequences from plant genomes were not amplified and the primers appear to have strong selectivity for the Eumycota.

The goal in designing these primers was to be able to characterize the diversity of fungi in orchid mycorrhizas with as little bias as possible (i.e. without excluding any potential basidiomycete associates). The ITS1-OF/ITS4-OF primer pair fulfills these objectives. Note that the two versions of the primer (one with a C and a C, one with a T and a T) must be synthesized separately, then mixed, to create the working ITS1-OF primer. If degenerate primers are synthesized with both bases in the two variable positions (i.e. producing C-T and T-C forms), serious primer-dimer artifacts are likely. Lastly, we wish to emphasize that this primer pair was designed to perform well with as wide a spectrum of Basidiomycota as possible, but was not designed to exclude other Eumycota. On the other hand, they are not a perfect fit to all Eumycota, and so should be used with caution in any studies focusing on, for example, the Ascomycota.

Tulasnella ITS primer: ITS4-Tul

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 distribution 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. calospora* 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.

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Thelephoraceae ITS primers: SSU1318-Tom and LSU-Tom4

Some mycoheterotrophic orchids, including *Cephalanthera austinae* (Taylor & Bruns, 1997) and *Corallorhiza 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*, *Chalara*, a wide variety of zygomycetes including *Rhizopus* and *Mucor*, and the basidiomycetes *Bensingtonia*, *Donkioporia*, *Kondoa*, and *Sporobolomyces*, and the following members of the Thelephoraceae: *Bankera*, *Boletopsis*, *Hydnellum*, *Phellodon*, *Polyozellus*, *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 *Piedraria* (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 order-specific 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; Shefferson *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 Ceratobasidiaceae 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. cornigerum* complex, as was predicted by Roberts (Roberts, 1998a), but is interesting

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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 '*Ceratorhiza 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 *Uthatobasidium 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 putative taxa such as *T. calospora*, *T. violea* and *C. cornigerum*. 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 we have 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 Tulasnellales with accelerated evolution, has recently been described by Lynch & Thorn (2006). Because a few orchids, such as *Epipactis helleborine*, have recently been shown to associate predominantly with the Ascomycota genus *Tuber* (Bidartondo *et al.*, 2004; Selosse *et al.*, 2004), we also recommend screening orchids with the previously published primers ITS1-F and ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993). The Basidiomycota-specific primer ITS4-B (Gardes & Bruns, 1993) is a poor choice for orchid mycorrhizal work, since many species of the Tulasnellaceae and Sebacinaceae amplify poorly with this primer (Taylor *et al.*, 2002, 2003).

The other primers described here should prove useful in targeting particular orchid-associated fungal lineages to explore their distribution and ecological dynamics in the environment. Members of the *Tomentella* + *Thelephora* clade within the Thelephoraceae have proven to be dominant players in many ectomycorrhizal communities based upon below-ground, molecular studies (Gardes & Bruns, 1996b; Horton & Bruns, 1998; Jonsson *et al.*, 1999, 2000; Stendell *et al.*, 1999; Taylor & Bruns, 1999; Tedersoo *et al.*, 2003; Parrent *et al.*, 2006). Hence, the SSU1318-Tom/LSU-Tom4 primer pair, which shows very high specificity to a core group of *Tomentella* + *Thelephora* species, may prove useful in a wider field of research than orchid mycorrhizas alone. Quickly screening ECM roots for this *Tomentella* + *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. 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, ITS3Seb, 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.

Acknowledgements

Fruitbodies, fungal isolates or identifications were kindly provided by Robert Bandoni, Egon Horak, Michael Larsen (in memoriam), Gary Laursen and Ken Wells. We are grateful for DNA extracts provided by Seth Adams, József Geml, John Haight, David Hibbett, Sarah Hopkins, Jack McFarland and

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Lori Neufeld. Financial support was provided by grants DEB-0523092 and DEB-0316523 from the National Science Foundation.

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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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