Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon-specific primers

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Summary

• Ectomycorrhizal (ECM) symbiosis is a widespread plant nutrition strategy in Australia, especially in semiarid regions. This study aims to determine the diversity, community structure and host preference of ECM fungi in a Tasmanian wet sclerophyll forest.

• Ectomycorrhizal fungi were identified based on anatomotyping and rDNA internal transcribed spacer (ITS)-large subunit (LSU) sequence analysis using taxon-specific primers. Host tree roots were identified based on root morphology and length differences of the chloroplast trnL region.

• A total of 123 species of ECM fungi were recovered from root tips of *Eucalyptus regnans* (Myrtaceae), *Pomaderris apetala* (Rhamnaceae) and *Nothofagus cunninghamii* (Nothofagaceae). The frequency of two thirds of the most common ECM fungi from several lineages was significantly influenced by host species. The lineages of *Cortinarius*, *Tomentella–Thelephora*, *Russula–Lactarius*, *Clavulina*, *Descolea* and *Laccaria* prevailed in the total community and their species richness and relative abundance did not differ by host species.

• This study demonstrates that strongly host-preferring, though not directly specific, ECM fungi may dominate the below-ground community. Apart from the richness of *Descolea*, *Tulasnella* and Helotiales and the lack of *Suillus–Rhizopogon* and *Amphinema–Tylospora*, the ECM fungal diversity and phylogenetic community structure is similar to that in the Holarctic realm.

Key words: biogeography, community structure of ectomycorrhizal (ECM) fungi, *Eucalyptus grandis* (Myrtaceae), host specificity, *Nothofagus cunninghamii* (Nothofagaceae), *Pomaderris apetala* (Pomaderreae, Rhamnaceae), temperate rain forest.

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Introduction

Ectomycorrhizal (ECM) symbiosis plays an important role in nutrient cycling in many Australian ecosystems (Ashford & Allaway, 1982; Reddell & Milnes, 1992; Reddell *et al.*, 1999; Tommerup & Bougher, 1999). The semiarid Australian flora includes a substantial diversity of ECM host plants, including Leptospermoideae, Mimosoideae, Papilionoideae, Pomaderreae, members of Goodeniaceae, Asteraceae, Casuarinaceae, Euphorbiaceae, etc. (Pryor, 1956; Warcup, 1980; Kope & Warcup, 1986; Bellgard, 1991; Brundrett & Abbott, 1991; Reddell & Milnes, 1992). The closest relatives of these plants often form exclusively arbuscular mycorrhiza in other continents (Ducousso & Thoen, 1991; Reddell & Milnes, 1992).

Semiarid sclerophyll habitats are the most widespread ecosystems in Australia and thus, much of the mycorrhizal research has focused on these habitats. The ecology of ECM fungi in moist coastal and submontane forests has been relatively little studied (Reddell *et al.*, 1999), although rain forest habitats prevailed before 30–25 million yr ago (Mya). The opening of the Tasman sea and rapid northward movement of the Australian continent affected the ocean currents, which in turn caused progressive global cooling and drying particularly in the Southern Hemisphere (Crisp *et al.*, 2004; Hill, 2004). From 25 Mya, these changing climatic conditions have enabled the evolution and radiation of present-day sclerophyll communities from rain forest-inhabiting ancestors (Ladiges *et al.*, 2003, 2005; Steane *et al.*, 2003; Crisp *et al.*, 2004). Wet forests dominated by gymnosperms and ECM *Nothofagus* were successively replaced by sclerophyll and scrub vegetation dominated by ECM Leptospermoideae (including *Eucalyptus*), Casuarinaceae and Mimosaceae (Crisp *et al.*, 2004; Hill, 2004). Therefore, certain *Nothofagus*-associated ECM fungi were hypothesized to have switched to Leptospermoideae and other plants following major changes in vegetation (Horak, 1983; Bougher & Malajczuk, 1985; Bougher *et al.*, 1994).

Accumulating information from fungal fruit-body surveys suggests that Australian ECM fungi are highly diverse (May & Simpson, 1997; Bougher & Lebel, 2001; Glen *et al.*, 2008), comprising an estimated number of 6500 species (Bougher, 1995). Recent studies on soil mycelia support such high estimates of ECM diversity in New South Wales and Queensland (Bastias *et al.*, 2006; Midgley *et al.*, 2007). Extensive fruit-body surveys involving epigeous or hypogeous fruiting taxa reveal that the ECM lineages of *Cortinarius*, *Descolea*, and *Russula–Lactarius* are the most species rich (Claridge *et al.*, 1999; Lu *et al.*, 1999; Bougher & Lebel, 2001; Gates *et al.*, 2005; Ratkowsky & Gates, 2005), whereas the *Russula–Lactarius* and *Tomentella–Thelephora* lineages, followed by *Cortinarius* and *Inocybe*, dominate soil mycelial communities (Bastias *et al.*, 2006; Midgley *et al.*, 2007).

Many stipitate, 'agaricoid' genera comprise a large number of secotioid or hypogeous-fruiting members in Australia (Bougher & Lebel, 2001). A number of these Australian hypogeous taxa have been described as entirely new genera or families (e.g. Trappe *et al.*, 1996). Their high abundance in Australian semiarid woodlands has been attributed to seasonal climate and coevolution with small marsupials that consume and distribute these taxa (Johnson, 1996; Trappe & Claridge, 2005). Surprisingly, most nonhypogeous Australian ECM fungal genera are shared with the Holarctic realm, indicating vicariance/dispersal events (May & Simpson, 1997). Watling (2001) hypothesized that many ECM boletes may have followed the migrating vegetation from Indo-Malay and New Guinea to Australia via Pleistocene and earlier land bridges, which explains their wide distribution both in Southeast Asia and Australia. Conversely, other fungal parasites and symbionts are shared between *Nothofagus* forests in New Zealand and southern South America, suggesting ancient vicariant distribution or more recent long-distance dispersal (Bougher *et al.*, 1994; Watling, 2001; Moyersoen *et al.*, 2003). Certain taxa such as Mesophelliaceae (Trappe *et al.*, 1996), *Descolea* (Bougher & Malajczuk, 1985) and *Rozites* (Bougher *et al.*, 1994) are far more diverse in Australia compared with other continents, suggesting their Australian origin.

The most common ECM fungi are usually associated with multiple host plants in the Holarctic realm (Horton & Bruns, 1998; Kennedy *et al.*, 2003). Exceptions include the closely related, Pinaceae-specific genera *Suillus* and *Rhizopogon* (the Boletaceae–Sclerodermataceae lineage; Molina & Trappe, 1982) as well as ECM symbionts of *Alnus* (Betulaceae; Molina, 1979). Both *Alnus* and Pinaceae are absent from the Australian indigenous flora. Most *in vitro* ECM synthesis experiments suggest that Australian plants and fungi associate with multiple symbiotic partners (Chilvers, 1973; Warcup, 1980, 1990; Kope & Warcup, 1986; Reddell *et al.*, 1999; but see Malajczuk *et al.*, 1982). By contrast, Chambers *et al.* (2005) argued that *Pisonia grandis* R. Br. (Nyctaginaceae) forms specific ECM associations with just two *Tomentella–Thelephora* spp. in nutrient-rich soils of coral cays in the Great Barrier Reef. Australian native fungi are usually incompatible with the introduced pines (Chilvers, 1973; Malajczuk *et al.*, 1982), but colonize European hardwoods (Diez, 2005). Similarly, several fungal taxa native to African hardwoods or American conifers form ECM with eucalypts (Malajczuk *et al.*, 1982; Tedersoo *et al.*, 2007; but see Chen *et al.*, 2007).

Based on the history of Australian vegetation, we hypothesize that ECM fungal communities are highly diverse and lack host specificity in a Tasmanian wet sclerophyll forest that comprises both temperate rain forest and true sclerophyll elements. This study further aims to uncover the relative importance of host species (i.e. direct root association) and host vicinity (the identity of nearest host tree) effects on ECM fungal community structure. Combining anatomotyping and sequencing, we demonstrate that the ECM fungal community of this forest is species-rich, phylogenetically diverse and substantially influenced by host trees.

Materials and Methods

Study site

Sampling was performed at Tall Trees Walk, Mt Field National Park, Tasmania (geocode 42°40.9′S, 146°42.2′E) in August, 2006. Mt Field National Park has a long history of conservation and recreational management, being first reserved in 1885 and proclaimed a national park in 1917. The vegetation of the study site forms a tall open forest with no logging history. *Eucalyptus regnans* F. Müll. (ECM host) forms a canopy at approx. 60 m. The subdominant canopy layer consists of *Pomaderris apetala* Labill. (ECM host), *Acacia*

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verniciflua A. Cunn. (Confirmed nonECM at this site), *Nothofagus cunninghamii* (Hook.) Oerst (ECM host), *Atherosperma moschatum* Labill., *Olearia argophylla* (Labill.) Benth. and a few *Acacia melanoxylon* R. Br. (Putative ECM host). The understorey is covered by tree ferns (*Dicksonia antarctica* Labill.), *Pittosporum bicolor* Hook. and *Coprosma quadrifida* (Labill.) Rob. Forest floor is covered by ferns, including *Histiopteris incisa* (Thunb.) J. Sm., *Hypolepis rugosula* (Labill.) J. Sm. and *Blechnum* spp., and various bryophytes. Decaying boles and branches of all decomposition stages are abundant. Soils are derived from Permian mudstone and siltstone parent material and are deep gradational clay loam over light brown clay. The mean annual rainfall averages 1224 mm and mean daily minimum and maximum temperatures range between 5.3°C and 16.2°C (Maydena Post Office Station # 095063, 1992–2004).

Three 1-ha plots were established 100–500 m apart in sites where at least three ECM host trees – *E. regnans*, *N. cunninghamii* and *P. apetala* – co-occurred. Plots 1 and 2 were situated on a south-easterly aspect of a slope of approx. 1–5°, whereas plot 3 was situated on steep (slope 10–25°), eastern and western banks of a stream. In plots 1 and 2, all three hosts grew mixed, whereas in plot 3, *N. cunninghamii* and *E. regnans* inhabited low river banks and elevated sites, respectively. From each plot, five root samples (15 × 15 cm to 5 cm depth) were collected from 0.2–1.5 m distance to trunks of each three host species. Roots were separated from remaining soil particles in tap water. After careful examination, ECM roots from each sample were sorted by plant species based on colour, ramification pattern and thickness. Using a stereomicroscope, ECM morphotypes were distinguished based on colour, roughness of mantle surface, occurrence of rhizomorphs, emanating hyphae and cystidia. The ECM root tips were assigned to morphotypes and scored for relative abundance (percentage of all ECM root tips) on each putative host species separately. Several ECM clusters of each morphotype were mounted into 1% CTAB (cetyltrimethylammonium bromide) DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 1% CTAB) for storage and transportation. Roots were processed within 5 d of collection. Several CTAB-stored root tips from each morphotype per host were further anatomotyped. To improve precision, anatomotypes were determined in each plot separately. One or more root tips of each anatomotype per host and plot were carefully cleaned from adhering debris, dense clumps of extraradical mycelium and rhizomorphs, and frozen until molecular analyses.

Molecular analyses

The DNA extraction was performed using a High Pure polymerase chain reaction (PCR) Template Preparation Kit for Isolation of Nucleic Acids from Mammalian Tissue (Roche Applied Science, Indianapolis, IN, USA) as outlined in Tedersoo (2007). Microscopical examination revealed frequent ascomycete hyphae in the ECM mantle and PCR often resulted in multiple or no product using universal fungal primers. Therefore, new taxon-specific primers were developed in rDNA nuclear large subunit (nLSU) to selectively amplify the putative ECM mycobiont. All primers were manually designed based on a common alignment comprising plant, ascomycete and basidiomycete sequences and to perform specifically at 55°C (calculated TM 56–60°C).

The rDNA internal transcribed spacer (ITS) and ca. 350 bp of nLSU were amplified using a primer ITS1F (5′-cttggtcatttagaggaagtaa-3′) in combination with a newly designed basidiomycete-specific primer LB-W (5′-cttttcatctttccctcacgg-3′) or ascomycete-specific primer LA-W (5′ cttttcatctttcgatcactc 3′) following the protocol of Tedersoo (2007). *Tomentella–Thelephora*, *Sebacina* and *Tulasnella* morphotypes were amplified using a primer ITS1F or ITS5 (5′-ggaagtaaaagtcgtaacaagg-3′) in combination with newly designed primers LR5-Tom (5′-ctaccgtagaaccgtctcc-3′), LR5-Seb (5′-attcgcttaccgcacaagg-3′) or LR3-Tul (5′-bactcgcatgcaaggtgca-3′), respectively. Later, ITS1F was substituted by another primer 1–2 bp downstream (5′-acttggtcatttagaggaagt-3′) that apparently differs from the recently published primer ITS0F-T (Taylor & McCormick, 2008) by lacking a single nucleotide in the 5′-terminus and is similarly experimentally proven to amplify fungi, including Tulasnellales. The nLSU was amplified using a primer LR0R (5′-acccgctgaacttaagc-3′) in combination with newly designed basidiomycete-specific primers LB-Y (5′-tttgcacgtcagaatcgcta-3′) or LB-Z (5′-aaaaatggcccactagaaact-3′), ascomycete-specific primer LR3-Asc (5′-cacytactcaaatccwagcg-3′) or fungal-specific primer LR5-F (5′-cgatcgatttgcacgtcaga-3′). All these primers are characterized in Fig. 1 and the Supporting Information Text S1. Since the ITS and nLSU of several anatomotypes could not be amplified, mitochondrial LSU and nuclear rDNA Small Subunit amplifications were attempted instead, using a combination of several primers, but these recovered just a few extra species.

The PCR products were checked on 1% agarose gels under UV-light and purified using Exo-Sap enzymes (Sigma, St Louis, MO, USA). Sequencing was performed using primers ITS4 (5′-tcctccgcttattgatatgc-3′), ITS5 and/or a newly designed universal sequencing primer LF340 (5′-tacttgtkcgctatcgg-3′) for the ITS region; ctb6 (5′-gcatatcaataagcggagg-3′) and/or LR5 (5′-tcctgagggaaacttcg-3′) for the nLSU. Contigs were assembled using Sequencher 4.7 (GeneCodes Corp., Ann Arbor, MI, USA). A value of 97.0% ITS region identity (excluding flanking 18S and 28S rDNA sequences) was used as a DNA barcoding threshold (molecular species criterion; Tedersoo *et al.*, 2003). For *Cortinarius* and *Laccaria*, a 98.0% threshold was used instead, because the ITS region is more conserved in these genera (Frøslev *et al.*, 2005; L. Tedersoo, pers. obs.). All unique ITS sequences were submitted to UNITE (Kõljalg *et al.*, 2005) and the European Molecular Biology Laboratory (EMBL) databases. BLASTn searches were

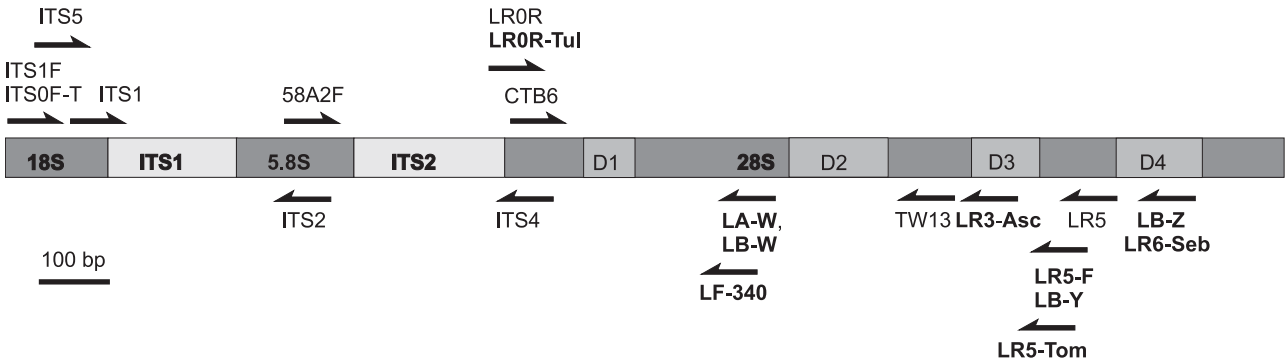


Fig. 1 Relative positions of rDNA internal transcribed spacer (ITS) and nuclear large subunit (nLSU) primers used in this study. Newly designed primers are indicated in bold type.

performed against public sequence databases NCBI, etc., and UNITE to provide as precise identification for the ECM fungi as possible.

To confirm the identity of a host tree, the plastid *trnL* region of root tip DNA was amplified using primers trnC (5′-cgaaatcggtagacgctacg-3′) and trnD (5′-ggggatagagggactt-gaac-3′). Two or more root tips per putative host and soil sample were checked to confirm host identification. Size differences of the trnL region, as revealed from 1% agarose gels, effectively distinguished the three host trees from each other (not shown).

Statistical analyses

Using a computer program ESTIMATES ver. 8 (Colwell, 2006), sample-based rarefaction curves and minimal total species richness estimates Chao2, Jackknife2 and ACE were calculated for each host species and the whole community. In these analyses, root samples were used as sampling units and fungal species were sampled randomly without replacement.

To study the effects of host species and host vicinity on frequency of ECM fungal species, Fisher's Exact tests were calculated at significance level $\alpha = 0.05$. Differences in species richness (square root-transformed) and relative abundance (arcsine square root-transformed) of ECM fungal lineages by host species were tested using ANOVA. To control false discovery rate and reduce familywise error rate associated with multiple testing, a sharpened procedure of Benjamini–Hochberg correction was used instead of classical Bonferroni correction, as implemented in Verhoeven *et al.* (2005). The combination of these tests required at least three and four observations of each species to obtain statistically significant results for studying host species and host vicinity effects, respectively.

Using PC-ORD ver. 5.04 (McCune & Mefford, 2006), Detrended correspondence analysis (DCA) was employed to unravel the effects of host species, host vicinity and plot on ECM fungal community structure. Root samples with binary-encoded species data and downweighting rare species were used in the analysis. All factors were transformed to dummy variables because of multiple levels and unordered status.

Results

Combining DNA barcoding and anatomotyping, 123 taxa of putative ECM fungi were recovered from root tips of the three host species (see the Appendix). Among these, six distinct anatomotypes remained unamplified using various primers on several nuclear and mitochondrial DNA regions. Certain species of *Tulasnella*, *Endogone* and *Amanita* proved most difficult to amplify. Many additional sequence types identified as members of Helotiales or Sordariales of the ascomycetes were recovered from ECM root tips, but excluded from the analyses, because they were considered root endophytes or saprobes. These 'contaminants' were separated from putatively ECM Helotiales by comparing mantle anatomy and phylogenetic affinities (not shown).

Neither rarefaction curves nor minimal species richness estimates (except Chao2) approached an asymptote with increasing sample size (Fig. 2), indicating the need for enhanced sampling effort. Of the estimators, Chao2 produced more stable and consistent estimates than Jackknife2. The total ECM root-associated fungal community at Tall Trees Walk was estimated to comprise between 210 (Chao2) and 247 (ACE) species (Fig. 2).

The ECM fungal community comprised a few frequent species and a large number of rare species (Fig. 3). In particular, 68 (55.3%) of the taxa were found only once. *Laccaria* sp1 and *Lactarius eucalypti* were the most frequent, colonizing 44.4% and 42.2% of root samples, respectively. *Cortinarius* (incl. hypogeous members; 28 spp.), *Tomentella–Thelephora* (18 spp.), *Russula–Lactarius* (10 spp.), *Clavulina* (9 spp.), *Descolea* (including *Setchelliogaster* and *Descomyces*; 8 spp.) and *Laccaria* (5 spp.) were the most species-rich lineages of ECM fungi. Of ascomycetes, both *Cenococcum* sp., members

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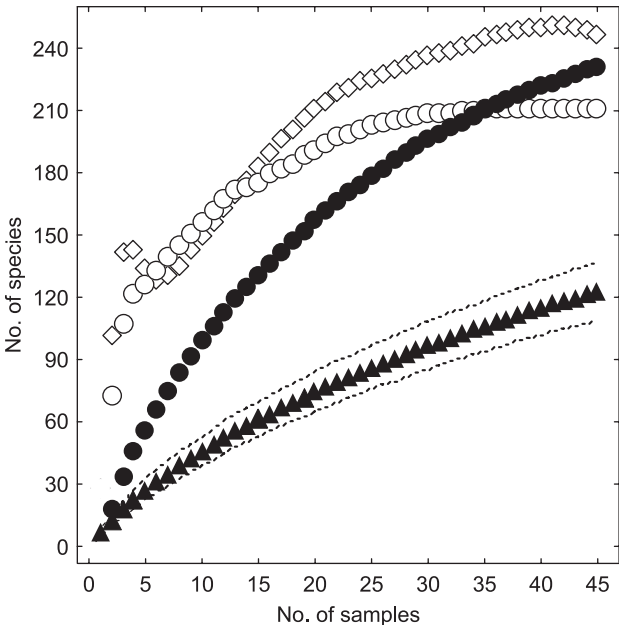


Fig. 2 Rarefaction curve (triangles), its 95% confidence intervals (dotted lines); Jackknife2 (closed circles), Chao2 (open circles) and ACE (open diamonds) minimal species richness estimates of ectomycorrhizal fungi in the study site by increasing sample size.

of Pezizales (2 spp.) and Helotiales (5 spp.) were detected as ECM symbionts, but, in total, they colonized just 9.0% of root tips.

All three host tree species supported multiple ECM fungi and displayed no significant specificity for any fungal lineage in terms of species richness or abundance on root tips (see the Supporting Information, Fig. S1a,b). Based on rarefaction curves, roots and soil of *P. apetala* supported more species of fungi compared to *E. regnans* and *N. cunninghamii*, but overlapping confidence intervals suggested that this effect was nonsignificant (not shown).

The strongly overlapping host species and host vicinity effects of *P. apetala* respectively contributed 69.3% and 55.7% to the primary axis of DCA (eigenvalue 0.80) that explained 14.4% of variance in species data (see the Supporting Information, Fig. S2). The DCA main axis effectively separated ECM fungal communities of *P. apetala* root tips from these of *E. regnans* and *N. cunninghamii*. Based on arrow length, the combined host effects appeared more important than plot effect in explaining the ECM fungal community structure.

Most ECM fungi (56.4%) that were observed more than once colonized root tips of a single host species (Fig. 3). Differential presence of species on root tips and in the vicinity of host trees and plots could be statistically tested in 28, 19 and 19 of the species, respectively, given the integrated power of Fisher's exact test and sharpening of the reduced familywise error rate. The frequency of 10 species (52.6%) was significantly different in the vicinity of a certain host tree, whereas 19 (65.5%) species were significantly affected by roots of a host plant (Fig. 3). *Pomaderris apetala*, *E. regnans* and *N. cunninghamii* accounted for significant differences in 13, 4 and 2 of these host-biased fungal species, respectively. Host species effect was substantially stronger than host vicinity effect in 12 out of 15 cases (Fisher's exact test: $n = 12$, $P = 0.030$; see the Appendix). Plot had no significant effect on any species.

Discussion

The ECM fungi formed a phylogenetically diverse community in a Tasmanian wet sclerophyll forest that compares well with Holarctic ecosystems (Horton & Bruns, 2001; Richard *et al.*, 2005; Tedersoo *et al.*, 2006; Ishida *et al.*, 2007). The minimal species richness estimates of 210–247 spp. are probably strong underestimates of the local community richness, because richness estimators and rarefaction curves had not begun to approach an asymptote and the low sample size. *Cortinarius*, *Tomentella–Thelephora*, *Russula–Lactarius*, *Clavulina*, *Descolea* and *Laccaria* lineages were the most species-rich and abundant in the ECM fungal community of Tall Trees Walk in Tasmania. Except for *Descolea*, these lineages also form a substantial part of the Holarctic ECM fungal communities (Horton & Bruns, 2001; Izzo *et al.*, 2005; Tedersoo *et al.*, 2006; Ishida *et al.*, 2007). In paleotropical ecosystems, the Boletaceae–Sclerodermataceae (represented by two rare species in this study), *Tomentella–Thelephora* and *Russula–Lactarius* account for the majority of ECM fungal taxa (Sirikintaramas *et al.*, 2003; Riviere *et al.*, 2007; Tedersoo *et al.*, 2007), whereas *Cortinarius*, *Descolea* and *Laccaria* are uncommon (Peintner *et al.*, 2003 but see Onguene, 2000; Tedersoo *et al.*, 2007). In the Holarctic realm, individual species of *Cortinarius* and *Laccaria* usually occur below ground in low abundance and frequency owing to small genetic individuals and highly clumped distribution of ECM root tips (Gherbi *et al.*, 1999; Genney *et al.*, 2006). In Tasmania, however, *Laccaria* and *Cortinarius* were among the most abundant ECM taxa, colonizing, respectively 23.8%, and 10.9% of ECM root tips in this study, and 47% and 15% in the forest floor of an old-growth *Nothofagus* forest in Victoria state (Tedersoo, 2007). This phenomenon suggests that these lineages may have different ecological roles and importance compared with Holarctic ecosystems. The ECM fungal lineages of *Descolea* (Bougher & Malajczuk, 1985), *Tulasnella* and members of Helotiales (except *Meliniomyces bicolor*) commonly observed in Tasmania are seldom recorded in the Northern Hemisphere. The putatively ECM-forming species of Helotiales are closely related with many endophytic and/or ericoid mycorrhizal taxa (Vrålstad *et al.*, 2002; Hambleton & Sigler, 2005) that rendered their distinction ambiguous solely based on DNA sequence data. Consistent features in mantle anatomy enabled

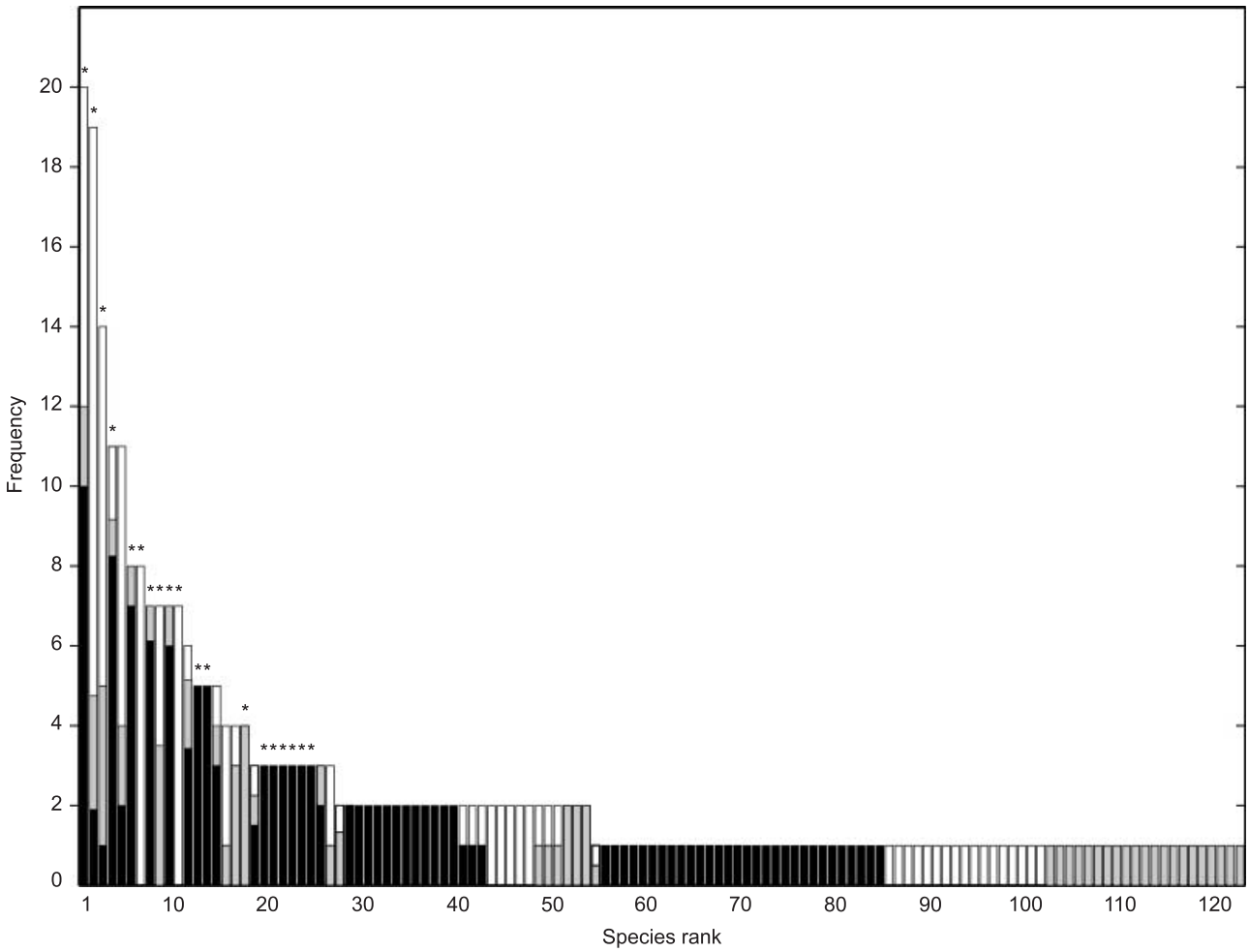


Fig. 3 Ranked frequency of ectomycorrhizal fungi on three host species in the study site. Differential shading demonstrates the weighted proportion of each host species (open columns, *Eucalyptus regnans*; shaded columns, *Nothofagus cunninghamii*; closed columns, *Pomaderris apetala*). Asterisks denote statistically significant host-biased frequency according to Fisher's Exact test and sharpened Benjamini–Hochberg corrections (Verhoeven *et al*., 2005). Species ranks are encoded in the Appendix.

us to separate these putative ECM taxa from root endophytes and saprobes, highlighting the importance of integrating these approaches when studying poorly known taxa such as Ceratobasidiaceae, Sebacinaceae, Tulasnellaceae and various ascomycetes that comprise closely related ECM and other root associated taxa. Based on this unreplicated study site, it is unwise to conclude on the absence of certain ECM lineages in Tasmanian wet sclerophyll forests or Australia in general. Indeed, two additional lineages, *Elaphomyces* and Sordariales, were found from Victoria despite the substantially lower sampling effort (Tedersoo, 2007). Nevertheless, the *Suillus–Rhizopogon* group of the Boletaceae–Sclerodermataceae lineage and *Amphinema–Tylospora* (Atheliales), which are relatively abundant in the Holarctic realm (Taylor *et al.*, 2000; Horton & Bruns, 2001), were not observed below ground in Tasmania or Victoria. In agreement with this, none of these taxa are reported as native to Australia (May & Simpson, 1997).

Root morphology combined with length difference of chloroplast *trnL* region was successfully employed to distinguish the three host species below ground. Because most samples comprised roots of a single host species that grew the closest, separation of host root and host vicinity effects proved difficult using both ordination and statistical methods. Nonetheless, statistical analyses of individual species revealed that host preference was consistently stronger than host vicinity effect for individual species and the whole community. As suggested by DCA and the frequency of individual species, the ECM community of *P. apetala* differed from *E. regnans* and *N. cunninghamii* even in the vicinity of another host. Similar effects of host species on the ECM fungal community have been demonstrated in mixed forests of Japan (Ishida

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et al., 2007) and woodlands of California (Morris *et al.*, 2008). Ishida *et al.* (2007) demonstrated that host effects on ECM community are stronger with increasing taxonomic distance and successional status of hosts.

Unlike previous studies, as many as 66% of the common ECM fungal species displayed statistically significant host preference, although exclusive specificity was less common among the most frequent species. Similarly, Ishida *et al.* (2007: 430) concluded that 'a significant proportion of ECM fungi exhibited host specificity', demonstrating that eight of 55 common species (15%) had statistically significantly biased host preference in a Japanese mixed forest. In the Holarctic realm, strictly host-specific taxa are usually restricted within a few fungal lineages (e.g. *Alnicola*, *Leccinum*, *Rhizopogon*, *Suillus* and *Lactarius* sect. *Dapetes*), whereas lineages of *Laccaria*, *Tomentella–Thelephora*, *Cenococcum* and *Clavulina* usually comprise promiscuous species (Horton & Bruns, 1998; Kennedy *et al.*, 2003; Richard *et al.*, 2005; Nara, 2006; Ishida *et al.*, 2007; Morris *et al.*, 2008; Tedersoo *et al.*, 2008).

Host preference at any taxonomic level may provide new niches and hence support higher local species richness (Ishida *et al.*, 2007), whereas host generalist ECM fungi are considered important drivers of forest succession by facilitating seedling establishment of late-successional host trees (Horton *et al.*, 1999; Kennedy *et al.*, 2003; Dickie *et al.*, 2004; Richard *et al.*, 2005; Nara, 2006). Following this hypothesis, *P. apetala* and *E. regnans*, both pioneer, fire-dependent tree species may effectively exclude each other through priority effect and hardly compatible ECM symbionts. Kope & Warcup (1986) reported no apparent host specificity of various Australian ECM plants (Pomaderreae and *Nothofagus* not studied) and fungi from early successional habitats in pure culture synthesis trials. Late-successional *N. cunninghamii* may be facilitated especially by shared ECM symbionts of eucalypts.

The ECM fungi from different lineages had biased association with *P. apetala*. In agreement, a computer program UNIFRAC (Lozupone & Knight, 2005) revealed no significant phylogenetic difference in the frequency of ECM fungal species and lineages among the three hosts and plots (T. Suvi & K. Abarenkov, unpublished), indicating repeated, independent evolution towards host preference. The evolution of host preference may depend on ecological or genetic specificities such as substantial differences in phytochemistry and nutrient content between *Pomaderris aspera* Sieber ex DC. and *E. regnans* (Ashton, 1975a,b). Experimental synthesis trials involving more species of *Eucalyptus* and Pomaderreae are required to rule out the plant *species* effects on ECM specificity.

In conclusion, this study provides first evidence for the presence of plant communities with predominately host-biased, but not directly host specific ECM relationships. The underlying causes and mechanisms of this remain unknown, but deserve attention to learn the basic features of biogeography and host shifting in ECM symbiosis.

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Supporting Information

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

Fig. S1 Importance of ectomycorrhizal lineages as root associates of three host species based on (a) total species richness and (b) mean relative abundance on root tips (bars, 95% CI).

Fig. S2 Detrended correspondence analysis (DCA) ordination demonstrating the relative importance of host species, host vicinity and plot effects (arrows) on community composition of ectomycorrhizal fungi.

Text S1 Multiple sequence alignments of newly designed primers (in bold) with target and nontarget nuclear large subunit (nLSU) rDNA sequences

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Appendix Identification and host preference of ectomycorrhizal fungi

			Best BLASTn full-length ITS match		*P*-value of Fisher's exact test		
Taxon	Rank	UNITE accession	Specimen	% identity	Host species	Host vicinity	Plot
Lactarius eucalypti	1	UDB002671	*Lactarius eucalypti* UDB002270	100.0	**0.049**	0.274	0.651
Laccaria sp1	2	UDB002672	*Laccaria laccata* AJ699075	96.1	**< 0.001**	**0.001**	0.026
Descolea sp2	3	UDB002673	*Descolea recedens* AF325649	98.4	**0.002**	**0.027**	1.000
Laccaria sp3	4	UDB002674	*Laccaria laccata* AJ699074	99.0	**0.005**	**< 0.001**	0.311
Russula sp1	5	UDB002675	*Russula cremoricolor* DQ974755	91.0	0.170	**0.014**	0.130
Cenococcum sp	6	UDB002676	*Cenococcum geophilum* (Japan) AB251837	99.6	**0.001**	0.229	0.488
Tomentella sp1	7	UDB002677	*Tomentella fuscocinerea* DQ974776	91.5	**< 0.001**	**< 0.001**	0.488
Tomentella sp9	8	UDB002678	*Tomentella ramosissima* U83480	98.1	**0.001**	0.179	0.688
Tulasnella sp3	9	UDB002679	*Tulasnella eichleriana* AY373292	partial[4]	**0.057**	0.134	0.460
Tomentella sp4	10	UDB002680	*Tomentella lateritia* UDB000268	94.5	**0.004**	**0.010**	1.000
Cortinarius sp2	11	UDB002681	*Cortinarius rotundisporus* AF389127	99.0	**0.001**	**0.010**	0.463
Inocybe sp1	12	UDB002682	*Inocybe* cf. *glabripes* AJ889952	81.8	0.306	0.858	0.858
Clavulina sp4	13	UDB002683	*Clavulina* cf. *cristata* DQ974710	94.6	**0.004**	**0.007**	1.000
Tomentella sp8	14	UDB002684	*Tomentella badia* UDB000961	91.3	**0.004**	**0.007**	0.343
Tomentella sp7	15	UDB002685	*Tomentella stuposa* UDB000967	90.9	0.506	0.594	0.594
Tomentella sp2	16	UDB002686	*Tomentella subclavigera* AY010275	93.8	0.299	0.302	0.524
Sebacina sp4	17	UDB002687	*Sebacina helvelloides* AJ966750	90.5	0.093	0.302	1.000
Helotiales sp2	18	UDB002688	*Leptodontidium elatius* AY781230	86.0	**0.005**	**0.027**	1.000
Clavulina sp6	19	UDB002689	*Clavulina* cf. *cristata* DQ974710	89.0	0.826	0.762	0.096
Unidentified sp3	20	nd[1]	nd	nd	**0.055**	nd	nd
Laccaria sp6	21	UDB002690	*Laccaria laccata* AJ699075	96.0	**0.055**	nd	nd
Hysterangium sp1	22	UDB002691	*Hysterangium cassirhachis* DQ365633	82.2	**0.055**	nd	nd
Russula sp5	23	UDB002692	*Russula adusta* AY061652	89.2	**0.055**	nd	nd
Tomentella sp3	24	UDB002693	*Tomentella stuposa* AY010277	91.6	**0.055**	nd	nd
Tomentella sp10	25	UDB002694	*Tomentella lateritia* UDB000963	92.6	**0.055**	nd	nd
Helotiales sp1	26	UDB002695	*Solenopezia solenia* U57991	84.0	0.375	nd	nd
Cortinarius sp20	27	UDB002696	*Cortinarius delibutus* AY669587	92.2	0.496	nd	nd
Laccaria sp2	28	UDB002697	*Laccaria laccata* AJ699075	97.2	0.276	nd	nd
Unidentified sp2	29	nd	nd	nd	nd	nd	nd
Cantharellus sp1	30	UDB002698	*Craterellus tubaeformis*	partial	nd	nd	nd
Clavulina sp17	31	UDB002699	*Clavulina* cf. *cristata* DQ974710	75.9	nd	nd	nd
Clavulina sp1	32	UDB002700	*Clavulina* cf. *cristata* DQ974710	82.5	nd	nd	nd
Hydnum sp	33	UDB002701	*Hydnum albidum* AJ534709	70.2	nd	nd	nd
Cortinarius sp6	34	UDB002702	*Cortinarius teraturgus* AF389151	94.3	nd	nd	nd
Cortinarius sp19	35	UDB002703	*Cortinarius cephalixus* AY174784	92.2	nd	nd	nd
Inocybe sp5	36	UDB002704	*Inocybe* cf. *glabripes* AJ889952	81.3	nd	nd	nd
Piloderma sp2	37	UDB002705	*Piloderma byssinum* DQ365683	86.5	nd	nd	nd
Russula sp8	38	UDB002706	*Russula chloroides* AF418604	86.4	nd	nd	nd
Sebacina sp1	39	UDB002707	*Sebacina helvelloides* AJ966749	89.3	nd	nd	nd
Tomentellopsis larsenii	40	UDB002708	*Tomentellopsis larsenii* AF326980	99.2	nd	nd	nd
Helotiales sp5	41	UDB002709	*Hymenoscyphus immutabilis* AY348584	85.3	nd	nd	nd
Cortinarius sp9	42	UDB002710	*Cortinarius canthocephalus* UDB000674	95.0	nd	nd	nd
Pezizaceae sp1	43	UDB002711	*Terfezia arenaria* AF276674	76.7	nd	nd	nd
Cortinarius sp11	44	UDB002712	*Cortinarius obtusus* UDB000127	94.6	nd	nd	nd
Descolea sp3	45	UDB002713	*Descolea maculata* DQ192181	99.8	nd	nd	nd
Endogone sp	46	UDB002714	*Endogone pisiformis* AF006511	partial	nd	nd	nd
Inocybe sp2	47	UDB002715	*Inocybe lacera* AB211269	78.5	nd	nd	nd
Russula sp2	48	UDB002716	*Russula nigricans* AY061695	86.3	nd	nd	nd
Helotiales sp4	49	UDB002717	*Hyphodiscus hymenophilus* DQ227258	82.5	nd	nd	nd
Boletus sp1	50	UDB002718	*Boletus amygdalinius* DQ974705	78.2	nd	nd	nd
Laccaria sp4	51	UDB002719	*Laccaria amethystina* UDB001492	94.3	nd	nd	nd
Cortinarius sp8	52	UDB002720	*Cortinarius teraturgus* AF389151	96.0	nd	nd	nd
Cortinarius sp13	53	UDB002721	*Cortinarius cystideocatenatus* AY669651	94.8	nd	nd	nd
Descolea sp1	54	UDB002722	*Descolea phlebophora* AF325656	100.0	nd	nd	nd
Russula sp3	55	UDB002723	*Russula nigricans* AM113960	87.8	nd	nd	nd
Unidentified sp1	56	nd	nd	nd	nd	nd	nd
Unidentified sp4	57	nd	nd	nd	nd	nd	nd
Unidentified sp5	58	nd	nd	nd	nd	nd	nd

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Appendix *continued*

Taxon	Rank	UNITE accession	Best BLASTn full-length ITS match		*P*-value of Fisher's exact test		
			Specimen	% identity	Host species	Host vicinity	Plot
Thelephorales sp	59	UDB002724	Thelephorales sp AJ509798[2]	93.9	nd	nd	nd
Helotiales sp3	60	UDB002725	*Leohumicola minima* AY706329	89.9	nd	nd	nd
Clavulina sp2	61	UDB002726	*Clavulina* cf. *cristata* DQ974711	83.4	nd	nd	nd
Cortinarius sp1	62	UDB002727	*Cortinarius firmus* AF389163	86.8	nd	nd	nd
Cortinarius sp14	63	UDB002728	*Quadrispora tubercularis* DQ328113	92.7	nd	nd	nd
Cortinarius sp17	64	UDB002729	*Cortinarius olivaceobubalinus* AF539736	97.5	nd	nd	nd
Cortinarius sp21	65	UDB002730	*Quadrispora tubercularis* DQ328113	95.1	nd	nd	nd
Cortinarius sp24	66	UDB002731	*Cortinarius ombrophilus* AF389149	90.8	nd	nd	nd
Cortinarius sp34	67	UDB002732	*Cortinarius walkeri* AY669632	93.8	nd	nd	nd
Cortinarius sp35	68	UDB002733	*Cortinarius cannarius* AY669630	95.4	nd	nd	nd
Descolea sp5	69	UDB002734	*Descomyces albus* DQ328209	98.1	nd	nd	nd
Descolea sp6	70	UDB002735	*Descolea* sp. AF325658	100.0	nd	nd	nd
Inocybe sp3	71	UDB002736	*Inocybe lanuginsa* DQ367905	78.8	nd	nd	nd
Lactarius sp3	72	UDB002737	*Lactarius serifluus* AY332558	90.6	nd	nd	nd
Piloderma sp1	73	UDB002738	*Piloderma fallax* AY010282	85.1	nd	nd	nd
Piloderma sp3	74	UDB002739	*Piloderma fallax* DQ179125	86.0	nd	nd	nd
Hysterangium sp2	75	UDB002740	*Hysterangium cassirhachis* DQ365632	80.8	nd	nd	nd
Gautiera sp	76	UDB002741	*Gautiera caudata* AF377057	partial	nd	nd	nd
Sebacina sp2	77	UDB002742	*Sebacina helvelloides* AJ966750	90.0	nd	nd	nd
Sebacina sp3	78	UDB002743	*Sebacina* sp. AF440664	88.3	nd	nd	nd
Tomentella sp5c	79	UDB002744	*Tomentella lilacinogrisea* UDB000953	89.8	nd	nd	nd
Tomentella sp5e	80	UDB002745	*Tomentella fuscocinerea* DQ974776	93.1	nd	nd	nd
Tomentella sp11	81	UDB002746	*Tomentella cinerascens* UDB000232	96.2	nd	nd	nd
Tomentella sp12	82	UDB002747	*Tomentella stuposa* UDB000965	94.8	nd	nd	nd
Tricholoma sp	83	UDB002748	*Tricholoma scalpturatum* AF377201	85.3	nd	nd	nd
Tulasnella sp1	84	UDB002749	*Tulasnella tomaculum* AY373296	partial	nd	nd	nd
Tulasnella sp2	85	UDB002750	*Tulasnella violea* AY373293	partial	nd	nd	nd
Tulasnella sp5	86	nd	nd	nd	nd	nd	nd
Amanita sp	87	UDB002751	*Amanita* sp. AM117682[2]	96.2	nd	nd	nd
Boletus sp2	88	UDB002752	*Xerocomus chrysonemus* DQ066378	82.1	nd	nd	nd
Clavulina sp3	89	UDB002753	*Clavulina* cf. *cristata* DQ974712	94.1	nd	nd	nd
Clavulina sp7	90	UDB002754	*Clavulina* cf. *cristata* DQ974710	84.0	nd	nd	nd
Coltriciella sp	91	UDB002755	*Coltriciella dependens* AM412252	84.5	nd	nd	nd
Cortinarius sp4	92	UDB002756	*Cortinarius badiovinaceus* UDB002221	92.7	nd	nd	nd
Cortinarius sp5	93	UDB002757	*Cortinarius teraturgus* AF389151	95.2	nd	nd	nd
Cortinarius sp7	94	UDB002758	*Dermocybe olivaceopicta* U56050	97.1	nd	nd	nd
Cortinarius sp12	95	UDB002759	*Dermocybe olivaceopicta* U56050	95.7	nd	nd	nd
Cortinarius sp18	96	UDB002760	*Dermocybe olivaceopicta* U56050	95.9	nd	nd	nd
Cortinarius sp30	97	UDB002761	*Dermocybe olivaceopicta* U56050	95.7	nd	nd	nd
Inocybe sp4	98	UDB002762	*Inocybe fraudans* AJ889953	77.6	nd	nd	nd
Lactarius sp2	99	UDB002763	*Lactarius subdulcis* AF218552[3]	98.2	nd	nd	nd
Ramaria sp	100	UDB002764	*Ramaria ignicolor* AJ408386	72.3	nd	nd	nd
Russula sp4	11	UDB002765	*Russula nauseosa* AY061733	91.7	nd	nd	nd
Tomentella sp14	12	UDB002766	*Tomentella atramentaria* DQ974722	89.8	nd	nd	nd
Tulasnella sp4	13	UDB002767	*Tulasnella violea* AY382814	95.1	nd	nd	nd
Clavulina sp8	14	UDB002768	*Clavulina* cf. *cristata* DQ974710	79.1	nd	nd	nd
Clavulina sp9	15	UDB002769	*Clavulina* cf. *cristata* DQ974710	83.4	nd	nd	nd
Cortinarius sp3	16	UDB002770	*Thaxterogaster albocanus* AF325599	92.6	nd	nd	nd
Cortinarius sp10	17	UDB002771	*Cortinarius teraturgus* AF389151	92.4	nd	nd	nd
Cortinarius sp15	18	UDB002772	*Thaxterogaster* sp. DQ328121	95.7	nd	nd	nd
Cortinarius sp16	19	UDB002773	*Cortinarius cystideocatenatus* AY669651	96.8	nd	nd	nd
Cortinarius sp22	110	UDB002774	*Cortinarius collariatus* AY033115	96.7	nd	nd	nd
Cortinarius sp23	111	UDB002775	*Thaxterogaster albocanus* AF325599	93.0	nd	nd	nd
Cortinarius sp33	112	UDB002776	*Thaxterogaster levisporus* DQ328105	96.8	nd	nd	nd
Descolea sp4	113	UDB002777	*Descolea recedens* AF325649	91.5	nd	nd	nd
Pezizaceae sp2	114	UDB002778	*Terfezia arenaria* AF276674	76.8	nd	nd	nd
Russula sp7	115	UDB002779	*Russula littoralis* AY061702	83.5	nd	nd	nd
Descolea sp10	116	UDB002780	*Setchelliogaster* sp. DQ328087	99.5	nd	nd	nd

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Appendix *continued*

			Best BLASTn full-length ITS match		*P*-value of Fisher's exact test		
Taxon	Rank	UNITE accession	Specimen	% identity	Host species	Host vicinity	Plot
Descolea sp11	117	UDB002781	*Setchelliogaster* sp. DQ328214	99.6	nd	nd	nd
Tomentella sp5b	118	UDB002782	*Tomentella fuscocinerea* DQ974776	91.2	nd	nd	nd
Tomentella sp5d	119	UDB002783	*Tomentella fuscocinerea* DQ974776	91.4	nd	nd	nd
Tomentella sp6	120	UDB002784	*Tomentella lateritia* UDB000963	90.3	nd	nd	nd
Tomentella sp13	121	UDB002785	*Tomentella coerulea* UDB000266	91.4	nd	nd	nd
Tomentellopsis sp1	122	UDB002786	*Tomentellopsis larsenii* AF326980	92.7	nd	nd	nd
Tomentellopsis sp3	123	UDB002787	*Tomentellopsis bresadoliana* AJ410779	86.8	nd	nd	nd

Best BLASTn/FASTA3 matches of the entire internal transcribed spacer (ITS) region to database sequences are shown. The *P*-values for host root and host soil preference are indicated. Statistically significant effects following the sharpening procedure of Benjamini–Hochberg correction are shown in bold type.

[1]nd, not determined because of consistent failure of polymerase chain reaction (PCR) or insufficient statistical power; [2]Identification based on rDNA mitochondrial large subunit (mtLSU) sequence; [3]Identification based on rDNA nuclear large subunit (nLSU) sequence; [4]Partial, identification based on partially alignable internal transcribed spacer (ITS) sequence match.



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