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The host bias of three epiphytic Aeridinae orchid species is reflected, but not explained, by mycorrhizal fungal associations[1]

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- *Premise of the study:* The three co-occurring epiphytic orchid species, *Sarcochilus hillii*, *Plectorrhiza tridentata*, and *Sarcochilus parviflorus* vary in host specificity; all are found predominantly on the tree *Backhousia myrtifolia* but some also associate with a broad range of species. Despite this specialization, no fitness advantage has been detected for adult orchid plants growing on the preferred host. Therefore, we predicted that the host specialization of these orchid species is a consequence of a bias toward particular orchid mycorrhizal fungi, which are in turn biased toward particular woody plant species.
- *Methods:* To test this hypothesis, we sampled representatives of each orchid species on *B. myrtifolia* and other host species across sites. *Rhizoctonia*-like fungi were isolated from orchid roots and identified using molecular markers.
- *Key results:* Three groups of fungi were identified, and the orchid species varied in their specificity for these. All fungal groups were found on the host *B. myrtifolia*; yet at all sites, only one orchid species, *S. hillii*, associated with all three groups.
- *Conclusions:* Our results demonstrate that these orchid species did vary in their mycorrhizal specificity; however, the distribution of their mycorrhizal associates did not directly explain their host associations. Rather, we propose that the mycorrhizal relationship of these orchid species is complex and have suggested future avenues of research.

Key words: *Ceratobasidium*; epiphytic orchid; internal transcribed spacer region (nrITS); mitochondrial large subunit (mtLSU); multiple nrITS copies; multispecies interactions; orchid mycorrhizal fungi; Orchidaceae; specialization.

Species do not exist in isolation: interactions between organisms typically occur within a complicated web of interactions (reviewed by Margulis, 1998; Thompson, 1999; Polis et al., 2000; Sapp, 2004; Strauss and Irwin, 2004; Bennett et al., 2006). It is with this understanding in mind that we asked why three related epiphytic orchid species exhibit varying degrees of phorophyte (the supporting substrate e.g., host tree, rock (Tremblay, 1997)) specificity in the same habitat (Gowland et al., 2011). Epiphytic orchids provide interesting systems in which to examine specialist interactions because they require both a substrate for growth and persistence and endosymbionts for germination and establishment. We have previously shown that among three co-occurring epiphytic Aeridinae orchid species of Australia, *Sarcochilus hillii* (F.Muell.) F.Muell., shows little host specificity, *Plectorrhiza tridentata* (Lindl.) Dockrill, is intermediate, and *Sarcochilus parviflorus*, Lindl. has significantly biased host associations at all sites investigated (Gowland et al., 2011). For all three orchid species we studied, the dominant host was the tree species *Backhousia myrtifolia* Hook. & Harv. (Myrtaceae). However, we could detect no fitness benefit of the nonrandom distribution.

Because seeds of epiphytic orchid species are typically wind-dispersed and are presumed to be widely distributed, the absence of an orchid species on particular tree species implies that seed of that orchid species has failed to establish there, rather than failed to disperse there. These three epiphytic orchid species are thought to be largely independent of their host trees, obtaining their moisture and nutrient requirements from rain and debris. Thus, without any clear fitness trade-off for specialization or nutritional benefit from their host tree, the biased distribution of these epiphytic orchid species would appear to be counterintuitive, and as such, we hypothesized that the host bias would be an indirect effect of orchid dependence on a specialized mycorrhizal relationship (Gowland et al., 2011).

For an orchid to establish, its seed must be colonized by a suitable fungus (orchid mycorrhizal fungi, OMF), from which the developing embryo obtains its nutrition (Burges, 1939; Rasmussen, 1995). Hence, the observed phorophyte specificity could reflect associations between fungal symbionts and particular woody plant species (Clements, 1987; Hietz and Hietz-Seifert, 1995; Tremblay, 1997; Zotz and Schultz, 2008). Investigations of protocorms (germinating orchid seeds) on twigs demonstrated that fungi can be found in the orchids' cells, at the interface between the orchid and the supporting twig, and in the bark of the twig itself (Gowland, 2009). This observation led us to propose that the bias in the tree host used by these orchid species

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might be due to variation in associations between either the fungi and the orchid species, or the mycorrhizal fungi and the trees.

Species-specific fungal associations have not commonly been found among epiphytic orchid species, (but see Tremblay et al., 1998; Otero et al., 2002; Graham and Dearnaley 2012; Martos et al., 2012). Mycorrhizal associations, like most mutualisms, can range from mutualistic interactions between the plant and fungus to parasitic interactions (Smith and Read, 1997; Hibbett et al., 2000; Brundrett, 2004). Among OMF, the role of the orchid in the relationship has been likened to that of a parasite (Burges, 1939; Taylor and Bruns, 1997; Roberts, 1999). The fungus is necessary for providing essential nutrients to the orchid embryo for germination, but until recently little return to the fungus has been detected from its role in orchid germination (Burges, 1939; Rasmussen, 1995). Cameron et al. (2006) demonstrated carbon exchange between a green orchid species and its fungal symbionts, as well as a fungus-dependent pathway for nitrogen acquisition by the orchid. We suggest that the extent of specificity in the orchid fungus relationship may well depend upon the nature of the relationship between the organisms: be that parasitic or mutualistic.

The distribution of OMF themselves is thought to be independent of orchids; OMF are often found where orchids are not (Masuhara and Katsuya, 1994; Batty et al., 2001; Taylor et al., 2004). Among photosynthetic orchid species, OMF typically belong to the form-genus *Rhizoctonia* (Warcup and Talbot, 1980; Warcup, 1981; Rasmussen, 1995; Currah et al., 1997; Brundrett, 2002, 2007; Rasmussen, 2002; Weston et al., 2005), a genetically diverse group of fungi that share similar anamorph (vegetative) morphology (Stalpers and Andersen, 1996; Roberts, 1999; González et al., 2006). These OMF are thought to be primarily saprotrophic or parasitic on plants other than the Orchidaceae (Smith and Read, 1997; Roberts, 1999; Rasmussen, 2002). Therefore, the distribution of the fungus may reflect that of some other component of the ecosystem, such as a suitable nonorchidaceous plant to colonize.

Here we examine the relationships between each of these three species of epiphytic orchid, their *Rhizoctonia*-like OMF, and their host species. We hypothesized that the patterns of host association of the three orchid species, *S. hillii* (widest host range), *P. tridentata* (intermediate host range), and *S. parviflorus* (narrowest host range) would reflect differences in their symbioses with *Rhizoctonia*-like fungi. We expected *S. hillii* to have the broadest mycorrhizal niche, *P. tridentata* to be intermediate, and *S. parviflorus* to have the narrowest niche. To test this hypothesis, we used molecular tools to determine the number of *Rhizoctonia*-like fungal lineages that associate with each of the three orchid species. We estimated the genetic relationships between the isolated fungal lineages and determined whether the three orchid species are associated with specific or different fungal lineages and whether the fungal lineages are associated with specific tree species or geographic sites. This system provides a valuable opportunity to examine the complexity of interacting species webs.

MATERIALS AND METHODS

Multiple mature individuals of each orchid species (*Sarcochilus hillii* (F.Muell.) F.Muell., *Plectorrhiza tridentata* (Lindl.) Dockrill, and *Sarcochilus parviflorus* Lindl.) were sampled from two sites (Wagonga State Forest, 36°11.512′S, 150°03.466′E, and Wanderra State Forest, 35°52.519′S, 149°59.229′E) in southern New South Wales, Australia. These sites were previously surveyed as part of an investigation to determine the phorophyte associations of the three orchid species across four sites (Gowland et al., 2011). Voucher specimens of each of the three orchid species were obtained from Wagonga State Forest and Wanderra State Forest and are held at the Australian National Herbarium (Vouchers CANB808825–CANB808830).

Roots were collected from eight to 10 individuals of each orchid species from each site between March 2002 and October 2004. At least five individuals of each orchid species were sampled from the most common tree host of all three orchid species, *Backhousia myrtifolia* at each site. At least one root was sampled from an orchid of each species on *Notelaea venosa* F.Muell, also a phorophyte of all three orchid species at each of the sites (Gowland et al., 2011). The remaining individuals of each orchid species were taken from the second most common host of each orchid species at each site. The identity of this host varied among orchid species and site (see Table 1, Gowland et al., 2011). Where these numbers could not be achieved (e.g., because the orchids were not present or out of reach), orchids on other phorophytes were sampled. Because *S. parviflorus* is frequently found on rock, we sampled roots from one plant on a rock substrate of that species at each site. Finally, additional fungal isolates were taken from one *S. parviflorus* individual from Chichester State Forest (32°14.150′S, 151°43.576′E), two *S. hillii* individuals from Buckenbowra State Forest (35°38.9′S, 150°03.0′E), and a single sample was taken from one individual of the closely related orchid species *Sarcochilus falcatus* R.Br. from Sassafras (35°06.213′S, 150°15.859′E).

We found that fleshy, green roots have the most actively growing fungi (K. M. Gowland, personal observations), and these were therefore preferentially targeted for isolation. Roots were transported to the laboratory in separate plastic bags with a few drops of sterile water.

***Fungal isolations*—**Because earlier work on species of *Sarcochilus* and *Plectorrhiza* found the orchids were associated with *Rhizoctonia*-like fungi (Warcup and Talbot, 1980; Warcup, 1981), we targeted our research at understanding the diversity of *Rhizoctonia*-like associates of these orchid species. We also have verified the capacity of the *Rhizoctonia*-like fungi found in these orchid species to stimulate germination (Gowland, 2009). Multiple fungi can be found in an individual orchid (Andersen and Rasmussen, 1996; Kristiansen et al., 2001; Suárez et al., 2006; Porras-Alfaro and Bayman, 2007); hence, the identification of potential OMF is complex.

To understand the diversity of OMF associations for each of these orchid species, we collected one fungus sample from each of several orchid individuals on different hosts at different sites for each orchid species. In this way, we hoped to sample the diversity of fungal associates across orchid species and sites. In two cases, insufficient samples of the initial fungus were available for multiple analyses; therefore, more than one fungal isolate was sampled from a single orchid. The different isolates were treated as different fungi and never used in the same molecular analyses to avoid repeat sampling.

Fungi were isolated within 72 h of root collection. Roots were washed in sterile water to remove debris such as remnants of bark and dissected in a Petri dish using a Wild Heerbrugg M5 dissecting microscope. Cells containing fungi were teased apart to release intracellular fungal hyphae (pelotons). Excess root tissue was removed, and lukewarm fungal isolation media (based on Clements, 1982) was poured over the remaining material.

The *Rhizoctonia*-like isolates generally grew sufficiently within 24 h to be identified morphologically and isolated (subcultured) from the parent plate. The isolated section of the plate was placed a few millimeters from fresh media, forcing the hyphae to grow across a sterile section of the plate before reaching a fresh food source. Repetition of this procedure served to isolate the *Rhizoctonia*-like fungi from bacteria and other fungi. Pure cultures were maintained at ambient temperature (approximately 25°C) and then used to verify their capacity to stimulate orchid germination and hence confirm their status as potential OMF (Gowland, 2009).

***DNA extraction and gene amplification*—**Fungal DNA was extracted from lyophilized fungal isolates using a modification of the CTAB extraction protocol of Doyle and Doyle (1987). See Gowland (2009) for details.

Sequences were generated for the large subunit gene of the mitochondrial ribosomal DNA (mtLSU) using primers ML7 and ML8 (White et al., 1990) and for the internal transcribed spacer regions and 5.8S gene of the nuclear ribosomal DNA (nrITS) using primers ITS1-F and ITS4-B (Gardes and Bruns, 1993).

Genetic fingerprints were obtained using repetitive element sequence-based PCR (repPCR) using primers Rep1R-I and ERIC-2 (Versalovic et al., 1991). This method enables rapid fingerprinting of microorganisms (Versalovic et al.,

TABLE 1. Genetic identification of fungal isolates and GenBank accessions. Dashes indicate that the marker was not applied or was not successful. nrITS copy refers to isolates represented as terminal taxa or within collapsed clades in Fig. 2, see figure for clarification on terminology. The number of colonies sequenced from isolates indicating multiple nrITS sequences given along with the number of distinct copies detected.

		mtLSU		nrITS			No. colonies	No. copies	Rep-PCR
Fungal species and site	Isolate no.	Clade	Accession	Clade	Copy	Accession	sequenced	detected	group
A) *Sarcochilus hillii* (Sh)									
Wagonga State Forest (Wg)									
Backhousia myrtifolia (Bm)	1	A	HQ914192	K	i_12	HQ914030	18	2	1
					i_8	HQ914086			
	2	B	HQ914166	L	i_*S.hillii*2BmWg	HQ914157	—	1	4
	3	A	HQ914172	J	i_*S.hillii*3BmWg_a	HQ914150	13	7	—
					i_*S.hillii*3BmWg_b	HQ914149			
					i_*S.hillii*3BmWg_c	HQ914146			
					i_*S.hillii*3BmWg_d	HQ914151			
					i_*S.hillii*3BmWg_e	HQ914154			
					i_*S.hillii*3BmWg_f	HQ914145			
					i_*S.hillii*3BmWg_g	HQ914148			
	4	B	HQ914165	L	i_*S.hillii*4BmWg	HQ914156	—	1	—
	5	A	HQ914183	K	i_12	HQ914101	—	1	—
Notolaea venosa (Nv)	6	A	HQ914187	J	i_2	HQ914134	—	1	—
	7	A	HQ914177	K	i_12,	HQ914057	9	3	—
					i_8,	HQ914083			
					i_8	HQ914099			
Rapanaea howittiana (Rh)	8	—		K	i_11	HQ914070	12	3	1
					i_*S.hillii*8RhWg_b	HQ914115			
					i_*S.hillii*8RhWg_c	HQ914111			
Wanderra State Forest (Wd)									
Backhousia myrtifolia (Bm)	10	—		K	i_8,	HQ914082	9	4	1
					i_10,	HQ914097			
					i_12, i_12	HQ914058, HQ914112			
	11	—		J	i_1, i_2	HQ914127, HQ914131	12	3	—
					i_4	HQ914130			
	12	A	HQ914204	K	i_12	HQ914033	15	3	—
					i_12	HQ914068			
					i_12	HQ914048			
	13	A	HQ914206	K	i_8, i_13	HQ914090, HQ914102	16	4	1
					i_7	HQ914108			
					i_*S.hillii*13BmWd_d	HQ914114			
	14	B	HQ914168	L	i_15	HQ914159	—	1	—
	15	B	HQ914164	L	i_*S.hillii*15BmWd_a	HQ914161	13	3	3
					i_*S.hillii*15BmWd_b	HQ914155			
Elaeocarpus obovatus (Eo)					i_*S.hillii*15BmWd_c	HQ914162			
Notelaea venosa (Nv)	16	A	HQ914186	J	i_2	HQ914136	—	1	2
	17	A	HQ914174	K	i_12,	HQ914069	15	3	1
					i_8, i_*S.hillii*17NvWd_c	HQ914084, HQ914074			
	18	A	HQ914181	J	i_4	HQ914147	—	1	1
	19	A	HQ914207	K	i_12	HQ914035	13	3	—
					i_8	HQ914091			
					i_*S.hillii*19NvWd_c	HQ914080			
B) *Plectorrhiza tridentata* (Pt)									
Wagonga State Forest (Wg)									
Backhousia myrtifolia (Bm)	1	—	—	K	i_11	HQ914041	10	4	1
					i_12	HQ914059			
					i_11, i_12	HQ914073, HQ914047			
	2	A	HQ914176	—	—	—	—	—	—
	3	A	HQ914185	J	i_5	HQ914139	12	2	1
				K	i_12	HQ914064			
	4	A	HQ914169	J	i_*P.tridentata*4BmWg_a	HQ914141	16	4	—
					i_3, i_6	HQ914128, HQ914143			
					i_*P.tridentata*4BmWg_d	HQ914137			
	5	A	HQ914184	—	—	—	—	—	—
	6	A	HQ914198	K	i_12	HQ914061	—	1	1

TABLE 1. Continued.

Fungal species and site	Isolate no.	mtLSU Clade	mtLSU Accession	nrITS Clade	nrITS Copy	nrITS Accession	No. colonies sequenced	No. copies detected	Rep-PCR group
Notelaea venosa (Nv)	7	A	HQ914190	J	i_2	HQ914133	—	1	2
	8	A	HQ914182	K	i_12 i_10 i_12	HQ914060, HQ914096, HQ914117	6	3	—
Wanderra State Forest (Wd)									
Backhousia myrtifolia (Bm)	9	A	HQ914188	J	i_1	HQ914126	—	1	2
	10	A	HQ914214	J	i_2	HQ914135	—	1	2
	11	A	HQ914189	—	—	—	—	—	—
	12	A	HQ914205	K	i_*P.tridentata*12BmWd_a I_12, i_12	HQ914049 HQ914066 HQ914034	15	3	—
	13	A	HQ914178	J	i_2, i_*P.tridentata*13BmWd_b i_4	HQ914132 HQ914153 HQ914152	11	3	2
Notolaea venosa (Nv)	14	A	HQ914213	—	—	—	—	—	—
	15	A	HQ914201	K	i_8	HQ914087	—	1	1
	16a	A	HQ914208	—	—	—	—	—	—
	16b	—	—	K	i_14, i_10, i_12 i_9, i_9	HQ914079, HQ914044 HQ914036 HQ914095, HQ914094	7	5	—
	16c	—	—	—	—	—	—	—	1
	17	A	HQ914210	K	i_8	HQ914088	—	1	1
	18	A	HQ914179	—	—	—	—	—	—
C) *Sarcochilus parviflorus* (Sp)									
Wagonga State Forest (Wg)									
Backhousia myrtifolia (Bm)	1	—	—	K	i_12, i_7, i_7, i_12	HQ914119, HQ914104 HQ914116, HQ914120	16	4	1
	2	A	HQ914195	—	—	—	—	—	—
	3	A	HQ914196	J	i_*S.parviflorus*3BmWg_a* i_*S.parviflorus*3BmWg_b i_6, i_5 i_3	HQ914129 HQ914144 HQ914142, HQ914140 HQ914138	11	5	—
	4	A	HQ914171	K	i_12, i_13 i_11 i_*S.parviflorus*4BmWg_d i_*S.parviflorus*4BmWg_e, i_12 i_*S.parviflorus*4BmWg_g	HQ914065, HQ914100 HQ914071 HQ914093 HQ914113, HQ914103 HQ914075	16	7	1
	5a	—	—	—	—	—	—	—	1
	5b	—	—	K	i_12	HQ914043	—	1	—
Cissus hypoglauca (Ch)	6	A	HQ914170	K	i_7 i_12	HQ914107 HQ914032	14	2	—
Notelaea venosa (Nv)	7	A	HQ914194	K	i_12, i_*S.parviflorus*7NvWg_b i_*S.parviflorus*7NvWg_c	HQ914063, HQ914125 HQ914124	7	3	1
	8	A	HQ914212	—	—	—	—	—	1
Rapanaea howittiana (Rh)	9	—	—	K	i_7, i_12	HQ914110 HQ914067	8	2	1
Rock	10	A	HQ914197	K	i_12	HQ914062	—	1	1
Wanderra State Forest (Wd)									
Backhousia myrtifolia (Bm)	11	A	HQ914191	—	—	—	—	—	—
	12	A	HQ914200	—	—	—	—	—	—
	13	A	HQ914175	K	i_*S.parviflorus*13BmWd_a, i_11, i_12, i_*S.parviflorus*13BmWd_d	HQ914123 HQ914040, HQ914055 HQ914122	23	4	—

TABLE 1. Continued.

Fungal species and site	Isolate no.	mtLSU Clade	mtLSU Accession	nrITS Clade	nrITS Copy	nrITS Accession	No. colonies sequenced	No. copies detected	Rep-PCR group
	14	A	HQ914209	K	i_12, i_*S.parviflorus*14BmWd_b,	HQ914037, HQ914050	7	4	—
					i_*S.parviflorus*14BmWd_c,	HQ914072			
					i_12	HQ914039			
	15	A	HQ914180	K	i_8,	HQ914092	6	2	1
					i_12,	HQ914038			
	16	A	HQ914211	K	i_8	HQ914089	—	1	1
	17	—	—	K	i_7,	HQ914109	6	3	—
					i_*S.parviflorus*17BmWd_b,	HQ914076			
					i_*S.parviflorus*17BmWd_c	HQ914052			
Marsdenia rostrata (Mr)	18	—	—	K	i_7,	HQ914106	8	3	1
					i_*S.parviflorus*18MrWd_b,	HQ914046			
					i_*S.parviflorus*18MrWd_c	HQ914054			
Notelaea venosa (Nv)	19	A	HQ914202	K	i_12, i_12,	HQ914030, HQ914118	8	5	1
					i_14,	HQ914078			
					i_*S.parviflorus*19NvWd_d,	HQ914042			
					i_*S.parviflorus*19NvWd_e	HQ914056			
	20	A	HQ914203	K	i_7,	HQ914029	16	4	—
					i_12,	HQ914051			
					i_*S.parviflorus*20NvWd_c,	HQ914105			
					i_*S.parviflorus*20NvWd_d,	HQ914121			
					i_12	HQ914053			
Pittosporum undulatum (Pu)	21	—	—	K	i_8,	HQ914085	8	2	1
					i_*S.parviflorus*21PuWd_b	HQ914098			
Rock	22	A	HQ914173	K	i_12,	HQ914028	14	4	—
					i_*S.parviflorus*22RockWd_b,	HQ914081			
					i_*S.parviflorus*22RockWd_c,	HQ914045			
					i_*S.parviflorus*22RockWd_d	HQ914077			
D) Additional samples									
S. hilllii at Buckenbowra State Forest (Bb)									
Backhousia myrtifolia (Bm)	20	B	HQ914163	L	i_15	HQ914160	—	1	3
	21	B	HQ914167	L	i_*S.hillii*21BmBb	HQ914158	—	1	4
S. parviflorus at Chichester Hill									
Backhousia myrtifolia (Bm)	23	A	HQ914199	—	—	—	—	—	—
S. falcatus at Sassafras									
Acacia melanoxylon (Am)	1	A	HQ914193	—	—	—	—	—	—

* Where multiple fungi have been sampled from one orchid, the distinct isolates are denoted by suffixes a, b, c.

1991) including *Rhizoctonia*-like fungi (Linde et al., 2005) and was used here to verify the genetic clades identified from the nrITS and mtLSU analyses.

PCR reactions for the mtLSU and nrITS were performed in 50-μL volumes, each containing 0.2 nmol of each dNTP, 2.5 units of *Taq* DNA polymerase (New England BioLabs, Ipswich, Massachusetts, USA), and 3 μL of DNA template. For mtLSU, 10 nmol of $MgCl_2$ and 0.25 pmol of each primer were used, and for nrITS, 7.5 nmol of $MgCl_2$ and 0.2 pmol of each primer were used. The repPCRs were carried out in a total volume of 25 μL with a final $MgCl_2$ concentration of 3.75 nmol, 2.5 μl of dimethyl sulfoxide (DMSO; Sigma, St. Louis, Missouri, USA), 0.125 nmol of each dNTP, 0.2 pmol of each primer, 1 unit of *Taq* DNA polymerase, and 1.5 μL DNA. Cycling conditions for mtLSU and nrITS were similar with 1 min at 94°C; followed by 35 cycles of 1 min at 94°C, 30 s at 53°C and 1 min at 72°C; with a final extension of 7 min at 72°C. For the rep-PCR, an initial step of 2 min at 95°C denaturation was followed by 35 cycles of 30 s at 94°C 1 min at 52°C, and 2.5 min at 65°C; and a final extension of 8 min at 65°C. Reactions were performed on a PCR Express (Thermo Hybaid, Ashford, Middlesex, UK) and Corbett PC-960C cooled thermal cycler (Corbett Research Australia, Mortlake, NSW, Australia). PCR products were cleaned using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) prior to sequencing. Sequences were obtained using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, Mulgrave, VIC, Australia), on an ABI 3730 Capillary Genetic Analyser at the Biomolecular Resource Facility at the John Curtin School of Medical Research at the Australian National University. Sequences were aligned and manually corrected using the program Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Michigan, USA). All sequences were BLAST searched (Altschul et al., 1997) against GenBank. Sequences were deposited in GenBank (nrITS: HQ914028 to HQ914162; and mtLSU: HQ914163 to HQ914214).

***Cloning and sequencing PCR products*—**We detected multiple copies of the nrITS region. Therefore, PCR products were purified using the Wizard SV Gel Clean Up kit (Promega) and cloned using the pGEM-T Vector System (Promega) according to the manufacturer's specifications. Colony PCRs were conducted using the nrITS primers and between six and 23 colonies per isolate were sequenced.

***Molecular identification of fungal isolates*—**Differences between sequences obtained through cloning and subsequent sequencing could be due to multiple copies of the region within the study organism, PCR recombinants and errors incorporated during the PCR reaction. To discern distinct nrITS copies within a fungal isolate, we aligned the sequences using the program Sequencher obtained from the cloning with sequences of isolates where only single nrITS copies were detected. We then recorded where each of the cloned sequences obtained from one isolate differed from each other and whether the differences were indels, transitions, or transversions. This comparison allowed us to identify distinct nrITS copies among the clones. Uninformative mutations (autapomorphies) present in only one cloned copy were edited out of consensus sequences of these distinct copies.

***Detecting potential recombinant sequences*—**To detect potential recombinant sequences (Bradley and Hillis, 1997) among the nrITS copies, we conducted neighbor-joining analyses separately for ITS1 and ITS2 using the program MEGA 3.1 (Kumar et al., 2004). A sequence was considered to be a recombinant if different genetic relationships were found with the two nrITS regions. Smaller regions of similarity between otherwise distinct nrITS copies from the same fungal isolate were identified through investigations of aligned copies and considered to be indicative of potentially multiple crossovers.

***Alignment and analysis*—**A nrITS alignment was constructed containing all the distinct nrITS copies, the potential recombinant sequences, and the sequences from isolates where only a single nrITS copy was found; this alignment was used in the data analyses. Sequences were aligned using the FFT-NS-i algorithm in the program MAFFT version 6 (Katoh et al., 2005) and manually edited in the program BioEdit 7.0.5.3 (Hall, 1999). Gaps at the ends of the sequences were scored as missing data, and indels were excluded from the analysis because sequences could not be aligned with confidence with them.

We implemented phylogenetic methods to address the question whether fungal isolates from the three different orchids are differentiated into different clades. Maximum parsimony and Bayesian inference were used to infer sequence relationships. The program Modeltest 3.7 (Posada and Crandall, 1998) was used to infer an appropriate evolutionary model of the sequence alignments prior to running the analysis using the program MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

For the parsimony analysis, we used equally weighted parsimony with a heuristic search strategy (options: tree-bisection-reconnection (TBR) and MULPARS, three random addition sequence (RAS) replicates, holding a maximum of 100 trees per replicate) to identify the most parsimonious trees (MPTs) in the program PAUP* version 4.0b10 (Swofford, 2003). The MPTs were used as starting trees for a second search using TBR and MULPARS, saving a maximum of 10 000 trees. Bootstrapping (Felsenstein, 1985) was implemented with 1000 bootstrap pseudoreplicates and three RAS replicates, saving a maximum of five trees per replicate.

For the mtLSU data analysis, we considered branches with bootstrap support (BS) values of 50–70% to be weakly supported, 70–90% moderately supported, and >90% to be robust. Due to the high level of sequence similarity among the nrITS copies, we considered branches with BS values of 60–90% to be moderately supported.

Bayesian analyses were run over 5 million generations using 10 chains, saving every 5000 generations. The analyses were repeated to ensure that runs converged on the same topology. Majority rule consensus trees were generated in PAUP*, excluding trees generated in burn-in time (first 10% of saved trees). Only nodes with posterior probabilities higher than 0.94 were considered as well supported.

Isolates sequenced with the nuclear or mitochondrial markers were used in the fingerprinting analysis. 13.5 μL of its repPCR product was electrophoresed on 1.5% agarose SB (sodium boric acid) gels. Gels were stained with 0.3% ethidium bromide and photographed and lane background stain minimized using Universal Hood II and Quantity One 4.6.2 software (Bio-Rad). To allow for comparison, we ran duplicates on all gels. A presence–absence matrix was constructed for the repPCR gels. Only bands (amplicons) that were of consistently high density (>1000 carbon nanotubes, CNT) among multiple samples were scored. Bands of consistently low density (<1000 CNT) or restricted to a single sample were excluded from the analysis. Low-density bands that were present in other samples as high density bands were scored as present. A neighbor-joining analysis was used to infer a phenogram from the binary data matrix using PAUP*. Clade support was assessed using 1000 bootstrap replicates.

Based on the results from the three different molecular markers (mtLSU, nrITS, repPCR), robustly supported clades representing the same individual isolates supported by at least two of these markers were recognized as distinct taxa. Having established the identity of the fungal isolates, we compared the distribution of fungal lineages identified across site, phorophyte, and orchid species.

RESULTS

In total, 65 fungal isolates were included in our analysis (Table 1). Forty isolates were sequenced using both mtLSU and nrITS markers; 23 of these were also used in the repPCR analysis. BLAST searches of the nrITS against the GenBank database revealed that the isolates had a high similarity to *Rhizoctonia*-like fungi in the genus *Ceratobasidium*. No similar sequences of the mtLSU region were found through BLAST searches.

The evolutionary model of the sequence alignments inferred by the program Modeltest 3.7 (Posada and Crandall, 1998) estimated a HKY+Γ model for the mtLSU data and a HKY+I+Γ model for the nrITS data.

Diversity and sequence analyses*—mtLSU*—In total, 13 different mtLSU haplotypes were sequenced among the fungal isolates from the three orchid species. Six mtLSU haplotypes were isolated from *S. hillii*, nine from *P. tridentata*, and two from *S. parviflorus*. Two of the 13 haplotypes were shared among all three orchid species. Seven haplotypes were only found in *P. tridentata* isolates and four only in *S. hillii* isolates. No unique haplotypes were identified among the *S. parviflorus* isolates. An alignment of 384 bp with 26 variable sites, of which 17 were parsimony informative, was constructed with the mtLSU sequences (Appendix S1, see Supplemental Data with the online version of this article).

Parsimony and Bayesian phylogenetic analysis of the mtLSU were highly congruent, and branch support from the two methods indicated robust support for two distinct mitochondrial clades: mtLSU clade A and mtLSU clade B (Fig. 1). Isolates from all three orchid species were found in clade A, while the smaller clade B contained only isolates from *S. hillii*.

nrITS—Initial sequencing of 51 isolates for the nrITS region indicated that multiple copies were present in 33 (65%) of the fungal isolates. The source of these putative recombinants could be the product of polymerase error, PCR recombinants, or real additional nrDNA copies present in the fungal DNA due to homoplasy; thus, the sequences were not removed from the analysis. Consequently, PCR products of these 33 isolates were cloned and sequenced. After rigorous screening for PCR recombinants and polymerase error, up to seven nrITS copies were found from a single fungal isolate (Table 1), and 28 distinct nrITS sequences were found among all 51 isolates. Of the 33 sets of cloned PCR products, 21 included at least one putative recombinant sequence.

We found 17, nine, and 16 nrITS genotypes among the *S. hillii*, *P. tridentata*, and *S. parviflorus* isolates. Five nrITS genotypes were shared among the three orchids, and these genotypes were at both sites and on multiple host species.

Examination of the regions of recombination revealed that 17 of the 21 recombinant sequences indicated a switch had occurred somewhere between the ITS1 and the ITS2. Switching of templates by the polymerase has been shown to occur during PCR when partially homologous templates are present, producing chimeric sequences of these homologues (Pääbo et al., 1990; Odelberg et al., 1995). The other four recombinant sequences indicated short regions of similarity to other copies inserted within either the ITS1 or ITS2, potentially indicative of multiple recombination events.

Recombinant nrITS sequences can occur naturally (Buckler et al., 1997; Álvarez and Wendel, 2003), and because of template switching during the PCR amplification reactions (Pääbo et al., 1990; Odelberg et al., 1995). Additionally, the cloning process provides ample opportunity for the production of chimeric sequences given that template switching can occur in the first PCR replication cycle. Logistical issues prevented further analysis of the cause of the recombinants detected here.

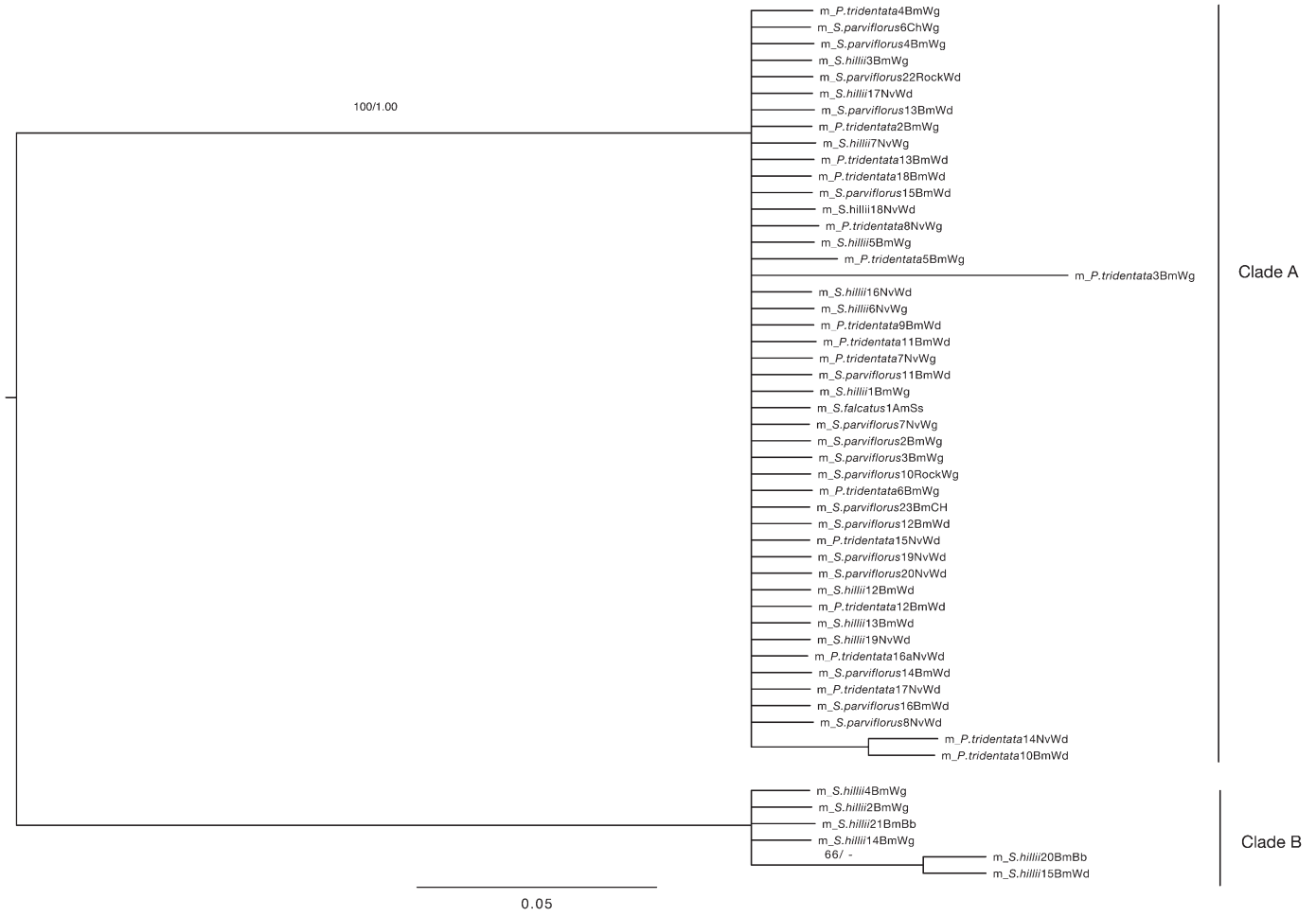


Fig. 1. A midpoint rooted Bayesian phylogram of mtLSU data. Branch support values indicated where either the parsimony analyses had bootstrap values >50% or Bayesian analysis had posterior probabilities ≥0.90. Bootstrap values and/or posterior probabilities are indicated above the line, separated by a slash. Branches that are robustly supported by both analyses are recognized as clades A and B. Terminal taxa representative of individual sequences are depicted by "m_" followed by the orchid species, orchid number, phorophyte, and site information; all abbreviations are provided in Table 1.

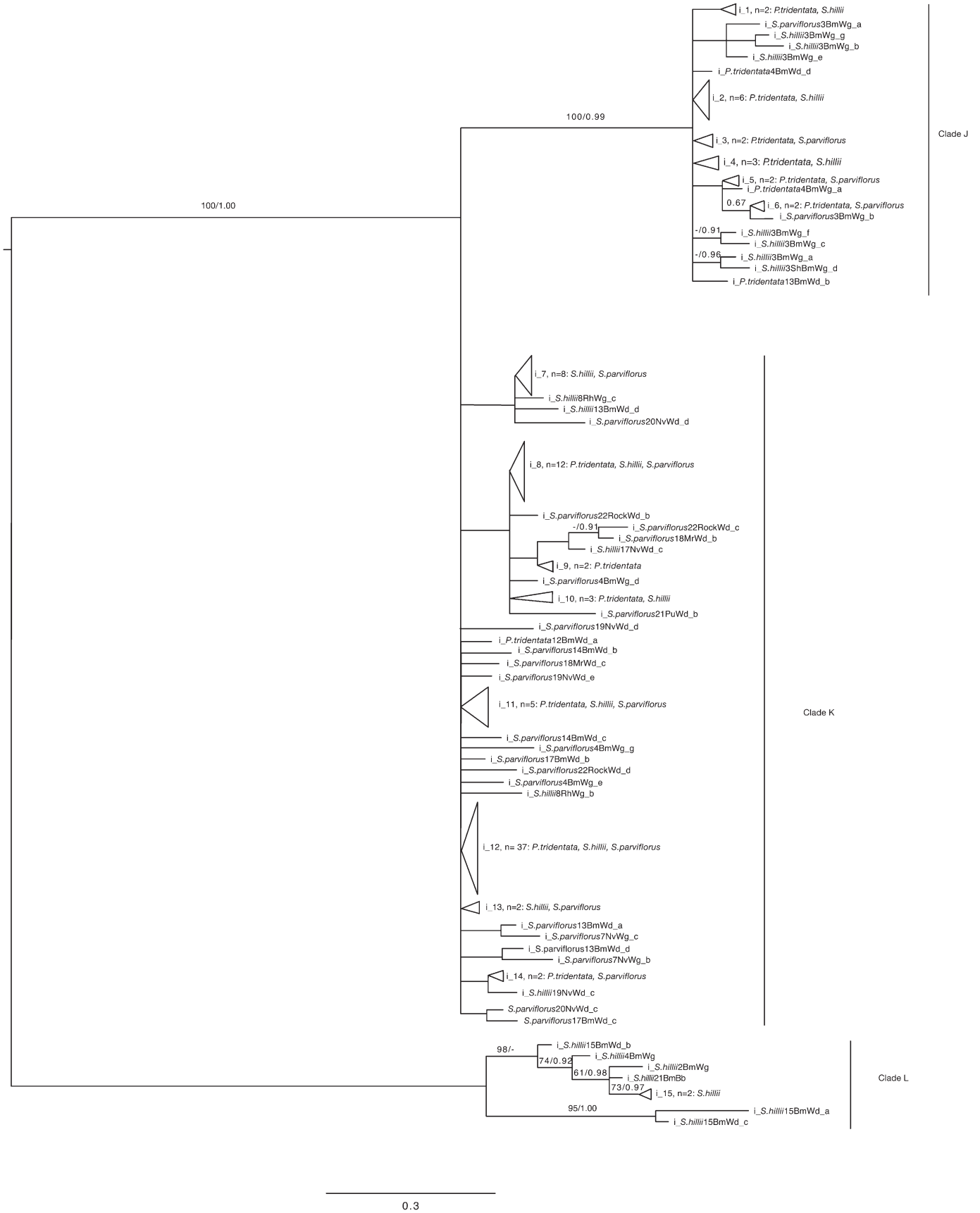
This investigation sought to identify the diversity of distinct fungal taxa/lineages associated with these three orchid species to identify their role in affecting orchid distribution. We implemented phylogenetic methods to assess the number of genetic groups that each orchid species associates with and not necessarily to determine phylogenetic relationships among the fungi. With phylogenetic analysis recombination violates the assumption that all parts of the sequence under investigation have undergone the same phylogenetic history (Posada, 2000). Nevertheless, we found that despite the high occurrence of switching between the ITS1 and ITS2, separate analysis of these regions did not alter the group assignment.

The nrITS alignment consisted of 616 nucleotides (Appendix S1), of which 460 sites were conserved and 92 were parsimony informative. The ITS1 was the most variable with 12 sites, indicating a single nucleotide insertion or deletion event, and 42 sites were parsimony informative.

Both analyses of the nrITS relationships indicated robust support for three clades: nrITS clade J, nrITS clade K, and nrITS clade L (Fig. 2). Clades J and K both consisted of isolates from all three orchid species, while clade L only contained genotypes from *S. hillii*. One isolate from *P. tridentata* contained nrITS copies from clade J and clade K (Table 1). The nrITS copies of all other isolates were found only within one of the three major clades: J, K, and L. Among the potential recombinant sequences,

→

Fig. 2. A midpoint rooted Bayesian phylogram of nrITS copies. Branch support values are indicated where either parsimony analysis had bootstrap values ≥60% or Bayesian analysis had posterior probabilities ≥0.95. Branches were considered to be moderately supported by parsimony analyses with bootstrap values between 60–90% and significant with values greater than 90%. Terminal taxa represented as i_1 to i_15 refer to nrITS copy number and indicate that multiple nrITS sequences were found within the clade. The number of sequences (n=) and orchid species from which these sequences were obtained are listed after the clade number. Terminal taxa representative of individual sequences are depicted by "i_" preceding the fungal isolate information (as per mtLSU data, Fig. 1); a lowercase letter after this information is indicative of a unique copy identified in the cloning analysis. Identification of all sequences and their copy information are given in Table 1.


i_1, n=2: P.tridentata, S.hillii
i_S.parviflorus3BmWg_a
i_S.hillii3BmWg_g
i_S.hillii3BmWg_b
i_S.hillii3BmWg_e
i_P.tridentata4BmWd_d
i_2, n=6: P.tridentata, S.hillii
100/0.99
i_3, n=2: P.tridentata, S.parviflorus
i_4, n=3: P.tridentata, S.hillii
i_5, n=2: P.tridentata, S.parviflorus
i_P.tridentata4BmWg_a
0.67
i_6, n=2: P.tridentata, S.parviflorus
i_S.parviflorus3BmWg_b
-/0.91
i_S.hillii3BmWg_f
i_S.hillii3BmWg_c
-/0.96
i_S.hillii3BmWg_a
i_S.hillii3ShBmWg_d
i_P.tridentata13BmWd_b
Clade J
100/1.00
i_7, n=8: S.hillii, S.parviflorus
i_S.hillii8RhWg_c
i_S.hillii13BmWd_d
i_S.parviflorus20NvWd_d
i_8, n=12: P.tridentata, S.hillii, S.parviflorus
i_S.parviflorus22RockWd_b
-/0.91
i_S.parviflorus22RockWd_c
i_S.parviflorus18MrWd_b
i_S.hillii17NvWd_c
i_9, n=2: P.tridentata
i_S.parviflorus4BmWg_d
i_10, n=3: P.tridentata, S.hillii
i_S.parviflorus21PuWd_b
i_S.parviflorus19NvWd_d
i_P.tridentata12BmWd_a
i_S.parviflorus14BmWd_b
i_S.parviflorus18MrWd_c
i_S.parviflorus19NvWd_e
i_11, n=5: P.tridentata, S.hillii, S.parviflorus
i_S.parviflorus14BmWd_c
i_S.parviflorus4BmWg_g
i_S.parviflorus17BmWd_b
i_S.parviflorus22RockWd_d
i_S.parviflorus4BmWg_e
i_S.hillii8RhWg_b
i_12, n= 37: P.tridentata, S.hillii, S.parviflorus
i_13, n=2: S.hillii, S.parviflorus
i_S.parviflorus13BmWd_a
i_S.parviflorus7NvWg_c
i_S.parviflorus13BmWd_d
i_S.parviflorus7NvWg_b
i_14, n=2: P.tridentata, S.parviflorus
i_S.hillii19NvWd_c
S.parviflorus20NvWd_c
S.parviflorus17BmWd_c
Clade K
98/-
i_S.hillii15BmWd_b
74/0.92
i_S.hillii4BmWg
i_S.hillii2BmWg
61/0.98
i_S.hillii21BmBb
73/0.97
i_15, n=2: S.hillii
95/1.00
i_S.hillii15BmWd_a
i_S.hillii15BmWd_c
Clade L
0.3

the suspected contributing copies were within the same robustly supported clade. Most commonly, recombination was detected among sequences in the largest clade, clade K.

***Genetic fingerprinting*—**Fingerprinting results were obtained for 32 isolates. UPGMA analysis of the repPCR data revealed four distinct groupings among the isolates (Fig. 3; Appendix S2, see online Supplemental Data).

Groups 1 and 2 corresponded well with mtLSU clade A, while groups 3 and 4 corresponded well with mtLSU clade B. Further delimitation within the repPCR groups did not completely correspond with the subdivision inferred from the mtLSU or nrITS data. The nrITS clades J and K predominantly corresponded with the repPCR groups 2 and 1, respectively; however, one isolate from *S. hillii* had sequences from nrITS clade J and repPCR group 1. Furthermore, an isolate from *P. tridentata* had nrITS copies from both nrITS clades J and K, but was represented by fingerprinting analysis as belonging to repPCR group 1.

***Isolate identification and associations*—**All three genetic markers (mtLSU, nrITS, and repPCR) were congruent on the separation of these fungal isolates into two distinct molecular groups: group A and group B (Fig. 4). The nrITS and repPCR data supported the divergence of group A into two subgroups (A1 and A2), while the repPCR results gave support for further separation within group B.

Group A isolates made up 91% (59) of the 65 isolates identified across all five sites and were common at both Wagonga and Wanderra state forests (Table 2A). Of the 25 lineage A isolates at Wagonga State Forest, 14 had group A1 copies, five had group A2 copies, and one had nrITS copies belonging to both groups A1 and A2. The remaining five lineage A isolates detailed in Table 1 were not identified using the nrITS marker, although one was isolated from the same *S. parviflorus* individual that a group A1 isolate was sequenced from and may be the same individual fungus. A similar proportion of group A1 and A2 isolates were found among the 32 lineage A isolates at Wanderra State Forest; group A1 was represented by 19 isolates, and group A2 was represented by six isolates, with seven isolates not identified by the nrITS marker, although two of these were isolated from the same *P. tridentata* individual in which a group A1 and a repPCR group 1 isolate were found.

More than half (58%) of all fungal isolates identified in this investigation were sampled from orchids on *B. myrtifolia* and 18 (28%) from *Notelaea venosa* (Table 2B). Representatives from all three lineages were found on *B. myrtifolia*, but lineage B isolates were only isolated from *S. hillii* orchids on *B. myrtifolia* despite isolates on seven other phorophyte species and rock being investigated (Table 2B). The fungal community of the host *N. venosa* was sampled six times at Wagonga State Forest and 12 times at Wanderra State Forest (although three of these 12 samples were from the same *P. tridentata* individual—one for each genetic marker). The nrITS marker was successfully applied to five of the six fungal isolates at Wagonga State Forest on *N. venosa,* two of which had group A2 copies and three had group A1 copies (Table 3). The nrITS marker was successfully applied to eight of the 10 potential isolates at Wanderra State Forest, and only one of these isolates had group A2 copies. The majority (five of the seven) of the lineage A isolates found on other phorophytes (not *B. myrtifolia* nor *N. venosa*) had group A1 copies. The fungi isolated from *S. parviflorus* individuals growing on rock at Wagonga State Forest and Wanderra State Forest also belonged to the most common isolates found: group A1 (Table 3).

Unfortunately, insufficient numbers of lineage B isolates were encountered to conduct formal contingency tests (Ramsey and Schafer, 2002); nevertheless, *S. hillii*, *P. tridentata*, and *S. parviflorus* did not appear to associate randomly with lineages A and B (and groups within) (Table 2C). Lineage A, the most common *Ceratobasidium* fungus found, occurred in all three of the target orchid species (and was also found in association with the related species *Sarcochilus falcatus*). The most common group encountered was group A1; this group was found in all three of the target orchid species. The only orchid species found in association with lineage B isolates was *S. hillii*, making it the only one of the three target orchid species found in association with all three groups A1, A2, and B (Table 2C). Additionally, the two fungi isolated from *S. hillii* at Buckenbowra State Forest both belonged to lineage B.

In comparison, *P. tridentata* was found in association with groups A1 and A2 (Table 3). Isolates of eight individuals of *P. tridentata* were identified from Wagonga State Forest; six of these were sequenced with the nrITS marker (Table 3). Of the six, one isolate had nrITS copies from both clades K and J (group A1 and A2, respectively), while three isolates only had nrITS copies from group A1 and one had copies from group A2. The remaining 12 isolates from *P. tridentata* were from Wanderra State Forest, seven of which were sequenced using the nrITS marker; three belonging to group A2 and four belonging to group A1. Three *P. tridentata* isolates from Wanderra State Forest were from the same individual, and the three molecular markers placed these in lineage A (the isolate sequenced with the nrITS marker was in group A1).

Like *P. tridentata*, *S. parviflorus* was only found with lineage A isolates (Table 2C), but it was predominantly associated with isolates within group A1 (Table 3); only one orchid was found with an isolate with nrITS copies from group A2 (Table 3). Six isolates, however, were not sequenced using the nrITS marker. Of the 11 individuals of *S. parviflorus* from Wagonga State Forest, two were lineage A isolates from the same individual orchid; the isolate sequenced with the nrITS marker indicated it was from group A1 (nrITS clade J: Table 3).

DISCUSSION

Do the mycorrhizal associations of *Sarcochilus hillii*, *Plectorrhiza tridentata*, and *Sarcochilus parviflorus* underlie their patterns of phorophyte association? Our work identified two lineages: A (containing subgroups A1 and A2) and B (Fig. 4) of *Ceratobasidium* fungi among the mycorrhizal fungi of these orchid species. As predicted, *S. hillii* was the most generalist among the three orchid species in fungal associates, and *P. tridentata* was intermediate. *Sarcochilus parviflorus* is the species most biased in its tree host association, and it was also the most specialized in its fungal associations: almost exclusively with one mycorrhizal lineage. Fungal groups A1 and A2 appeared not to be restricted in their distribution with regard to tree species, orchid species, or site; group B was found at both of the main sites and at Buckenbowra, but only on one tree species and with one orchid species (Tables 2, 3). Thus, these results support the hypothesis that the mycorrhizal bias of these orchid species is similar in pattern to each species' phorophyte associations; however, it is not clear whether the mycorrhizal specializations are driving the phorophyte specializations.

Having identified these fungal lineages (and verified their physiological capacity to stimulate germination in these orchid

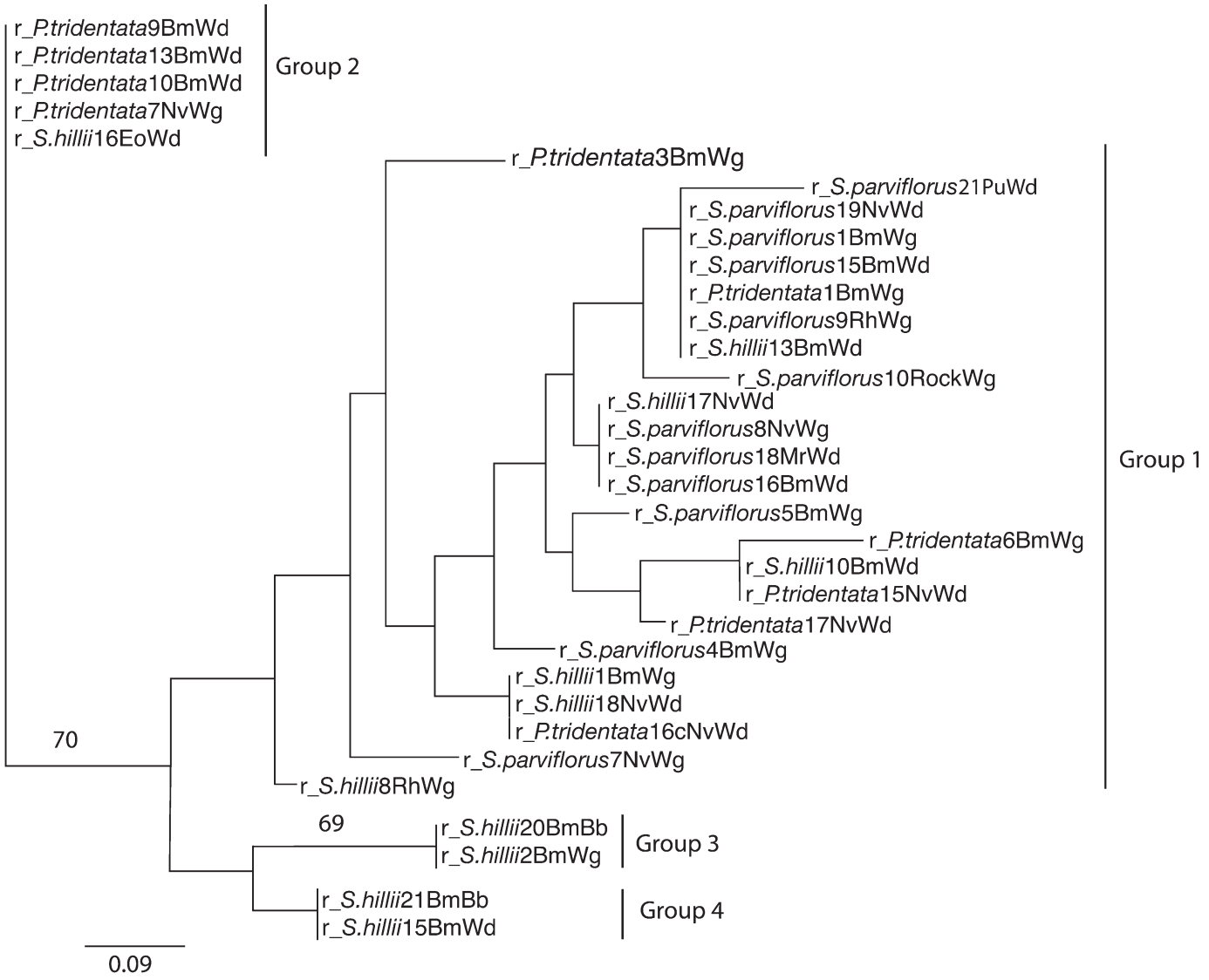


Fig. 3. An unrooted neighbor-joining phenogram of RepPCR results. Bootstrap values greater than 50% indicated above branches. Terminal taxa are representative of individual sequences and are described by "r_" before the orchid species, orchid number, phorophyte, and site information; all abbreviations are provided in Table 1. The letter "c" after *P.tridentata*16 distinguishes this fungal isolate from other isolates of this individual orchid that were analyzed with the other genetic markers.

species in vitro (Gowland, 2009)) a caveat must be stated: natural associations can only be inferred. All current techniques for identification of potential OMF are biased toward particular fungi (Rasmussen, 1995, 2002; Bidartondo et al., 2003; Porras-Alfaro and Bayman, 2007; Taylor and McCormick, 2008) and do not identify bacteria (Wilkinson et al., 1989; Frey-Klett et al., 2007) and/or other fungi that may be involved in the germination process. Furthermore, symbiotic germination in vitro does not necessarily mean that the association would be possible and competitive in situ (Hadley, 1970; Rasmussen, 2002).

In addition to potential identification biases, this investigation confronted considerable technical difficulties uncovering multiple distinct nrITS copies within an isolate. The nrITS region is the marker of choice for OMF research (Dearnaley, 2007); however, our research and that of others (Buckler et al., 1997; Álvarez and Wendel, 2003; Bougoure et al., 2009), dispute its proposed intragenomic uniformity (Bruns et al., 1991; Gardes and Bruns, 1993; Álvarez and Wendel, 2003). In this investigation, up to seven distinct nrITS copies were found in a single isolate (Table 1).

The mycorrhizal fungal community of these orchid species—The results demonstrate that *S. hillii*, *P. tridentata*, and *S. parviflorus* associate with different subsets of the mycorrhizal fungal community, and their pattern of mycorrhizal specificity was similar to their pattern of host specificity; however, the mycorrhizal niches of these orchid species do not adequately explain their host tree niches. All three fungal groups were found on *B. myrtifolia* at the two main sites surveyed (Tables 1, 3) and were also represented in the few samples from other sites (Tables 2, 3). Thus, the biased mycorrhizal associations of *P. tridentata* and *S. parviflorus* do not appear to be due to lack of exposure to all potential OMF. Furthermore, *S. parviflorus*, the orchid species with the narrowest phorophyte and mycorrhizal

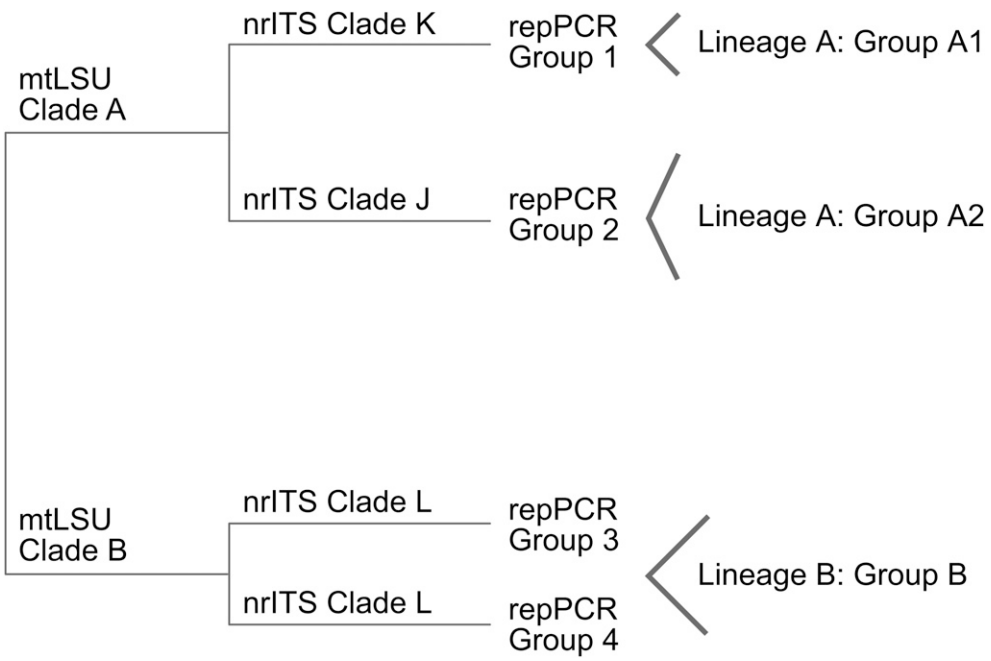


Fig. 4. Cladogram of 23 isolates for which all three genetic markers were used, depicting relationship among robust clades defined by each marker. Robust clades and groups identified in Figs. 1–3 are listed here according to the mtLSU, nrITS, and repPCR data analyses. On the right side of the diagram are the fungal lineage and group assignments.

niche, associated with the most widely distributed group detected, group A1. Together the evidence suggests that like other orchid species (Taylor and Bruns, 1999; Otero et al., 2002, 2004; Taylor et al., 2003, 2004; Irwin et al., 2007, Bonnardeaux et al., 2007), *P. tridentata* and *S. parviflorus* are selectively excluding nontarget mycorrhizal lineages or simply not including them.

In contrast, the ability of *S. hillii* to associate with all lineages of OMF detected suggests that the breadth of its phorophyte associations is due to an ability to associate with a genetically diverse array of fungi. However, lineage B, the lineage that only *S. hillii* associates with, were only isolated from the dominant phorophyte, *B. myrtifolia* (Table 2B). Thus, lineage B isolates do not appear to broaden the host range of this orchid species, although this interpretation may be refuted on broader host sampling.

TABLE 2. Distribution of *Ceratobasidium* fungal lineages across (A) sites, (B) phorophytes, and (C) orchid species. Data are summaried from all three markers: mtLSU, nrITS, and repPCR (Fig. 4).

Level of assessment	Lineage A	Lineage B	Total
A) Site			
Wagonga	25	2	27
Wanderra	32	2	34
Chichester Hill	1	0	1
Buckenbowra	0	2	2
Sassafras	1	0	1
B) Phorophyte			
Backhousia myrtifolia	32	6	38
Notelaea venosa	18	0	18
Other woody phorophyte	7	0	7
Rock	2	0	2
C) Orchid species			
Sarcochilus hillii	14	6	20
Plectorrhiza tridentate	20	0	20
Sarcochilus parviflorus	24	0	24
Sarcochilus falcatus	1	0	1

Interpreting the mycorrhizal associations of these orchid species—We propose that observations of mycorrhizal niche breadth can aid interpretation of the nature of the orchid–mycorrhizal relationship. Obligate species-specific pairwise mutualisms are rare (Howe, 1984; Stanton, 2003); mutualisms are typically predicted to involve generalists (Howe, 1984; Borowicz and Juliano, 1991). Conversely, parasitic, or more generally antagonistic, interactions are thought to occur between a narrower selection of partners, or pairs of species (Borowicz and Juliano, 1991; but see Cameron et al., 2008 and Shefferson et al., 2010 for a potential specialized mutualism), and maintained by reciprocal evolution of the "host" defense and the "parasite" attack mechanisms, a theory known as the Red Queen hypothesis (van Valen, 1973).

In light of these theories, interpretation of the mycorrhizal niches detected here suggests that these orchid–OMF relationships reflect a range of associations from mutualistic to parasitic. With further research, one might expect that the orchid species with the broadest range of associates, *S. hillii*, would have a more mutualistic relationship with its mycorrhizal fungi. Contrastingly, the narrow mycorrhizal niches of *P. tridentata* and *S. parviflorus* might be indicative of more antagonistic associations. Regardless of the nature of these associations, this variability in niche breadth suggests that these mycorrhizal lineages vary in their suitability as OMF for the different orchid species. In an ecological context, variability in partner quality might lead to evolution of partner choice mechanisms (Stanton, 2003), e.g., through chemical communication (Giovannetti and Sbrana, 1998; Bruns et al., 2002).

TABLE 3. Distribution of *Ceratobasidium* fungal groups across sites, phorophytes, and for each of three orchid species: *Sarcochilus hillii*, *Plectorrhiza tridentata*, and *Sarcochilus parviflorus.* In parentheses after fungal group is the number (*N*) of individual isolates for that group. Fungal groupings were defined using the nrITS and/or the repPCR markers (Fig. 4).

		Phorophytes			
Species	Site	*B. myrtifolia* fungal group (*N*)	*N. venosa* fungal group (*N*)	Other woody phorophyte fungal group (*N*)	Rock fungal group (*N*)
S. hillii	Wagonga	A1 (4)	A1 (1)	A1 (1)	—
		A2 (2)	A2 (1)		
		B (2)			
	Wanderra	A1 (3)	A1 (2)	A1 (1)	—
		A2 (1)	A2 (1)		
		B (2)			
P. tridentata	Wagonga	A1 (3)	A1 (1)	—	—
		A2 (2)	A2 (1)		
	Wanderra	A1 (1)	A1 (3)	—	—
		A2 (3)			
S. parviflorus	Wagonga	A1 (3)	A1 (1)	A1 (2)	A1 (1)
		A2 (1)			
	Wanderra	A1 (5)	A1 (2)	A1 (2)	A1 (1)

Mycorrhizal specialization is known to occur across a broad spectrum of orchid species, from mycoheterotrophic (Taylor and Bruns, 1999; Taylor et al., 2003, 2004) to photosynthetic terrestrial (McCormick et al., 2004; Shefferson et al., 2005; Brundrett, 2007), and tropical epiphytic orchid species (Otero et al., 2002, 2004; Martos et al., 2012). It has even been proposed that mycorrhizal specialization is driving speciation in some orchid species (Taylor and Bruns, 1999; Taylor et al., 2003; Otero and Flanagan, 2006, but see Roche et al., 2010). The mycorrhizal specialization detected here suggests that *P. tridentata* and *S. parviflorus* actively exclude lineage B isolates despite co-occurring with this lineage.

An explanation for the mycorrhizal specializations of these orchid species might be that the phorophytes are structurally partitioned and the fungal lineages occupy distinct niches on their phorophyte. Niche differentiation has been invoked to describe how ectomycorrhizal fungi can coexist in species rich assemblages (Bruns, 1995; Dickie, 2007) if the fungi have differing resource requirements and the resources fluctuate over time and space (Bruns, 1995). While different mycorrhizal associations cannot be directly compared (Brundrett, 2002), with the limited information available regarding the ecology of OMF (Otero and Flanagan, 2006), work on other mycorrhizal interactions becomes more pertinent. The occurrence of distinct fungal niches might explain how *S. parviflorus*, an orchid of the more humid niches of a phorophyte (Gowland et al., 2011), does not have as broad a mycorrhizal niche as the increasingly more desiccation-tolerant species, *P. tridentata* and *S. hillii* (Gowland et al., 2011). That is, the observed mycorrhizal specialization may occur because lineage B isolates occupy a drier ecological niche than do lineage A isolates, a niche in which predominantly *S. hillii* occurs.

***Conclusions*—**Our results demonstrate important aspects of the relationships of orchids and their mycorrhizal associates that raise new research possibilities. We found that the distribution of the mycorrhizal fungal community of the three epiphytic orchid species investigated here reflects but does not fully explain their biased phorophyte associations.

It is clear that within the Australian temperate rainforest system, complex patterns of association exist between orchids, their OMF, and their phorophytes. Species display different degrees of specificity at a spatial and organism level, and only through detailed studies can we untangle the complex web of interactions needed to understand broad scale patterns of coevolution among organisms engaged in mutualistic relationships.

We propose that future research into the cause of the specialized mycorrhizal associations detected here should investigate (1) niche differentiation among the mycorrhizal lineages across the ecological niche space on the phorophyte and (2) the nature of the interaction between these orchid species and their mycorrhizal associates.

LITERATURE CITED

ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHÄFFER, J. ZHANG, Z. ZHANG, W. MILLER, AND D. J. LIPMAN. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25: 3389–3402.

ÁLVAREZ, I., AND J. F. WENDEL. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution* 29: 417–434.

ANDERSEN, T. F., AND H. N. RASMUSSEN. 1996. The mycorrhizal species of *Rhizoctonia. In* B. Sneh, S. Jabaji-Hare, S. Neate, and G. Dijst [eds.], *Rhizoctonia* species: Taxonomy, molecular biology, ecology, pathology and disease control, 379–390. Kluwer, Dordrecht, Netherlands.

BATTY, A. L., K.W. DIXON, M. BRUNDRETT, AND K. SIVASITHAMPARAM. 2001. Constraints to symbiotic germination of terrestrial orchid seed in a mediterranean bushland. *New Phytologist* 152: 511–520.

BENNETT, A. E., J. ALERS-GARCIA, AND J. D. BEVER. 2006. Three-way interactions among mutualistic mycorrhizal fungi, plants, and plant enemies: Hypotheses and synthesis. *American Naturalist* 167: 141–152.

BIDARTONDO, M. I., T. D. BRUNS, M. WEISS, C. SERGIO, AND D. J. READ. 2003. Specialized cheating of the ectomycorrhizal symbiosis by an epiparasitic liverwort. *Proceedings of the Royal Society of London, B, Biological Sciences* 270: 835–842.

BONNARDEAUX, Y., M. BRUNDRETT, A. BATTY, K. DIXON, J. KOCH, AND K. SIVASITHAMPARAM. 2007. Diversity of mycorrhizal fungi of terrestrial orchids: Compatibility webs, brief encounters, lasting relationships and alien invasions. *Mycological Research* 111: 51–61.

BOROWICZ, V. A., AND S. A. JULIANO. 1991. Specificity in host–fungus associations: Do mutualists differ from antagonists? *Evolutionary Ecology* 5: 385–392.

BOUGOURE, J., M. LUDWIG, M. BRUNDRETT, AND P. GRIERSON. 2009. Identity and specificity of the fungi forming mycorrhizas with the rare mycoheterotrophic orchid *Rhizanthella gardneri. Mycological Research* 113: 1097–1106.

BRADLEY, R. D., AND D. M. HILLIS. 1997. Recombinant DNA sequences generated by PCR amplification. *Molecular Biology and Evolution* 14: 592–593.

BRUNDRETT, M. 2004. Diversity and classification of mycorrhizal associations. *Biological Reviews of the Cambridge Philosophical Society* 79: 473–495.

BRUNDRETT, M. C. 2002. Coevolution of roots and mycorrhizas of land plants. *New Phytologist* 154: 275–304.

BRUNDRETT, M. C. 2007. Scientific approaches to Australian temperate terrestrial orchid conservation. *Australian Journal of Botany* 55: 293–307.

BRUNS, T. 1995. Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *Plant and Soil* 170: 63–73.

BRUNS, T. D., M. I. BIDARTONDO, AND D. L. TAYLOR. 2002. Host specificity in ectomycorrhizal communities: What do the exceptions tell us? *Integrative and Comparative Biology* 42: 352–359.

BRUNS, T. D., T. J. WHITE, AND J. W. TAYLOR. 1991. Fungal molecular systematics. *Annual Review of Ecology and Systematics* 22: 525–564.

BUCKLER, E. S., A. IPPOLITO, AND T. P. HOLTSFORD. 1997. The evolution of ribosomal DNA: Divergent paralogues and phylogenetic implications. *Genetics* 145: 821–832.

BURGES, A. 1939. The defensive mechanism in orchid mycorrhiza. *New Phytologist* 38: 273–283.

CAMERON, D. D., I. JOHNSON, D. J. READ, AND J. R. LEAKE. 2008. Giving and receiving: Measuring the carbon cost of mycorrhizas in the green orchid, *Goodyera repens. New Phytologist* 180: 176–184.

CAMERON, D. D., J. R. LEAKE, AND D. J. READ. 2006. Mutualistic mycorrhiza in orchids: Evidence from plant–fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens. New Phytologist* 171: 405–416.

CLEMENTS, M. A. 1982. Developments in the symbiotic germination of Australian terrestrial orchids. *In* J. Steward and C. N. Van Der Merwe [eds.], Proceedings of the 10th World Orchid Conference, 1981, Durban, South Africa, 269–273. South African Orchid Council, Edenvale, South Africa.

CLEMENTS, M. A. 1987. Orchid–fungus–host associations of epiphytic orchids. *In* S. Kamezo and R. Tanaka [eds.], Proceedings of the 12th World Orchid Conference, 1987, Tokyo, Japan, 80–83. World Orchid Conference, Inc., Ft. Lauderdale, Florida, USA.

CURRAH, R. S., C. D. ZELMER, S. HAMBLETON, AND K. A. RICHARDSON. 1997. Fungi from orchid mycorrhizas. *In* J. Arditti and A. Pridgeon [eds.], Orchid biology: Reviews and perspectives VII, vol. VII, 117–170. Kluwer , Dordrecht, Netherlands.

DEARNALEY, J. D. W. 2007. Further advances in orchid mycorrhizal research. *Mycorrhiza* 17: 475–486.

DICKIE, I. A. 2007. Host preference, niches and fungal diversity. *New Phytologist* 174: 230–233.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783–791.

FREY-KLETT, P., J. GARBAYE, AND M. TARKKA. 2007. The mycorrhiza helper bacteria revisited. *New Phytologist* 176: 22–36.

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

GIOVANNETTI, M., AND C. SBRANA. 1998. Meeting a non-host: The behaviour of AM fungi. *Mycorrhiza* 8: 123–130.

GONZÁLEZ, D., M. A. CUBETA, AND R. VILGALYS. 2006. Phylogenetic utility of indels within ribosomal DNA and β-tubulin sequences from fungi in the *Rhizoctonia solani* species complex. *Molecular Phylogenetics and Evolution* 40: 459–470.

GOWLAND, K. M. 2009. Investigations of the phorophyte and fungal relationships of three epiphytic Aeridinae orchid species in southeastern Australia. Ph.D. dissertation, Australian National University, Canberra, Australia.

GOWLAND, K. M., J. WOODS, M. A. CLEMENTS, AND A. B. NICOTRA. 2011. Significant phorophyte (substrate) bias is not explained by fitness benefits in three epiphytic orchid species. *American Journal of Botany* 98: 197–206.

GRAHAM, R. R., AND J. D. W. DEARNALEY. 2012. The rare Australian epiphytic orchid *Sarcochilus weinthalii* associates with a single species of *Ceratobasidium. Fungal Diversity* 54: 31–37.

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

HALL, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.

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

HIETZ, P., AND U. HIETZ-SEIFERT. 1995. Intra- and interspecific relations within an epiphyte community in a Mexican humid montane forest. *Selbyana* 16: 135–140.

HOWE, H. F. 1984. Constraints on the evolution of mutualisms. *American Naturalist* 123: 764–777.

HUELSENBECK, J. P., AND F. RONQUIST. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.

IRWIN, M. K., J. J. BOUGOURE, AND J. D. W. DEARNALEY. 2007. *Pterostylis nutans* (Orchidaceae) has a specific association with two *Ceratobasidium* root-associated fungi across its range in eastern Australia. *Mycoscience* 48: 231–239.

KATOH, K., K. KUMA, H. TOH, AND T. MIYATA. 2005. MAFFT version 5: Improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* 33: 511–518.

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

KUMAR S, TAMURA K, NEI M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinformatics* 5: 150–163.

LINDE, C. C., M. ZALA, R. S. D. PAULRAJ, B. A. MCDONALD, AND S. S. GNANAMANICKAM. 2005. Population structure of the rice sheath blight pathogen *Rhizoctonia solani* AG-1 IA from India. *European Journal of Plant Pathology* 112: 113–121.

MARGULIS, L. 1998. The symbiotic planet: A new look at evolution. Phoenix, London, UK.

MARTOS, F., F. MUNOZ, T. PAILLER, I. KOTTKE, C. GONNEAU, AND M. A. SELOSSE. 2012. The role of epiphytism in architecture and evolutinoary constraint within mycorrhizal networks of tropical orchids. *Molecular Ecology* 21: 5098–5109.

MASUHARA, G., AND K. KATSUYA. 1994. *In situ* and *in vitro* specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames. var. *amoena* (M. Bieberstein) Hara (Orchidaceae). *New Phytologist* 127: 711–718.

MCCORMICK, M. K., D. F. WHIGHAM, AND J. O'NEILL. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist* 163: 425–438.

ODELBERG, S. J., R. B. WEISS, A. HATA, AND R. WHITE. 1995. Template-switching during DNA synthesis by *Thermus aquaticus* DNA polymerase I. *Nucleic Acids Research* 23: 2049–2057.

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

OTERO, J. T., J. D. ACKERMAN, AND P. BAYMAN. 2004. Differences in mycorrhizal preferences between two tropical orchids. *Molecular Ecology* 13: 2393–2404.

OTERO, J. T., AND N. S. FLANAGAN. 2006. Orchid diversity—Beyond deception. *Trends in Ecology & Evolution* 21: 64–65.

PÄÄBO, S., D. M. IRWIN, AND A. C. WILSON. 1990. DNA damage promotes jumping between templates during enzymatic amplification. *Journal of Biological Chemistry* 265: 4718–4721.

POLIS, G. A., A. L. W. SEARS, G. R. HUXEL, D. R. STRONG, AND J. MARON. 2000. When is a trophic cascade a trophic cascade? *Trends in Ecology & Evolution* 15: 473–475.

PORRAS-ALFARO, A., AND P. BAYMAN. 2007. Mycorrhizal fungi of *Vanilla*: Diversity, specificity and effects on seed germination and plant growth. *Mycologia* 99: 510–525.

POSADA, D. 2000. How does recombination affect phylogeny estimation? *Trends in Ecology & Evolution* 15: 489–490.

POSADA, D., AND K. A. CRANDALL. 1998. MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

RAMSEY, F. L., AND D. W. SCHAFER. 2002. The statistical sleuth: A course in methods of data analysis, 2nd ed. Duxbury, California, USA.

RASMUSSEN, H. N. 1995. Terrestrial orchids from seed to mycotrophic plant, 1st ed. Cambridge University Press, Melbourne, Australia.

RASMUSSEN, H. N. 2002. Recent developments in the study of orchid mycorrhiza. *Plant and Soil* 244: 149–163.

ROBERTS, P. 1999. *Rhizoctonia*-forming fungi: A taxonomic guide. Royal Botanic Gardens, Kew, UK.

ROCHE, S., R. J. CARTER, R. PEAKALL, L. M. SMITH, M. R. WHITEHEAD, AND C. C. LINDE. 2010. A narrow group of monophyletic *Tulasnella* (Tulasnellaceae) symbiont lineages are associated with multiple species of *Chiloglottis* (Orchidaceae): Implications for orchid diversity. *American Journal of Botany* 97: 1313–1327.

RONQUIST, F., AND J. P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

SAPP, J. 2004. The dynamics of symbiosis: An historical overview. *Canadian Journal of Botany* 82: 1046–1056.

SHEFFERSON, R. P., C. C. COWDEN, M. K. MCCORMICK, T. YUKAWA, Y. OGURA-TSUJITA, AND T. HASHIMOTO. 2010. Evolution of host breadth in broad interactions: Mycorrhizal specificity in East Asian and North American rattlesnake plantains (*Goodyera* spp.) and their fungal hosts. *Molecular Ecology* 19: 3008–3017.

SHEFFERSON, R. P., M. WEISS, T. KULL, AND D. L. TAYLOR. 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Molecular Ecology* 14: 613–626.

SMITH, S. E., AND D. J. READ. 1997. Mycorrhizal symbiosis, 2nd ed. Academic Press, London, UK.

STALPERS, J. A., AND T. F. ANDERSEN. 1996. A synopsis of the taxonomy of teleomorphs connected with *Rhizoctonia* s.l. *In* B. Sneh, S. Jabaji-Hare, S. Neate, and G. Dijst [eds.], *Rhizoctonia* species: Taxonomy, molecular biology, ecology, pathology and disease control, 49–63. Kluwer, Dordrecht, Netherlands.

STANTON, M. L. 2003. Interacting guilds: moving beyond the pairwise perspective on mutualisms. *American Naturalist* 162: S10–S23.

STRAUSS, S. Y., AND R. E. IRWIN. 2004. Ecological and evolutionary consequences of multispecies plant–animal interactions. *Annual Review of Ecology and Systematics* 35: 435–466.

SUAREZ, J. P., M. WEISS, A. ABELE, S. GARNICA, F. OBERWINKLER, AND I. KOTTKE. 2006. Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. *Mycological Research* 110: 1257–1270.

SWOFFORD, D. L. 2003. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer, Sunderland, Massachusetts, USA.

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

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

TAYLOR, D. L., T. D. BRUNS, AND S. A. HODGES. 2004. Evidence for mycorrhizal races in a cheating orchid. *Proceedings of the Royal Society of London, B, Biological Sciences* 271: 35–43.

TAYLOR, D. L., T. D. BRUNS, T. M. SZARO, AND S. A. HODGES. 2003. Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. *American Journal of Botany* 90: 1168–1179.

TAYLOR, D. L., AND M. K. MCCORMICK. 2008. Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. *New Phytologist* 177: 1020–1033.

THOMPSON, J. N. 1999. The evolution of species interactions. *Science* 284: 2116–2118.

TREMBLAY, R. L. 1997. Distribution and dispersion patterns of individuals in nine species of *Lepanthes* (Orchidaceae). *Biotropica* 29: 38–45.

TREMBLAY, R. L., J. K. ZIMMERMAN, L. LEBRON, P. BAYMAN, I. SASTRE, F. AXELROD, AND J. ALERS-GARCIA. 1998. Host specificity and low reproductive success in the rare endemic Puerto Rican orchid *Lepanthes caritensis*. *Biological Conservation* 85: 297–304.

VAN VALEN, L. 1973. A new evolutionary law. *Evolutionary Theory* 1: 1–30.

VERSALOVIC, J., T. KOEUTH, AND J. R. LUPSKI. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* 19: 6823–6831.

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

WARCUP, J. H., AND P. H. B. TALBOT. 1980. Perfect states of *Rhizoctonias* associated with orchids. III. *New Phytologist* 86: 267–272.

WESTON, P. H., A. J. PERKINS, AND T. J. ENTWISLE. 2005. More than symbioses: Orchid ecology, with examples from the Sydney region. *Cunninghamia* 9: 1–15.

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

WILKINSON, K. G., K. W. DIXON, AND K. SIVASITHAMPARAM. 1989. Interaction of soil bacteria, mycorrhizal fungi and orchid seed in relation to germination of Australian orchids. *New Phytologist* 112: 429–435.

ZOTZ, G., AND S. SCHULTZ. 2008. The vascular epiphytes of a lowland forest in Panama—Species composition and spatial structure. *Plant Ecology* 195: 131–141.