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A NARROW GROUP OF MONOPHYLETIC *TULASNELLA* (TULASNELLACEAE) SYMBIONT LINEAGES ARE ASSOCIATED WITH MULTIPLE SPECIES OF *CHILOGLOTTIS* (ORCHIDACEAE): IMPLICATIONS FOR ORCHID DIVERSITY[1]

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- *Premise of the study:* The Orchidaceae is characterized by exceptional species diversity. Obligate orchid mycorrhizae are predicted to determine orchid distributions, and highly specific relationships between orchids and fungi may drive orchid diversification. In this study, mycorrhizal diversity was examined in the terrestrial, photosynthetic orchid genus *Chiloglottis* to test the hypothesis of mycorrhizal-mediated diversification in the genus *Chiloglottis.* This orchid genus secures pollination by sexual deception, an obligate and highly specific pollination strategy. Here we asked whether the obligate orchid–fungal interactions are also specific.
- *Methods:* Two sequenced loci, the internal transcribed spacer region (ITS) and mitochondrial large subunit (mtLSU), were used to identify fungal isolates and assess fungal species diversity. Symbiotic germination of two species *Chiloglottis* aff. *jeanesii* and *C. valida* were used to assess germination potential of isolates and confirm mycorrhizal association.
- *Key results:* Phylogenetic analyses revealed that six representative *Chiloglottis* species spanning a broad survey of the genus were all associated with a narrow group of monophyletic *Tulasnella* fungal lineages.
- *Conclusions:* The *Chiloglottis–Tulasnella* interaction appears to be the first known case of such a narrow symbiont association across a broadly surveyed orchid genus. It appears that the specific pollination system of *Chiloglottis*, rather than specific orchid–fungal interactions has been the key driving force in the diversification of the genus. These findings also indicate that plant groups with highly specific mycorrhizal partners can have a widespread distribution.

Key words: ectomycorrhizae; *Chiloglottis*; ITS; mtLSU; molecular phylogeny; orchid mycorrhizal fungi; Orchidaceae; Tulasnellaceae.

Coevolution involving intimate interactions between two different groups of organisms is proposed to be a key driving force in speciation, especially when relationships display a high level of specificity such as host–parasite relationships and specific pollination systems (Barrett, 1986; Thompson, 1986; Weiblen, 2004). The Orchidaceae represents one of the most diverse plant families, and it has been proposed that this diversity could, in part, be driven by the often-specific interactions with animal pollinators (Peakall, 2007; Scopece et al., 2007; Peakall et al., 2010). It has been predicted that this diversity has been strongly influenced by the unique features of orchid reproduction and the associated specific interactions with insect pollinators (Cozzolino and Widmer, 2005). The below-ground orchid interactions involving symbiotic fungi, which in some cases demonstrate high levels of specificity, may have also played a key role in speciation within certain orchid groups (Taylor et al., 2003; Otero and Flanagan, 2006; Waterman and Bidartondo, 2008). Documenting the underlying patterns of orchid-fungal interactions is therefore critical for a more complete understanding of the evolution of orchid diversity.

The minute, wind-dispersed seeds of orchids are undifferentiated and do not store sufficient nutrition for unaided development of the embryo (Arditti, 1992). Thus, all orchids require mycorrhizal fungi, at least for germination and early seedling phases (Warcup, 1990; Arditti, 1992). During the early seedling phase, they may be considered parasitic on their fungal symbionts because nutrients are directed exclusively to the plant (Hadley, 1970; Waterman and Bidartondo, 2008; Rasmussen and Rasmussen, 2009). Even as photosynthetic adults, many terrestrial species may still rely on fungal-mediated transfer of nutrients and carbon, especially in forests where light can be limited (Bidartondo, 2005; Cameron et al., 2006). Whether this relationship remains parasitic in later growth stages may vary according to the photosynthetic ability of the orchid (Cameron et al., 2006, 2009).

Most orchids are photosynthetic, and there is no evidence that they obtain a majority of their energy from the fungi, except at the early stages in development following germination (Taylor, 2004; Rasmussen and Rasmussen, 2009). However, mutualistic orchid–fungal associations have been confirmed by radioactively labeled carbon and phosphorous studies, where bidirectional flow of carbon has been observed between fungal symbionts and the orchid *Goodyera repens* (Cameron et al., 2006, 2007). More species need to be investigated to determine whether this bidirectional nutrient flow is a general pattern in

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the trophic interactions between green orchids and fungi. If so, an association of mycorrhizae with orchids may provide a long-term benefit to both partners, and the exchange of limiting compounds may broaden the ecological range of either partner and overcome physiological stresses (Arditti, 1992; Bidartondo, 2005). Interactions between photosynthetic orchids and their mycorrhizae may therefore represent a complex and dynamic relationship between parasitism and mutualism, which can change across the life cycle of the host/symbiont and in response to changes in environmental conditions (Harley and Smith, 1983; Arditti, 1992; Hynson et al., 2009).

Highly specific relationships with obligate fungal partners have been proposed as a possible driving force in the diversification of some photosynthetic orchids (Otero and Flanagan, 2006; Shefferson et al., 2007), nonphotosynthetic orchids (Taylor et al., 2003), and members of the Monotropoideae (Bidartondo and Bruns, 2001). However, the occurrence of species-specific orchid–fungal relationships is unlikely to be a consequence of cospeciation (reciprocal speciation as a consequence of the interaction) (Thompson, 1986; Taylor et al., 2003) because orchid mycorrhizae are not dependent on orchids for reproduction and dispersal and can survive as either saprophytes (e.g., *Tulasnella* species [Roberts, 1999]) or parasites (e.g., *Armillaria mellea* [Rasmussen, 2002]). One proposed mechanism for fungal-driven speciation is analogous to "mixed-process coevolution" (Thompson, 1986), by which changes in specificity of orchid species for compatible fungi are predicted to result in reproductive isolation among plant hosts with different specific fungi. However, in their recent review, Waterman and Bidartondo (2008) stated that a direct link between mycorrhizal specificity and reproductive isolation of the plant host is hard to envision.

A second mechanism for fungal driven speciation has been suggested in which mycorrhizal specialization has indirect consequences for the diversity of host species by determining orchid distributions (Otero et al., 2004; Otero and Flanagan, 2006; Waterman and Bidartondo, 2008). Under this scenario, it is postulated that the often widespread but patchy distributions of orchid populations are an indirect consequence of narrow mycorrhizal associations and patchy fungal distributions. If gene flow between such populations is limited, as evident for some epiphytic species (Tremblay and Ackerman, 2001), a combination of drift and episodic selection may drive speciation (Otero et al., 2004; Waterman and Bidartondo, 2008).

The first step in testing whether fungal interactions play a role in orchid speciation is to investigate whether there is fungal specificity between orchids and their obligate mycorrhizal partners. Furthermore, teasing out the role of fungi in speciation is best done in those systems for which both fungal and pollinator interactions are well understood (Waterman and Bidartondo, 2008). In this study, we targeted the much-studied photosynthetic, Australian, terrestrial orchid genus *Chiloglottis*, a genus that uses the unusual pollination strategy of sexual deception, attracting male pollinators by mimicry of their sex pheromones (Schiestl et al., 2003; Franke et al., 2009). The pollination strategy of sexual deception is particularly well developed within Australian sexually deceptive orchids with more than 150 species across multiple genera involved (Peakall, 1990; Peakall and Beattie, 1996; Bower and Brown, 2009; Phillips et al., 2009). *Chiloglottis* is one of the largest and most widespread Australian sexually deceptive orchid genera with 23 described species and likely more than 30 species when cryptic taxa are taken into account (Peakall et al., 2002; Bower, 2006; Bower and Brown, 2009).

As is generally the case for sexually deceptive orchids, pollinator interactions with *Chiloglottis* are highly specific; a one to one relationship between orchid and pollinator is typical for a given site (Bower, 1996, 2006; Bower and Brown, 2009), although some pollinator sharing the edge of a species range is known (Peakall et al., 2002). The discovery of the specific chemicals representing a new class of compounds used by *Chiloglottis* orchids to lure their male pollinators (Schiestl et al., 2003; Franke et al., 2009; Peakall et al., 2010) confirms that pollinator specificity has a strong chemical basis. It has been hypothesized that changes in floral chemistry that enable pollinator switching, combined with subsequent pollinator-mediated selection, have played a key role in the evolution and diversification of *Chiloglottis* (Mant et al., 2002, 2005; Peakall et al., 2010).

Despite this extensive work on pollinator specificity, it is important that the potential role of below-ground interactions—the obligate interaction between orchid and fungal mycorrhizal associates (Waterman and Bidartondo, 2008)—is not neglected in studies of orchid diversification. Therefore, the overarching question we ask in this study is what role, if any, might specific fungal relationships have played in the diversification of the orchid genus *Chiloglottis?* We attempted to answer this by addressing four specific questions: (1) Which fungi and how many fungal species are involved in partnership with six *Chiloglottis* species strategically chosen to represent the phylogenetic breadth across the genus? (2) What is the germination potential of the fungal isolates associated with *Chiloglottis* species? (3) What are the phylogenetic relationships of the fungal symbionts? (4) Finally, what are the implications of our findings for the current hypotheses on the diversification of *Chiloglottis* and for other orchids more generally?

MATERIALS AND METHODS

Sample collection—All members of the genus *Chiloglottis* are small terrestrial herbs that grow as colonies in moist, sheltered locations in the open forests of the coast and the Great Dividing Range of eastern Australia. Several species are also found in Tasmania. Plants are characterized by two opposite leaves that are usually prostrate on the soil surface. Single flowers are inconspicuous and dull-colored on a stem 3–15 cm high. All species go through a dormancy phase over either summer or winter, depending on the flowering time of the species. Clonal colonies are formed as a result of vegetative reproduction when more than one daughter tuber is produced in a season (Peakall et al., 1997).

For this study, six species were selected to span the phenological and phylogenetic diversity across *Chiloglottis* s.l. as revealed by phylogenetic analysis (Mant et al., 2002; Peakall et al., 2010). Note that in some recent taxonomic treatments (e.g., Jones, 2006), *Chiloglottis* has been split into three genera. However, for nomenclatural continuity among the many publications on this group and because these taxonomic changes are not universally accepted, we retain the genus name *Chiloglottis*. Three species represented the autumn-flowering Reflexa group: *C. diphylla* R.Br., *C. reflexa* (Labill.) Druce, *C. seminuda* D.L. Jones. One species represented the spring-flowering Formicifera group: *C. trapeziformis* Fitzg. Two species represented the spring/summer-flowering Valida group: *C. valida* D.L. Jones and *C.* aff. *jeanesii*. Adult *Chiloglottis* plants were collected from field sites in New South Wales (NSW) and the Australian Capital Territory (ACT), Australia (Table 1, Fig. 1). Voucher specimens of the fungi are stored in the culture collection of the author's laboratory at the Australian National University, Canberra (ANU; see Appendix 1).

The species selected are broadly sympatric in at least parts of their range and at some sites multiple species may co-occur. For example, at least two species each within the Reflexa and Valida groups may co-occur. Furthermore, both groups may be found at the one site, for example *C. valida* and *C.* aff. *jeanesii* frequently co-occur and flower together in October.

Fungal isolation—Isolations were conducted within 1 to 7 d after the plants were collected from the field. Peloton-rich zones at the collar region of the tuber

TABLE 1. *Chiloglottis* species and collection details from which mycorrhizal pelotons were cultivated: Colony = plant colony code; Abbrev. = site name abbreviation; No. of plants = number of plants from which pelotons were successfully germinated; No. of mtLSU haplyptes = number of mtLSU haplotypes obtained from/number of fungal isolates sequenced; No. of ITS clonal types = number of ITS clonal types obtained from/number of fungal isolates sequenced; * = indicates fungal DNA amplified from plant tissue for *Tulasnella* isolates; ACT = Australian Capital Territory; NSW = New South Wales

Colony	Site name	Abbrev.	*Chiloglottis* species	GPS	Collection date	Region	No. of plants	No. of mtLSU haplotypes	No. of ITS haplotypes
0505	Bilpin Park Site 1	BP1	*C. diphylla*	S33.51549 E150.48861	31/3/07	Blue Mtns., Bells Line Rd, NSW	1	1/2	1/1
07054	Bilpin Park Site 1	BP1	*C. diphylla*	S33.51549 E150.48861	31/3/07	Blue Mtns., Bells Line Rd, NSW	3	2/3	—
07053	Bilpin Park Site 2	BP2	*C. diphylla*	S33.49537 E150.51754	31/3/07	Blue Mtns., Bells Line Rd., NSW	1	1/1	—
07050	Kurrajong Heights	KH	*C. diphylla*	S33.54073 E150.63308	31/3/07	Blue Mtns., Bells Line Rd., NSW	1	1/1	—
07048	Kurrajong Heights	KH	*C. diphylla*	S33.54073 E150.63308	31/3/07	Blue Mtns., Bells Line Rd., NSW	1	1/1	—
07071	Mt. Irvine Rd. Mt. Wilson	MI	*C. reflexa*	S33.49979 E150.41446	1/4/07	Mt Wilson, Blue Mtns., NSW	1	1/1	—
07072	Mt. Irvine Rd. Mt. Wilson	MI	*C. reflexa*	S33.49979 E150.41446	1/4/07	Mt Wilson, Blue Mtns., NSW	1	1/2	—
07061	Wynnes Lookout, Mt Wilson	WL	*C. reflexa*	S33.52118 E150.37075	1/4/07	Mt Wilson, Blue Mtns., NSW	2	1/3	1/1
07033–45	Hanging Rock Rd.	HR	*C. seminuda*	S34.62842 E150.19429	31/3/07	Penrose Forest, Exeter, NSW	1	1/1	1/1
07033	Hanging Rock Rd.	HR	*C. seminuda*	S34.62842 E150.19429	31/3/07	Penrose Forest, Exeter, NSW	4	1/8	2/2
SRBG01	Australian National Botanic Gardens	ANBG	*C. trapeziformis*	S35.27514 E149.10973	5/9/07	Black Mountain, ACT	2	1/5	3/3
SRBG03	Australian National Botanic Gardens	ANBG	*C. trapeziformis*	S35.27514 E149.10973	18/9/08	Black Mountain, ACT	4	—	6/9*
SRBM01	Black Mountain	BM	*C. trapeziformis*	S35.27496 E149.09765	18/9/08	Black Mountain, ACT	1	—	2/2*
CM07	Black Mountain	BM	*C. trapeziformis*	S35.27496 E149.09765	29/12/06	Black Mountain, ACT	2	1/4	2/4
CP0835	Boyd River fire trail	BRFT	C. aff. *jeanesii*	S33.94092 E150.05520	29/10/07	Kanangra Boyd Natl. Park, NSW	4	2/8	11/12
06082	Lowden Rd.	LR	*C.* aff. *jeanesii*	S35.50567 E149.53510	4/10/06	Tallaganda State Forest, NSW	1	1/2	3/4
CV0836	Boyd River fire trail	BRFT	*C. valida*	S33.94092 E150.05520	29/10/07	Kanangra Boyd Natl. Park, NSW	2	1/2	1/1
CV0627	Lowden Rd.	LR	*C. valida*	S35.50567 E149.53510	4/10/06	Tallaganda State Forest, NSW	1	1/2	1/1
Total		10	6				33	3	35

and rhizome were rinsed successively with sterilized distilled water in three petri dishes to remove surface contaminants. Rinsed tissue was then macerated in sterile distilled water containing 50 mg/mL streptomycin sulfate. Single pelotons as well as small plant tissue sections containing pelotons were transferred to fungal isolation media (FIM) agar plates (Clements and Ellyard, 1979) and incubated at room temperature in the dark. After 1 wk, germinating pelotons were transferred to new FIM plates. Even though tubers were not surface-sterilized before peloton extraction, only 5% of isolations were deemed contaminated by obvious contaminants such as *Penicillium*. All colonies were hyphal-tipped after three weeks to ensure single colonies. Cultures were stored on sterilized wheat and FIM agar slants covered with mineral oil at 5°C.

DNA extraction and sequence analysis—For DNA extraction, fungal isolates were grown in FIM (Clements and Ellyard, 1979) broth in petri dishes. Petri dishes were inoculated with 10–20, 1 mm agar blocks cut from colony edges of isolates grown on FIM agar and incubated at room temperature in the dark for 2 wk. In cases where isolates did not grow in FIM broth, DNA was extracted directly from agar cultures. The DNA extraction followed a protocol for lyophilized mycelium, using cetyltrimethylammonium bromide (CTAB) (Lee and Taylor, 1990).

To investigate the presence of fungal species that could not be cultured in vitro, we extracted DNA from *C. trapeziformis* tissue ($N = 4$ from site ANBG, $N = 1$ from BM) (Table 1) using Qiagen DNeasy Plant mini kit (Amersham Biosciences, Hilden, Germany) according to the manufacturer's instructions. Soil contamination was minimized by cleaning and sectioning plants as described before, leaving peloton-dense sections for DNA extraction.

A range of primer combinations were initially assessed to amplify mycorrhizal nuclear and mitochondrial ribosomal DNA (rDNA). The nuclear rDNA loci, which included the internal transcribed spacer region (ITS), were initially amplified using universal primers ITS1 and ITS4 (White et al., 1990). ITS primers specifically designed to amplify certain groups of orchid mycorrhizae included genus-specific primers CeTh1 and CeTh4 (Porras-Alfaro and Bayman, 2007) for *Ceratobasidium* and *Thanatephorus*, ITS1OF and ITS4OF (Taylor and McCormick, 2008), as well as *Tulasnella*-specific ITS1 and ITS4-tul (Taylor, 1997) and Tul1 and Tul4 (Porras-Alfaro and Bayman, 2007). For the mitochondrial large subunit, primer combinations ML7 and ML8 (White et al., 1990), as well as MLin3 (Bruns et al., 1998) and ML6 (White et al., 1990) were tested. Each 20-μL PCR reaction contained 2 μL PCR buffer (Scientifix, Melbourne, Australia); 25 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer; 0.5 U *Taq* polymerase (Scientifix) and 20–100 ng template DNA.

The following thermal cycling parameters were used in an Eppendorf Mastercycler (Hamburg, Germany) for the loci that amplified consistently, i.e., ITS1-4-tul, ITS1OF-ITS4OF, and MLIN3-ML6: initial denaturing for 2 min at 95°C; followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, elongation for 1 min at 72°C; and a final extension for 10 min at

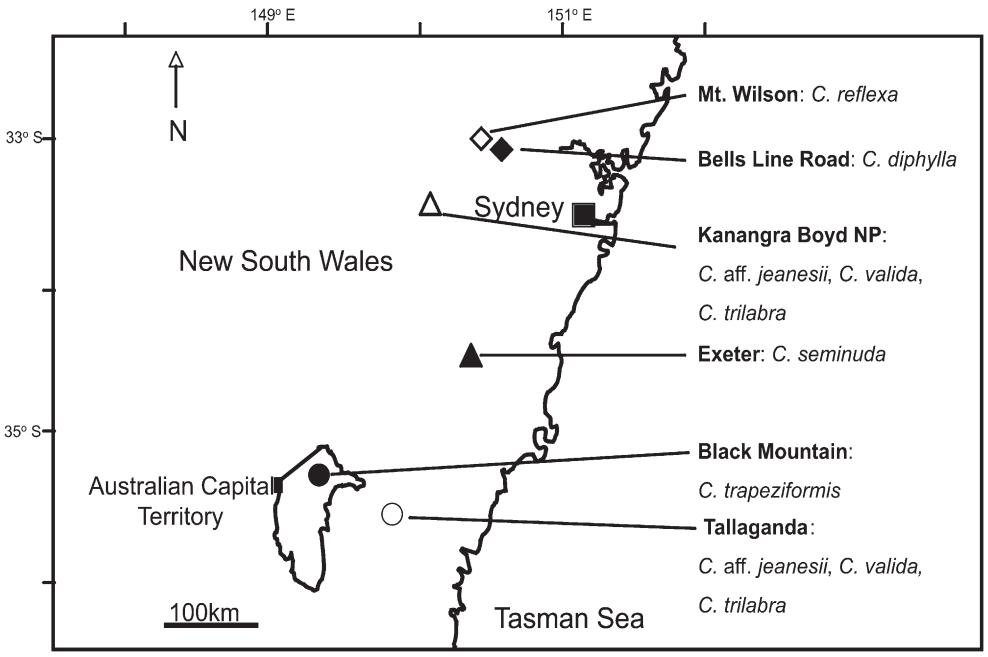

Fig. 1. Collection regions of *Chiloglottis* species from New South Wales (NSW) and the Australian Capital Territory (ACT), Australia, for mycorrhizal isolations.

72°C. Amplification products were electrophoresed on 1% agarose gels to verify the amplification of a single fragment of the appropriate length.

Because the ITS is a multicopy gene region and multiple copies were observed in the sequencing analysis, PCR products were cloned using pGem-T Easy Vector kit (Promega, Wisconsin, USA) according to the manufacturer's instructions. A list of isolates cloned in this study is provided in Table 2. Five white colonies per isolate were picked and cultured overnight in *E. coli* freezing medium [4.4% glycerol, 8.21 g K_2HPO_4/L, 1.80 g KH_2PO_4/L; 0.50 g Na_3 citrate /L; 0.10 g $MgSO_4 \cdot 7\ H_2O$ /L, 0.90 g $(NH_4)_2SO_4$/L; 100 mg ampicillin/L] to grow ample quantities for PCR. Colony PCR was carried out using 2-μL cell suspension as template and SP6 and T7 promoter primers in 20-μL reactions.

The PCR products were purified of inhibitory PCR reagents using Promega Wizard SV gel and PCR Clean-up System and ExoSAP-IT (GE Healthcare, Piscataway, New Jersey, USA) according to the manufacturer's instructions. Extension products were purified using an ethanol/EDTA/sodium acetate precipitation protocol according to the BigDye Terminator v3.1 sequencing kit instructions (Applied Biosystems, Foster City, California, USA). Products were sequenced bidirectionally with ABI PRISM BigDye Terminator v3.1 sequencing kit (Applied Biosystems) on an ABI-3100 automated sequencer. Sequences were edited using the program Sequencher v. 4.8 (GeneCodes, Ann Arbor, Michigan, USA) to correct for base read ambiguities. BLAST searches were conducted on all obtained sequences to establish the closest relatives represented in the GenBank database.

Phylogenetic analysis—BLAST searches were conducted on the consensus sequences of the obtained mtLSU and ITS data set (http://www.ncbi.nlm.nih.gov/BLAST), and GenBank sequences of the top BLAST hits were downloaded and included in the alignment. The multiple sequence alignment was conducted using an anchored multiple alignment with the program DIALIGN (Morgenstern et al., 2006). The alignment was manually adjusted using the program SE-AL v2.0a11 (Rambaut, 1996). Because of rapid evolution in the ITS, alignments were conducted with only highly similar sequences from GenBank. In the ITS alignment, none of the regions were excluded; highly variable regions in the outgroups that shared very little sequence in common with the ingroup were assumed to be either an insertion or a deletion and were offset. These indels contained very little information; therefore, indel coding was not applied.

Phylogenies were estimated with a maximum likelihood analysis using the program RAxML 7.0.4 (Stamatakis et al., 2008) as implemented at the Cyberinfrastructure for Phylogenetic Research (CIPRES) web portal 1.0 (http://www.phylo.org/sub_sections/portal) and Bayesian inference using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). A GTR+G model was used for all analyses because all other models are nested inside this model. Support for nodes was assessed for ML trees using nonparametric bootstrapping in RAxML (Stamatakis et al., 2008) and with Bayesian posterior probabilities (PP). The default parameters were used for both methods, with convergence and burn-in assessed according to the authors' recommendations (Ronquist and Huelsenbeck, 2003; Stamatakis et al., 2008).

The null hypothesis of no association between ITS haplotypes and orchid species *C. trapeziformis* and *C.* aff. *jeanesii* was tested with a contingency χ^2 test (Everitt, 1977).

Orchid germination trials—To demonstrate germination potential of isolated fungi, we used three isolates for germination trials on seed pods of *C.* aff. *jeanesii* ($N = 6$) and *C. valida* ($N = 4$) collected from Kanangra Boyd National Park, NSW, Australia. Fungal isolates used in germination trials were 07061.III.7 from *C. reflexa* (Wynnes Lookout), CV0627.II.2 (Lowden Rd.) from *C. valida*, and 06082.II.1 from *C.* aff. *jeanesii* (Lowden Rd.).

Before plating out the seed for symbiotic germination, we assessed the percentage of testa with embryos using carmine acetate staining. All seed batches used for seed germination trials contained seed with a high presence of embryos (80% or higher).

To control for microbial contamination and enhance diffusion of water through removal of suberin, sodium hypochlorite solution was used to surface-sterilize seed. Seed from pods were mixed by inversion with 1 mL of a 1% NaOCl solution in 1.5 mL Eppendorf tubes for 10 s. After soaking the seeds in the sterilizing solution for four minutes with inversion, seeds were centrifuged at 13 000 × g, for three minutes and the supernatant removed. This process was repeated three times. Seed was left to incubate overnight in the last rinse before the rinse solution was removed by centrifugation. Sterile distilled water (1.2 mL) was added to the seed, and aliquots of 400 μL of seed suspended in solution were transferred to fungal oatmeal agar (OMA) (Clements and Ellyard, 1979). Two negative control plates were used for each seed treatment, which did not contain any fungal isolates. One plate was used for each fungal–seed-pod treatment. Plates were incubated at 22°C in the dark to assist seed germination.

After 6 mo, total seed numbers were counted on every OMA plate. Only seeds that contained an intact embryo were included in the total seed counts. Seeds that developed into protocorms with leaves were considered germinated (stage 4: enlargement of protocorm with first green leaf and stage 5: seedling with green leaf and initiation of dropper) (Ramsay et al., 1986) (Fig. 2). Percentage seed germinated was calculated by dividing the number of stage 4 and stage 5 seeds by the total number of seeds per plate. A contingency χ^2 test

TABLE 2. *Tulasnella* isolates and their ITS and mtLSU haplotype as associated with six *Chiloglottis* species collected from sites in New South Wales and the Australian Capital Territory, Australia. Roman numerals of isolates refer to individual plants from which fungi were collected, followed by isolate number, then ITS clone number where applicable**:** — = no sequence available; ■■■ = fungal sequences amplified from plant tissue; * = Non-*Tulasnella* sequence

Isolate	*Chiloglottis* species	Site	ITS haplotype no.	mtLSU haplotype no.
06082.I.1.1	*C.* aff. *jeanesii*	LR	21	2
06082.I.1.2	*C.* aff. *jeanesii*	LR	22	—
06082.I.1.3	*C.* aff. *jeanesii*	LR	22	—
06082.I.3	*C.* aff. *jeanesii*	LR	1	2
CP0835.I.1.1	*C.* aff. *jeanesii*	BRFT	33	3
CP0835.I.1.2	*C.* aff. *jeanesii*	BRFT	28	—
CP0835.I.1.3	*C.* aff. *jeanesii*	BRFT	28	—
CP0835.I.2	*C.* aff. *jeanesii*	BRFT	—	3
CP0835.III.1.1	*C.* aff. *jeanesii*	BRFT	33	3
CP0835.III.1.3	*C.* aff. *jeanesii*	BRFT	27	3
CP0835.III.2	*C.* aff. *jeanesii*	BRFT	—	3
CP0835.VIII.2.1	*C.* aff. *jeanesii*	BRFT	24	2
CP0835.VIII.2.2	*C.* aff. *jeanesii*	BRFT	26	—
CP0835.VIII.2.3	*C.* aff. *jeanesii*	BRFT	25	—
CP0835.IX.1.3	*C.* aff. *jeanesii*	BRFT	30	3
CP0835.IX.2.1	*C.* aff. *jeanesii*	BRFT	31	3
CP0835.IX.2.2	*C.* aff. *jeanesii*	BRFT	32	—
CP0835.IX.2.3	*C.* aff. *jeanesii*	BRFT	28	—
CP0835.IX.3.2	*C.* aff. *jeanesii*	BRFT	34	3
CP0835.IX.3.3	*C.* aff. *jeanesii*	BRFT	35	—
CV0836.I.1	*C. valida*	BRFT	20	3
CV0836.II.1	*C. valida*	BRFT	—	3
CV0627.II.1	*C. valida*	LR	23	2
CV0627.II.2	*C. valida*	LR	—	2
505.III.1	*C. diphylla*	BP1	—	2
505.III.5	*C. diphylla*	BP1	11	2
07054.I.1	*C. diphylla*	BP1	—	2
07054.II.1	*C. diphylla*	BP1	—	1
07054.VI.1	*C. diphylla*	BP1	—	2
07050.I.1	*C. diphylla*	BP1	10	1
07048.I.1	*C. diphylla*	KH	—	2
07053.III.1	*C. diphylla*	BP2	—	2
07061.I.1	*C. reflexa*	WL	9	2
07061.I.7	*C. reflexa*	WL	—	2
07061.III.7	*C. reflexa*	WL	—	2
07071.I.1	*C. reflexa*	MI	—	2
07072.II.3	*C. reflexa*	MI	—	2
07072.II.4	*C. reflexa*	MI	—	2
07033.I.1	*C. seminuda*	HR	—	2
07033.I.2	*C. seminuda*	HR	—	2
07033.II.1	*C. seminuda*	HR	—	2
07033.II.2.1	*C. seminuda*	HR	5	2
07033.II.2.2	*C. seminuda*	HR	7	—
07033.II.3	*C. seminuda*	HR	—	2
07033.III.1	*C. seminuda*	HR	—	2
07033-45.I.2	*C. seminuda*	HR	19	2
07033.IV.1	*C. seminuda*	HR	—	2
07033.IV.2	*C. seminuda*	HR	—	2
CM07.I.2	*C. trapeziformis*	BM	—	2
CM07.I.5	*C. trapeziformis*	BM	15	2
CM07.I.9	*C. trapeziformis*	BM	—	2
CM07.I.10	*C. trapeziformis*	BM	18	2
CM07.II.1.1	*C. trapeziformis*	BM	2	2
CM07.II.1.2	*C. trapeziformis*	BM	2	—
CM07.II.1.3	*C. trapeziformis*	BM	2	—
SRBG01.I.1	*C. trapeziformis*	ANBG	—	2
SRBG01.II.1	*C. trapeziformis*	ANBG	29	2
SRBG01.II.2	*C. trapeziformis*	ANBG	—	2
SRBG01.II.3.1	*C. trapeziformis*	ANBG	14	2
SRBG01.II.3.2	*C. trapeziformis*	ANBG	3	—
SRBG01.II.4	*C. trapeziformis*	ANBG	—	2
SRBM01.I.3.1■■■	*C. trapeziformis*	BM	12	—
SRBM01.I.3.2■■■	*C. trapeziformis*	BM	17	—
SRBG03.I.3■■■	*C. trapeziformis*	ANBG	8	—
SRBG03.I.6■■■	*C. trapeziformis*	ANBG	6	—
SRBG03.I.7■■■	*C. trapeziformis*	ANBG	16 [a]	—
SRBG03.I.8■■■	*C. trapeziformis*	ANBG	16	—
SRBG03.II.1■■■	*C. trapeziformis*	ANBG	4	—
SRBG03.II.2■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.II.3■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.III.1■■■	*C. trapeziformis*	ANBG	17	—
SRBG03.III.2■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.III.3■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.III.5■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.III.6■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.III.11■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.III.12■■■	*C. trapeziformis*	ANBG	8	—
SRBG03.III.13■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.IV.1■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.IV.2■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.IV.3■■■	*C. trapeziformis*	ANBG	*	—
SRBG03.IV.4■■■	*C. trapeziformis*	ANBG	17	—
SRBG03.IV.5■■■	*C. trapeziformis*	ANBG	13	—
SRBG03.IV.6■■■	*C. trapeziformis*	ANBG	17	—
Total	6		46	47

[a] Not included in Fig. 4.

(Everitt, 1977) was applied to test the null hypothesis of no associations among seed germination potential of the three fungal isolates and seed pods of *C.* aff. *jeanesii*.

RESULTS

Fungal species associated with Chiloglottis—A total of 47 isolates from 28 plants were obtained from six *Chiloglottis* species representing 10 sites (Table 1). For the ITS locus, 34 clones were sequenced (GenBank accessions HM196779–HM196813, Appendix 1) from fungal cultures obtained from six *Chiloglottis* species representing seven sites (Table 2). For the mtLSU locus, 47 symbionts from six *Chiloglottis* species were sequenced (GenBank accessions HM196772-HM196774, Appendix 1) representing all 10 sites and 28 plants. The closest BLAST hits on GenBank were *Tulasnella tomaculum* (KC429) isolated from decaying *Pinus* sp. wood, *T. eichleriana* and *T. violea,* both isolated from *P. sylvestris* (Table 3). The next closest sequenced relatives isolated from orchids were *Epulorhiza albertensis* (*Epulorhiza* = *Tulasnella* anamorph) ex *Platanthera orbiculata*.

In the mtLSU phylogenetic analyses, all *Chiloglottis* fungal sequences formed a monophyletic group of *Tulasnella* lineages, sister to *T. tomaculum* with high bootstrap (97%) and Bayes clade credibility (1.0) values (Fig. 3). For the ITS locus, *Chiloglottis* fungal sequences formed a highly supported group related to *T. tomaculum*, *T. violae* (AY382813), and *T. eichleriana* (Fig. 4). All the *Chiloglottis*-derived fungal sequences were

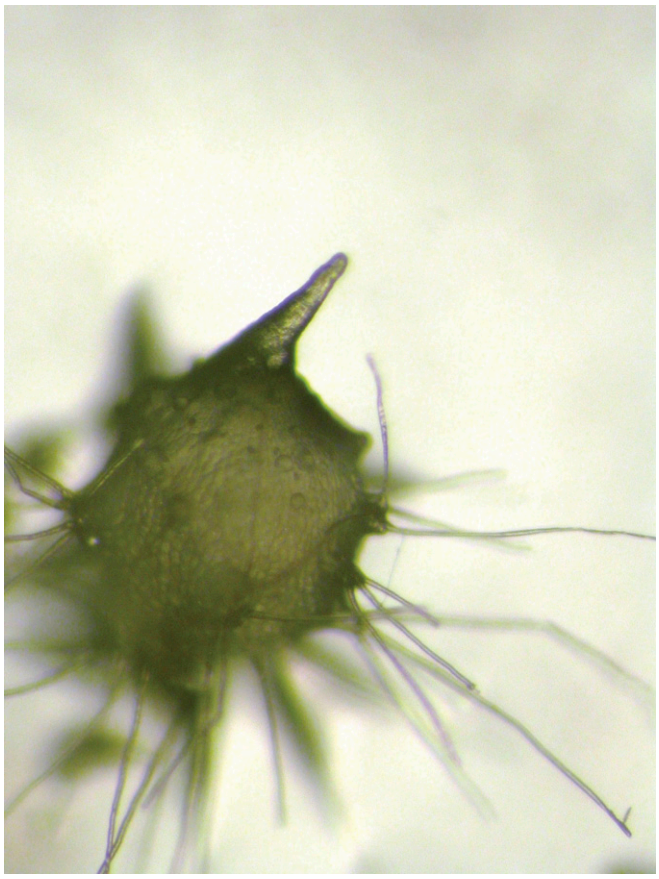

Fig. 2. A late stage 4 protocorm of *Chiloglottis* aff. *jeanesii*.

more closely related to each other than to any other *Tulasnella* species included in the analysis. The *Sebacina* sp. from GenBank (AD0016351) in the mtLSU tree (Fig. 3) was tentatively identified as a *Sebacina* sp. (Currah et al., 1990), but most likely it represents a misidentification, as all other sequences belong to *Tulasnella* or the *Tulasnella* anamorph genus *Epulorhiza*, and therefore *Tulasnella* is monophyletic. *Gloeotulasnella* is not recognized as different from *Tulasnella* (Olive, 1957; Roberts, 1994), which is supported by the mtLSU tree placing *Gloeotulasnella* (syn. *Tulasnella*) *cystidiophora* monophyletic within *Tulasnella*.

Little variation was recorded among mtLSU sequences from the *Chiloglottis* species, with only three haplotypes recorded among the 47 isolates sequenced (Fig. 3, Table 2). These haplotypes each differed by one polymorphic site only. For the ITS locus, 35 clonal types were identified among 46 ITS clones derived from 21 isolates and direct sequencing of five *C. trapeziformis* plants (Table 2). Clones derived from the same isolate generally belonged to different ITS haplotypes (e.g., clones of isolates 06082.I.1, CP0835.III.1, CP0835.VIII.2 and SRBG01.II.3). Furthermore, ITS clones derived from isolates associated with the same plant generally also belonged to different haplotypes (e.g., 06082.I.1 and 06082.I.3), although clones derived from isolates obtained from different plants were identical (e.g., haplotype 17 from SRBG03.IV and SRBG03.III from C. *trapeziformis* at the ANBG site). Haplotype 17 was also found associated with *C. trapeziformis* at the BM site, approximately 2 km away. In the one other case where the same ITS haplotype was associated with different plants, the individuals belonged to the same species from the same site (haplotype 28 from *C.* aff. *jeanesii*) (Fig. 4, Table 2).

There was little evidence for patterns of association between haplotypes and particular *Chiloglottis* species. However, isolates from *C.* aff. *jeanesii* plants at the BRFT site did form a group that included only one isolate from another *Chiloglottis* species, *C. trapeziformis* from site ANBG, SRBG01.II.1 when analyzed with the ITS locus (Fig. 4). This association was confirmed with a contingency χ^2 test ($\chi^2 = 29.4$, df = 1, $p = 0.001$), rejecting the null hypothesis of no association between haplotypes and the species *C. trapeziformis* and *C.* aff. *jeanesii*.

Fungal amplification from* Chiloglottis trapeziformis *tissue—Direct sequencing of the ITS locus with subsequent cloning of fragments (GenBank accessions HM196775–HM196778, Appendix 1) was conducted on five *C. trapeziformis* plants. Based on BLAST matches on GenBank, 12 of the 23 clones belonged to *Tulasnella,* with all 12 samples being genetically very similar to the isolates obtained by standard culturing methods (see bold accessions on Fig. 3*). Tulasnella* sequences were obtained from all five plants. Two plants yielded only *Tulasnella* sequences. In addition to *Tulasnella*, three non-*Tulasnella* or endophytic fungal sequences were found in the tissue of three *C. trapeziformis* plants, using the ITS1 and ITS4OF primer combination (Table 3). These were classified as uncultured soil fungi with BLAST searches, but when BLAST searches were conducted excluding uncultured samples, BLAST hits were obtained with *Trechispora subsphaerospora* ($N = 6$), *Scleroderma areolatum* ($N = 3$), and *Mycena hudsoniana* ($N = 2$) (Table 3).

Orchid germination trials—The three tested isolates derived from *C. valida*, *C.* aff. *jeanesii*, and *C. reflexa* were able to induce stage 4 and 5 protocorm development on seed of *C.* aff. *jeanesii*

TABLE 3. BLAST results showing the top sequence alignment match and corresponding statistics for fungal sequences selectively amplified from tissue segments of *Chiloglottis trapeziformis* and fungal cultures obtained from *Chiloglottis* species. * = Representative sequences, *N* = the number of sequences identical to the representative sequence obtained.

ITS clone sequence	Closest BLAST match	Length (bp)	Max score	Query coverage (%)	E -value	Max identity (%)
*06082.I.1, represents ITS ancestral group	AY373292 *Tulasnella eichleriana*	917	787	83	0	85
*CP835.III.1.1, represents ITS derived group	AY373292 *Tulasnella eichleriana*	918	780	83	0	85
*SRBG03.I.7, *N* = 1	AY373296 *Tulasnella tomaculum*	559	569	99	4e-159	85
*SRBG03.II.1 , *N* = 11	AY373292 *Tulasnella eichleriana*	862	774	87	0	85
*SRBG03.IV.3, *N* = 6	AF347080 *Trechispora subsphaerospora*	671	355	61	2e-94	83
*SRBG03.III.13, *N* = 3	EU718116 *Scleroderma areolatum*	742	902	88	0	92
*SRBG03.III.6, *N* = 2	EU846300 *Mycena hudsoniana*	457	715	99	0	94

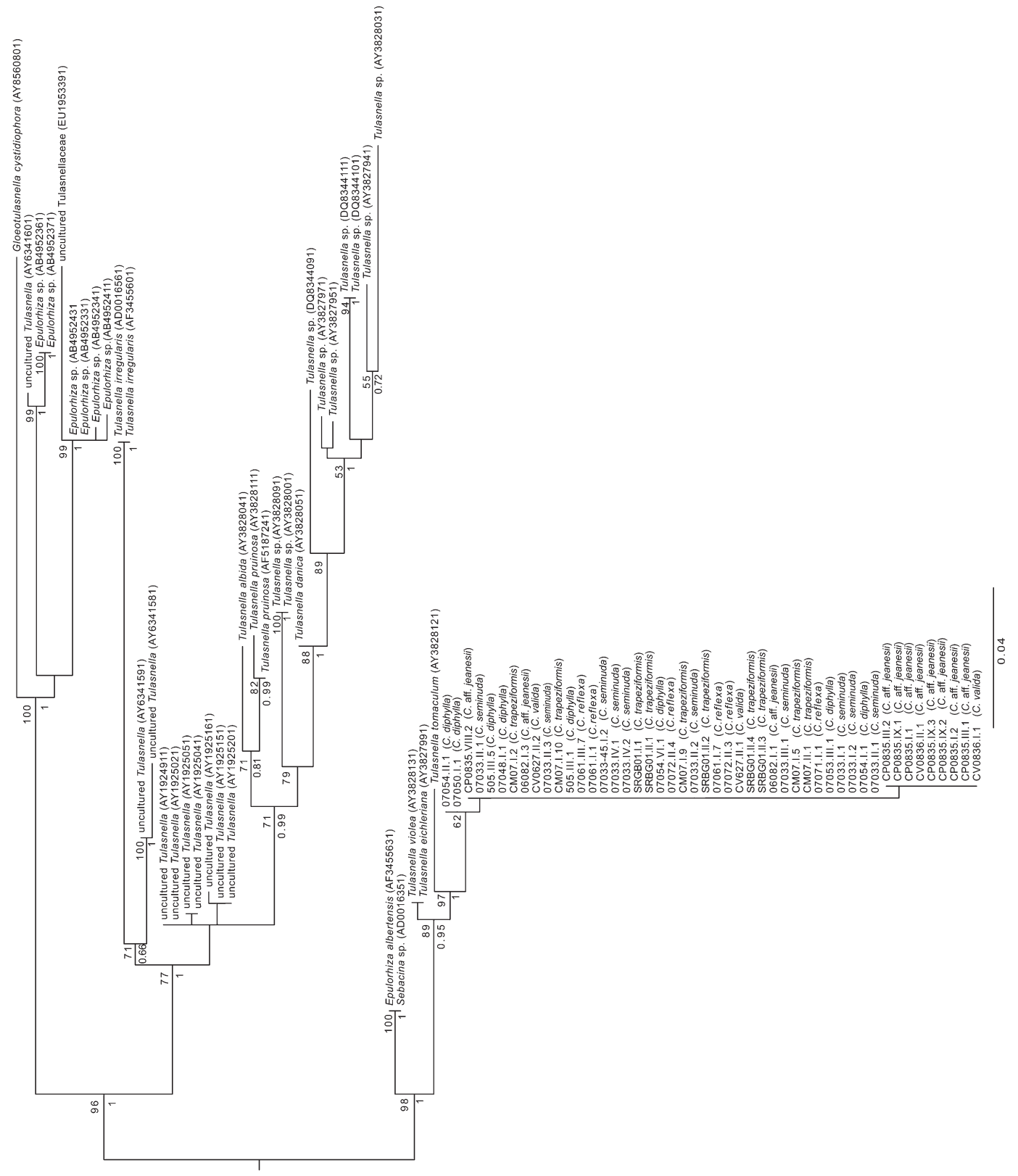

Fig. 3. Maximum likelihood of mtLSU sequences from fungal isolates associated with *Chiloglottis* species. Bootstrap values are provided above nodes and Bayes clade credibility values below nodes. Only bootstrap values >50 and Bayes clade credibility values >0.7 are shown.

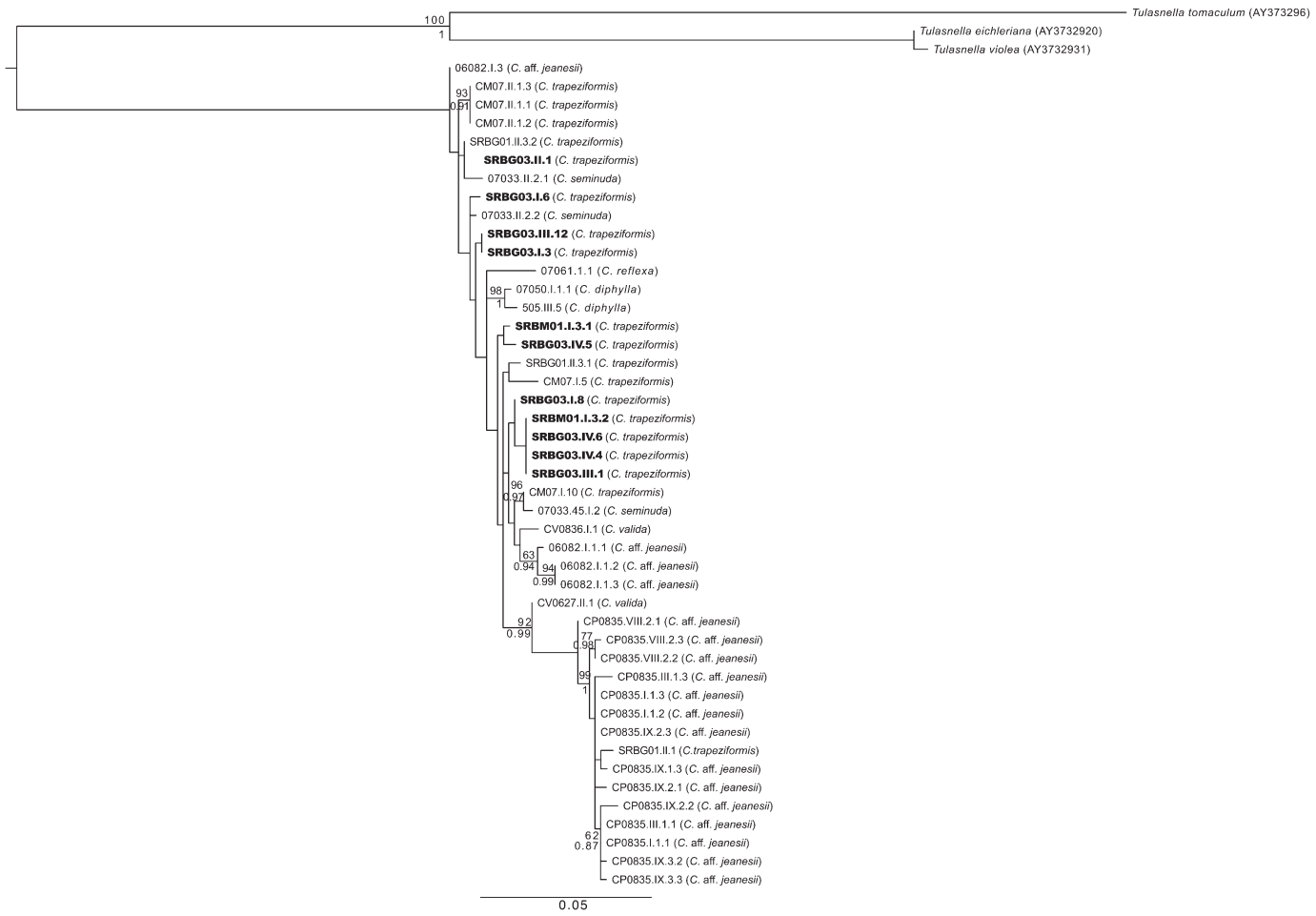

Fig. 4. Maximum likelihood of ITS sequences from fungal isolates associated with *Chiloglottis* species. Bootstrap values are provided above nodes and Bayes clade credibility values below nodes. Only bootstrap values >60 and Bayes clade credibility values >0.7 are shown. Fungal sequences obtained with direct sequencing from *Chiloglottis trapeziformis* are indicated in bold.

(Fig. 5) as well as *C. valida* (Fig. 6). Both *Chiloglottis* species varied in the number at stage 4 and 5 protocorm development within and between seed pods (Figs. 5, 6). The highest germination recorded was 23% on *C.* aff. *jeanesii* seed pod 7106 with the CV0627.II.2 (ex *C. valida*, Lowden Rd; derived ITS group—data not shown) isolate. This isolate also induced the highest recorded percentage germination (10%) of seed in *C. valida,* in this case on seed pod 7128 (Fig. 6). However, this same isolate failed to germinate seed of pod 7111 of *C.* aff. *jeanesii*. Isolate 06082.I.1 (ex *C.* aff. *jeanesii:* ancestral ITS group) also failed to germinate seed from pods 7118 and 7119. A contingency χ^2 test showed that the null hypothesis of no association in germination potential among the three tested fungal isolates and *C.* aff. *jeanesii* seed pods 7123, 7106, and 7108 was rejected ($\chi^2 = 9.2$, df = 4, $p = 0.001$). None of the seed on noncolonized OMA control treatments germinated.

DISCUSSION

Chiloglottis species have a wide distribution in southeastern Australia ranging from Queensland in the north to Tasmania in the south, and west to South Australia (Jones, 2006). Although we only sampled a subset of the total species from a portion of the genus' range, our sampling included the full breadth of phylogenetic diversity. We predicted that if fungal mycorrhizal associates have contributed to the diversification of this orchid genus, we would find narrow associations between orchid species and specific fungal species. In the discussion that follows, we evaluate the evidence for this hypothesis. First, we consider the evidence as to whether our sampling methods (via culture) underestimated mycorrhizal diversity in comparison to direct sequencing. Next, we consider the germination potential of the fungal isolates. We then explore the phylogenetic relationships and examine which species and how many are involved in the partnership. Finally, we consider the question of specificity and the evolutionary implications of our findings.

Does direct DNA amplification reveal additional diversity in orchid–fungal associations?—The current mycorrhizal fungal sampling methods employed in this and most other studies are biased toward fast-growing and easily cultured fungi. Consequently, the fungal diversity associated with green orchids may be underestimated (Taylor et al., 2002). Direct amplification of fungi from orchid tissue often reveals a diverse array of fungal species, including various ascomycetes (Otero and Bayman, 2006; Dearnaley, 2007). Therefore, direct sequencing from *C. trapeziformis* tissue was expected to reveal a wider diversity of associated fungal species than those found using our standard

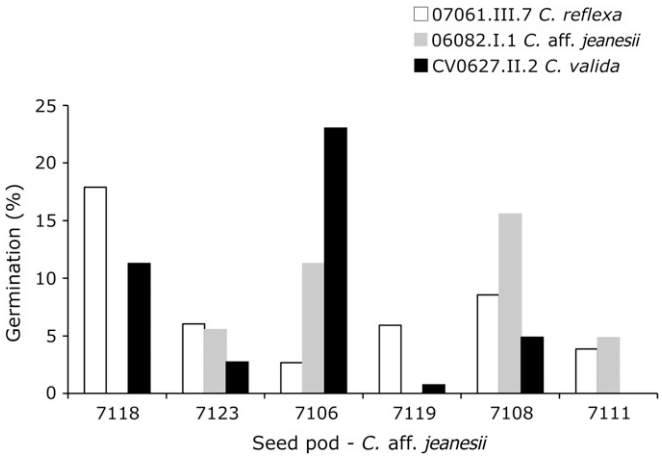

Fig. 5. Percentage germination of seed of *Chiloglottis* aff. *jeanesii* in association with three *Tulasnella* sp. isolates 07061.III.7 (ex *Chiloglottis reflexa*), 06082.I.1 (ex *C.* aff. *jeanesii* and CV0627.II.2 (ex *Chiloglottis valida*).

culture method. The majority of ITS clones obtained by direct sequencing in this study were very closely related to the *Tulasnella* lineage extracted via standard culture methods. BLAST searches did, however, indicate the presence of several other fungal symbionts as unidentified, uncultured species. Excluding uncultured samples from the BLAST searches resulted in hits with *Threchispora*, which is not considered mycorrhizal (Allen et al., 2003), *Scleroderma*, which is a common tree mycorrhizal symbiont (Dell et al., 1990), and *Mycena*, which is normally a free-living saprophyte exploited by *Gastrodia* orchids as a mycorrhizal symbiont (Ogura-Tsujita et al., 2009). Whether the uncultured fungal species from this study can indeed act as mycorrhizal symbionts in *Chiloglottis* is unknown and requires further investigation using techniques such as seed baiting (Batty et al., 2006) and reisolation, or direct amplification of protocormic fungi. The evidence here does not indicate a substantial underestimate of fungal diversity by our culture methods.

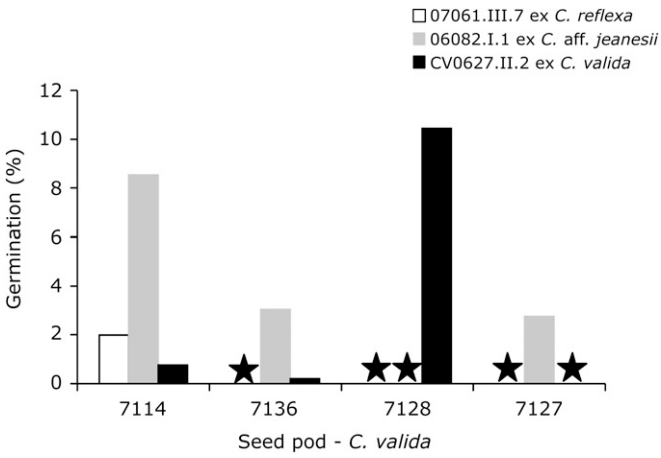

Fig. 6. Percentage germination of seed of *Chiloglottis valida* in association with three *Tulasnella* isolates 07061.III.7 (ex *Chiloglottis reflexa*), 06082.I.1 (ex *Chiloglottis* aff. *jeanesii*), CV627.II.2 (ex *C. valida*). Star = isolate treatments lost to contamination.

Direct sequencing is also useful to determine whether protocorm fungal composition is different for fungal symbionts established in adult plants (Batty et al., 2006). Mycorrhizal associations of *Cephalanthera damasonium, C. longifolia* (Bidartondo and Read, 2008), and *Caladenia formosa* (Huynh et al., 2009) have been shown to differ between adult and protocormic stages. Furthermore, in *Caladenia formosa,* fungi with the highest germination rates did not necessarily result in the greatest survival and growth 1 year after germination (Huynh et al., 2009). Because direct sequencing was only carried out on material collected from established adult plants, our study could have missed a portion of fungal diversity interacting at earlier orchid growth stages despite our cultured isolates nevertheless initiating germination of orchid seed.

What is the germination potential of the fungal isolates?—Seed germination trials confirmed the capability of the *Tulasnella* isolates to enable seed germination of two orchid species tested: *C. valida* and *C.* aff. *jeanesii*. Our experiments confirmed that all the tested *Tulasnella* fungi isolated from different plants at different sites could initiate germination, although possible isolate by *Chiloglottis* species or seed pod interaction was observed. Very few seeds reached advanced germination stages under the experimental conditions used in this study, with highest germination of seeds in *C.* aff. *jeanesii* being 23% and *C. valida* just 10%. These low seed germination rates are not unusual; low rates were also observed for *Caladenia* (4–21%) (Huynh et al., 2009) and for *Vanilla* and *Ionopsis* (2–7%) (Porras-Alfaro and Bayman, 2007), whereas germination rates ranged between approximately 37–90% for tropical epiphytes (Otero et al., 2004). Factors responsible for low seed germination rates are unknown and need further investigation.

The variation in germination among orchid seed pods and fungal isolates may simply be due to random error, or it may indicate that the *Tulasnella* isolates in this study represent genetically different strains that confer differences in mycorrhizal compatibility or fitness. Similar variation in germination ability of different *Ceratobasidium* isolates has been reported in the epiphytic orchid *Tolumnia variegata,* with only 5 of 10 seed pods germinating (Otero et al., 2005). Significant variation in germination effectiveness among fungal isolates of the same species on *Caladenia formosa* was also observed (Huynh et al., 2009). An expansion of the current study to investigate potential interactions among fungal isolates and *Chiloglottis* species and genotypes is required.

Which fungi and how many fungal taxa are involved in the partnership?—In our phylogenetic analysis of the mitochondrial (mtLSU) DNA locus, all *Chiloglottis*-derived fungal sequences formed a single clade sister to other *Tulasnella* species, with only three haplotypes represented among the 47 isolates. Compared with mtLSU, more sequence variation was encountered at the ITS locus with a total of 35 haplotypes detected among the 46 ITS clones. Some phylogeographic structure was evident, with the ITS haplotypes falling into two main groups. The first group representing the ancestral lineages in the tree included isolates from all six *Chiloglottis* species sampled, while the derived lineages, supported by 0.99 Bayes clade credibility values, represented a narrower range of species including isolates from *C.* aff. *jeanesii* (CP0835 from site BRFT) and a clone each from *C. valida* (CV0627.II.1) and *C. trapeziformis* (SRBG01.II.1). The grouping of *C.* aff. *jeanesii* in the derived lineages on the tree is of interest since some of the same isolates

also clustered together in the mtLSU analysis. Despite some genetic structure, the six *Chiloglottis* species are associated with a narrow monophyletic group of *Tulasnella* lineages.

While all six *Chiloglottis* species hosted a narrow monophyletic group of *Tulasnella* lineages, we did detect some genetic differences among fungal isolates, particularly in the ITS for which we identified 35 haplotypes from 46 clones representing 21 isolates. None of the haplotypes were shared among *Chiloglottis* species or sites, except for ITS haplotype 17, which was shared between the Black Mountain (BM) and Botanical Garden (ANBG) locations of *C. trapeziformis*. These sites are separated by approximately 2 km, and haplotype sharing may be explained by the possibility of plants at the BG site being artificially sourced from a colony on Black Mountain. *Chiloglottis* individuals were also often associated with more than one ITS clonal type, reflecting the diversity within the ITS locus. Sharing of haplotypes was rare even between plants of the same species at the same site with the only two examples being haplotype 17 on *C. trapeziformis* and haplotype 28 on *C.* aff. *jeanesii*.

Warcup suggested that *Chiloglottis* is associated with *T. cruciata* and *T. allantospora* as well as unidentified *Tulasnella* species (Warcup, 1981); however, in a later review, Warcup (1990) claimed the mycorrhizal symbiont of *Chiloglottis* as unknown. The present study confirms the preliminary results of Warcup (1981) that the mycorrhizal symbiont of *Chiloglottis* is a *Tulasnella* species. While all of our isolates formed a single ITS clade well separated from *Tulasnella tomaculum*, the identity of this taxon remains unknown because we are dependent on reference sequences in GenBank for sequence identification. This dependence on reference sequences to identify fungal species, highlights the need for a larger sampling to improve the phylogeny of *Tulasnella* to aid better identification of orchid mycorrhizal symbionts.

The question of species delineation, especially in the case of cryptic species that by definition lack obvious morphological differences, is a challenging one. Despite the promise of DNA-based taxonomy, the use of DNA evidence on its own to identify and discover plant and animal species remains highly controversial (Wheeler, 2005; Will et al., 2005). The problem of species delineation in fungi is even more problematic than for many plants and animals (Suarez et al., 2008) because some fungi may simply be unculturable and therefore can only be found by direct DNA sequencing. The problem is further compounded by a general lack of informative "species-level" genetic markers in fungi. One rule of thumb that has been applied to ITS fungal sequences is to apply a threshold of 3% sequence divergence to species boundaries (Nilsson et al., 2008; Peay et al., 2008). Indeed, *Tulasnella* clades claimed to be specific to their associated orchids *Goodyera* and *Liparis* in North America, were separated by ITS differences of approximately 8–10% (McCormick et al., 2004). By contrast, all of the *Tulasnella* isolates from *Chiloglottis* in this study differed at most by 1.6% sequence divergence. Therefore, by the 3% criterion (Nilsson et al., 2008; Peay et al., 2008), all of the *Tulasnella* isolates from *Chiloglottis* would be considered to belong to a single ITS-defined species if we were to strictly follow this rule.

An interesting anomaly noted in this study is that the GenBank ITS sequences attributed to *T. eichleriana* and *T. violea* are extremely similar and well below the 3% threshold to characterize different species (see Fig. 3). Two other studies (McCormick et al., 2004; Porras-Alfaro and Bayman, 2007) also found this pattern using GenBank sequences. In McCormick et al., (2004), it is evident that three putative *T. violea* sequences differed markedly from each other, with one sequence identical to *T. eichleriana*, as found in this study. We suggest three possible explanations for this anomaly: (1) *T. violea* as defined morphologically is polyphyletic; (2) ITS markers cannot distinguish between some species; or (3) the accessions were misidentified. It is beyond the scope of the current study to resolve this issue; nonetheless, it is evident that it will be helpful to develop additional informative genetic markers that provide resolution below the species level in *Tulasnella* and other fungi. While the taxonomic status of the *Chiloglottis* isolates cannot be fully resolved in this present study, there is no doubt that *Chiloglottis* species are associated with a narrow monophyletic group of *Tulasnella* lineages.

Patterns of orchid–fungal specificity: Ecological and evolutionary implications?—Many studies of orchid mycorrhizal associations suggest a specific association between orchid and symbiont and draw conclusions about specialization (Selosse et al., 2002; Girlanda et al., 2006; Suarez et al., 2006; Bonnardeaux et al., 2007; Shefferson et al., 2007). However, one problem in comparing among studies is that the terms specificity and specialization are often used differently. For example, specialization is sometimes taken to mean an association of phylogenetically closely related fungal species among different orchid species in the same genus. Specificity is also characterized as an association of a related group of fungi with one orchid species, e.g., 20 *Russula* spp. on *Corallorhiza maculata* (Taylor and Bruns, 1999). In other cases, it is concluded that a specific mycorrhizal association exists when the same phylogenetic group of fungi are recovered from repeated sampling of the orchid species over a geographic range (e.g., Rasmussen, 2002).

This *Tulasnella–Chiloglottis* association appears to be one of the first known cases in which such a narrow monophyletic group of fungal lineages is associated with multiple orchid species spanning a broad survey of the genus. In a recent study, Barrett and coworkers (Barrett et al., 2010) also found that a single fungal species, *Tomentella fuscocinerea*, associates with a species complex of the mycoheterotrophic orchid *Corallorhiza striata* (likely representing three orchid species and further varieties). Although not as many species of *Corallorhiza* were sampled as for our *Chiloglottis* study, it may suggest that future studies will find a similar trend of narrow fungal associations across a wide array of species. On the other hand, if the 3% (Nilsson et al., 2008; Peay et al., 2008) or 2.5% (Barrett et al., 2010) ITS divergence criterion is applied to this case of *Tomentella fuscocinerea*, up to 12 cryptic symbiont fungal species of *Corallorhiza* may be circumscribed within *T. fuscocinerea* (Barrett et al., 2010).

Mycorrhizal associations in the photosynthetic orchid genus *Cypripedium* have been investigated across the phylogenetic breadth of the genus with sampling spanning from North America to Europe (Shefferson et al., 2005, 2007). This research revealed narrow associations with mycorrhizal partners for some members such as *C. calceolus*; however, other orchid species associated with multiple clades within a fungal genus or even across several genera in the case of *C. californicum* (Shefferson et al., 2005, 2007). These studies used more slowly evolving markers compared to the current study: 5.8S, mtLSU (Shefferson et al., 2007) and nucLSU (Shefferson et al., 2005), and as a consequence, the extent of fungal diversity may have been underestimated.

A narrow phylogenetic breadth of orchid mycorrhizae has also been reported in several cases involving studies of a single

species of orchids, including *Eulophia zollingeri*, which associates with a fungal species complex across Japan, Myanmar, and Tawain (Ogura-Tsujita and Yukawa, 2008). A similar pattern in fungal diversity was found in epiphytic orchid species *Ionopsis utricularioides* that associate with very narrow clades within *Ceratobasidium* (Otero et al., 2007).

These examples demonstrate variation in the degree of specificity between orchid and fungal symbionts, ranging from apparent one to one relationships between orchid and a specific fungus, through cases such as *Chiloglottis* that has multiple orchid species associated with a narrow group of lineages, to cases of an orchid species associated with a diverse array of fungal species. It is clear that the *Chiloglottis–Tulasnella* example represents the most extreme case of specificity found so far, with a monophyletic group of lineages, possibly representing one species only, associated with a broad survey of *Chiloglottis* species.

It is unclear why specific mycorrhizal relationships exist and what the advantage of such a specific association would be. The advantages of specific mycocrrhizal relationships are elegantly discussed in Barrett et al. (2010) for the symbionts of *Corallorhiza striata,* proposing two hypotheses for this phenomenon. The first is that high specificity is selected for because it simplifies the interaction (e.g., orchids do not have to deal with different defense systems of symbionts) (Thompson, 1994) and provides an optimum food source (e.g., the chosen symbiont provides superior nutritional quality) (Rasmussen and Rasmussen, 2009). The second hypothesis posed by Barrett et al. (2010) is that the symbionts might have narrow geographic distributions, preventing association with the orchids, thereby forcing specificity by the orchid due to the absence of putative symbionts. However, if geographic ranges for orchids and fungi are patchy, then opportunities for symbiosis in areas of overlap will be few and might actually favor generalization, not specialization as suggested. In the absence of data concerning, for example, mycorrhizal fitness, differences in nutrient uptake ability of mycorrhizal strains and species and the distribution/availability of symbionts, it is impossible to discern between these two hypotheses. Indeed, very specific relationships with mycorrhizae may be dependent on the saprophytic fungal life cycle of some fungi, which could provide suitable habitat for terrestrial orchids by decomposing fallen debris from forest trees (Ogura-Tsujita and Yukawa, 2008).

Patterns of fungal specificity and sexual deception in Australian orchids?—It is of interest to ask whether an association of multiple sexually deceptive orchid species with a narrow group of fungal lineages, as discovered in *Chiloglottis*, might be a general pattern among Australian, sexually deceptive orchids. *Chiloglottis* belongs to the tribe Drakaeinae, which is a monophyletic clade of exclusively sexually deceptive genera including *Arthrochilus*, *Chiloglottis*, *Drakaea*, *Paracaleana, Caleana*, and *Spiculaea.* Isolate studies by Warcup (1981) indicated that *Arthrochilus*, *Drakaea*, and *Chiloglottis* (species were not listed) each associated with several species of *Tulasnella*, and *Caleana* with one *Tulasnella* species. However, the identity of these mycorrhizal symbionts and the pattern and degree of specificity have not yet been confirmed by molecular phylogenetic analysis. A pilot molecular study has indicated that multiple *Drakaea* species may also be associated with a narrow group of *Tulasnella* lineages (C. C. Linde, R. D. Phillips, and R. Peakall, unpublished data). Thus, mycorrhizal specificity in sexually deceptive orchids may be a more general pattern, at least in the tribe Drakaeinae.

The next closest related orchid tribes to the Drakaeinae are Thelymitrinae and Cryptostylidinae (Kores et al., 2001), although only *Cryptostylis* species are sexually deceptive. *Thelymitra* species are known to associate with a number of *Tulasnella* species. Beyond them, the next closest is *Caladenia* and *Diuris* in the Diuridinae-Caladeninae. *Caladenia* associates with a broad species complex within the Sebacinaceae (e.g., Bougoure et al., 2005; Bonnardeaux et al., 2007), while *Diuris* species associate with a narrow group of *Tulasnella* lineages (Smith et al., 2010). *Pyrorchis nigricans*, which belongs to a clade sister to Drakaeinae (Kores et al., 2001), was found to have some of the most diverse associations with multiple mycorrhizal genera including a relative of *Tulasnella danica* (Bonnardeaux et al., 2007).

The diverse Australian terrestrial orchid genus *Caladenia* with some 300 species is unique among orchid genera worldwide in that it contains species that employ pollination by food reward, food deception, and sexual deception (Phillips et al., 2009). As such, this genus may provide a unique model system to assess whether there is any relationship between patterns of fungal specificity and an orchid's pollination mechanism.

Implications of our findings for orchid diversification—In this study, we made the unexpected discovery that six species of *Chiloglottis*, representing a broad survey across the genus, were associated with a narrow monophyletic group of *Tulasnella* mycorrhizal symbiont lineages. This finding may explain the frequent observation that two or more *Chiloglottis* species can co-occur, in some cases even growing and coflowering in mixed colonies. It is further likely that the *Chiloglottis–Tulasnella* mycorrhizae are common and widespread in Australia, because *Tulasnella* spp. are known to survive saprophytically and are therefore not dependent on the orchid for survival. If true, this might contribute to *Chiloglottis* having such a wide distribution across southeastern Australia. There is evidence from other orchid–fungal studies that a widespread distribution of the fungal associations is correlated with orchid distribution and abundance (Ogura-Tsujita and Yukawa, 2008; Barrett et al., 2010). Furthermore, this study may have restoration implications for propagation of rare or threatened *Chiloglottis* species such as *C. platyptera* and *C. anaticeps* listed as vulnerable and endangered. Future research is needed to clarify the nature of mycorrhizal specificity with these species and whether the presence of compatible fungi is a significant limiting factor in contributing to their rarity.

Our finding that multiple species of sexually deceptive *Chiloglottis* are associated with a narrow group of fungi may be explained by the process of speciation in the genus. Peakall et al. (2010) have shown that pollinator specificity and, consequently, reproductive isolation, has a strong chemical basis, especially among sympatric *Chiloglottis* species that use different semiochemicals to attract their specific pollinators. Mapping of chemistry onto the phylogeny has further revealed that chemical change is frequently associated with speciation. However, the structural relatedness of the compounds involved suggests only minor modifications of a common biochemical pathway. Therefore, rapid pollinator-driven speciation appears plausible in this system (Peakall et al., 2010).

Thus, the specific pollination system of *Chiloglottis*, rather than specific orchid–fungal interactions, appears to have been the key driving force in the diversification of the genus. Nonetheless, the ability of multiple *Chiloglottis* species to associate with a narrow group of fungal lineages enables the sympatric distribution of multiple orchid species. Rapid pollinator-mediated

speciation might act in such a way as to quickly result in prezygotic reproductive barriers between incipient species without interrupting the existing associations between orchid and fungus lineages. Indeed, the maintained specialization of *Chiloglottis* on a narrow phylogenetic range of symbionts could be considered evidence that speciation has occurred rapidly.

The maintenance of orchid association with a specific and narrow lineage of fungi through speciation processes may therefore be contingent on the form of speciation involved and the rate of divergence. Rapid pollinator-mediated selection will be more likely to conserve orchid–fungus associations than more long-term processes such as ecological speciation or vicariance by which lineages might divide over a longer period of time so that both orchid and fungus species observed today have had more opportunity for drift, phyletic change, and adaptation to contribute to divergent mychorrizhal associations.

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APPENDIX 1. Voucher information for the present study. Culture collections are located in the author's laboratory at the Australian National University, Canberra (ANU).

Taxon; *Host*; GenBank accessions: ITS; mtLSU; *Voucher specimen*; Collection site.

***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196792; HM196774; 06082.I.1.1; Tallaganda State Forest, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196791; —; 06082.I.1.2; Tallaganda State Forest, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196791; —; 06082.I.1.3; Tallaganda State Forest, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196812; HM196774; 06082.I.3; Tallaganda State Forest, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196779; HM196773; CP0835.I.1.1; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196782; —; CP0835.I.1.2; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196782; —; CP0835.I.1.3; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; —; HM196773; CP0835.I.2; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196779; HM196773; CP0835.III.1.1; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196788; HM196773; CP0835.III.1.3; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; —; HM196773; CP0835.III.2; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196787; HM196774; CP0835.VIII.2.1; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196786; —; CP0835.VIII.2.2; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196783; —; CP0835.VIII.2.3; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196795; HM196773; CP0835.IX.1.3; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196785; HM196773; CP0835.IX.2.1; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196786; —; CP0835.IX.2.2; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196782; —; CP0835.IX.2.3; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196781; HM196773; CP0835.IX.3.2; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis* aff. *jeanesii*; HM196780; —; CP0835.IX.3.3; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis valida* D.L.Jones; HM196801; HM196773; CV0836.I.1; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis valida* D.L.Jones; —; HM196773; CV0836.II.1; Kanangra Boyd National Park, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis valida* D.L.Jones; HM196804; HM196774; CV0627.II.1; Tallaganda State Forest, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis valida* D.L.Jones; —; HM196774; CV0627.II.2; Tallaganda State Forest, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis diphylla* R.Br.; —; HM196774; 505.III.1; Blue Mountains—Bells Line Rd, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis diphylla* R.Br.; HM196803; HM196774; 505.III.5; Blue Mountains—Bells Line Rd, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis diphylla* R.Br.; —; HM196774; 07054.I.1; Blue Mountains—Bells Line Rd, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis diphylla* R.Br.; —; HM196772; 07054.II.1; Blue Mountains—Bells Line Rd, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis diphylla* R.Br.; —; HM196774; 07054.VI.1; Blue Mountains—Bells Line Rd, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis diphylla* R.Br.; HM196802; HM196772; 07050.I.1; Blue Mountains—Bells Line Rd, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis diphylla* R.Br.; —; HM196774; 07048.I.1; Blue Mountains—Bells Line Rd, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis diphylla* R.Br.; —; HM196774; 07053.III.1; Blue Mountains—Bells Line Rd, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis reflexa* (Labill.) Druce; HM196805; HM196774; 07061.I.1; Mt Wilson, Blue Mountains, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis reflexa* (Labill.) Druce; —; HM196774; 07061.I.7; Mt Wilson, Blue Mountains, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis reflexa* (Labill.) Druce; —; HM196774; 07061.III.7; Mt Wilson, Blue Mountains, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis reflexa* (Labill.) Druce; —; HM196774; 07071.I.1; Mt Wilson, Blue Mountains, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis reflexa* (Labill.) Druce; —; HM196774; 07072.II.3; Mt Wilson, Blue Mountains, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis reflexa* (Labill.) Druce; —; HM196774; 07072.II.4; Mt Wilson, Blue Mountains, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; —; HM196774; 07033.I.1; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; —; HM196774; 07033.I.2; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; —; HM196774; 07033.II.1; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; HM196798; HM196774; 07033.II.2.1; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; HM196797; —; 07033.II.2.2; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; —; HM196774; 07033.II.3; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; —; HM196774; 07033.III.1; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; HM196800; HM196774; 07033—45.I.2; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; —; HM196774; 07033.IV.1; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis seminuda* D.L.Jones; —; HM196774; 07033.IV.2; Penrose Forest, Exeter, NSW, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; —; HM196774; CM07.I.2; Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196794; HM196774; CM07.I.5; Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; —; HM196774; CM07.I.9; Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196799; HM196774; CM07.I.10; Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196789; HM196774; CM07.II.1.1; Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196789; —; CM07.II.1.2; Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196789; —; CM07.II.1.3; ANBG, Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; —; HM196774; SRBG01.I.1; ANBG, Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196796; HM196774; SRBG01.II.1; ANBG, Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; —; HM196774; SRBG01.II.2; ANBG, Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196793;

HM196774; SRBG01.II.3.1; ANBG, Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196790; —; SRBG01.II.3.2; ANBG, Black Mountain, ACT, Australia. ***Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; —; HM196774; SRBG01.II.4; ANBG, Black Mountain, ACT, Australia.

Uncultured *Tulasnella* sp.; *Chiloglottis trapeziformis* Fitzg.; HM196809; —; SRBM01.I.3.1; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196813; —; SRBM01.I.3.2; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196810; —; SRBG03.I.3; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196811; —; SRBG03.I.6; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196807; —; SRBG03.I.7; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196807; —; SRBG03.I.8; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196808; —; SRBG03.II.1; ANBG, Black Mountain, ACT, Australia.

Uncultured endophytic fungus; *Chiloglottis trapeziformis* Fitzg.; HM196778; —; SRBG03.II.2; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196778; —; SRBG03.II.3; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196813; —; SRBG03.III.1; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196778; —; SRBG03.III.2; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196778; —; SRBG03.III.3; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196775; —; SRBG03.III.5; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196775; —; SRBG03.III.6; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196777; —; SRBG03.III.11; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196810; —; SRBG03.III.12; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196776; —; SRBG03.III.13; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196777; —; SRBG03.IV.1; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196778; —; SRBG03.IV.2; ANBG, Black Mountain, ACT, Australia. **Uncultured endophytic fungus**; *Chiloglottis trapeziformis* Fitzg.; HM196778; —; SRBG03.IV.3; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196813; —; SRBG03.IV.4; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196806; —; SRBG03.IV.5; ANBG, Black Mountain, ACT, Australia. **Uncultured *Tulasnella* sp.**; *Chiloglottis trapeziformis* Fitzg.; HM196813; —; SRBG03.IV.6; ANBG, Black Mountain, ACT, Australia.