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ARTICLE

New species of *Tulasnella* associated with terrestrial orchids in Australia

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Abstract: Recent studies using sequence data from eight sequence loci and coalescent-based species delimitation methods have revealed several species-level lineages of *Tulasnella* associated with the orchid genera *Arthrochilus, Caleana, Chiloglottis,* and *Drakaea* in Australia. Here we formally describe three of those species, *Tulasnella prima, T. secunda,* and *T. warcupii* spp. nov., as well as an additional *Tulasnella* species associated with *Chiloglottis* growing in *Sphagnum*, *T. sphagneti* sp. nov. Species were identified by phylogenetic analyses of the ITS with up to 1.3 % sequence divergence within taxa and a minimum of 7.6 % intraspecific divergence. These new *Tulasnella* (*Tulasnellaceae*, *Cantharellales*) species are currently only known from orchid hosts, with each fungal species showing a strong relationship with an orchid genus. In this study, *T. prima* and *T. sphagneti* associate with *Chiloglottis*, while *T. secunda* associates with *Drakaea* and *Caleana,* and *T. warcupii* associates with *Arthrochilus oreophilus*.

Key words:
host specificity
ITS sequencing
orchid mycorrhizas
species delimitation

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INTRODUCTION

Tulasnella is a cosmopolitan saprotrophic fungal genus that often forms a mycorrhizal relationship with orchids. There are approximately 90 species epithets in *Tulasnella* (www.indexfungorum.org) with Kirk *et al.* (2008) indicating that there are approximately 50 accepted species in the genus. Asexual morphs of *Tulasnella* were formerly referred to in *Epulorhiza*. In earlier studies on the genus, Warcup and Talbot (Warcup & Talbot 1967, Warcup 1971, 1981) were able to induce formation of basidia and basidiospores from some Australian orchid-derived cultures by placing a casing of soil over cultures on agar. However, the spore-producing tissues were often slow to form and diffuse. Indeed, sporophores could only be detected by examination under a dissecting microscope. In some cases, such as in *T. calospora* (Warcup & Talbot 1967), only spores were visible above the casing soil surface. Unfortunately, subsequent studies on *Tulasnella* have not been able to generate basidiospore formation (Suarez *et al.* 2006, Cruz *et al.* 2011). For example, Ma *et al.* (2003) noted that "despite repeated attempts, none of the epulorhiza-like *Rhizoctonia* isolates produced hymenia or basidiospores on [various media] after two months". Although Warcup and Talbot (Warcup & Talbot 1967, Warcup 1971, Warcup & Talbot 1980) utilized morphological characters of the sporophores (such as the size and shape of basidiospores) for taxonomic treatments of *Tulasnella* from orchids, recent studies on the group have mostly designated operational taxonomic units (OTUs) based solely on phylogenetic analysis of DNA sequence data. Indeed, numerous molecular OTUs have been designated amongst *Tulasnella* associated with orchids (e.g. Smith *et al.* 2010, Jacquemyn *et al.* 2011, 2012, Pandey M *et al.* 2013, Cruz *et al.* 2014, Oja *et al.* 2015) or liverworts (Kottke *et al.* 2003, Bidartondo & Duckett 2010), without formally naming the species. Formal naming of the species is preferred and essential to prevent confusion of taxonomic units discovered in separate studies (Hibbett & Taylor 2013).

Molecular OTUs within *Tulasnella* have been designated by two methods. First, application of a sequence divergence threshold for a barcode DNA region such as the ITS; with thresholds ranging from 3–5 % (Suarez *et al.* 2006, Cruz *et al.* 2014, Jacquemyn *et al.* 2014, 2015). Second, application of a multi-gene concordance analysis utilizing coalescent theory that explicitly incorporates gene tree conflicts into a model of phylogenetic history for the populations or species concerned (Yang & Rannala 2010, Fujita *et al.* 2012) and utilizing a number of independent DNA loci (Linde *et al.* 2014). The second approach is more rigorous for delimiting species (Taylor *et al.* 2000) and the similarity within and between species delimited with coalescence can be used to calibrate the cut-off threshold used in the first method.

A study of *Tulasnella* isolates from Australian terrestrial orchids (*Orchidaceae*, tribe *Diurideae*, subtribe *Drakaeinae*) in the genera *Arthrochilus*, *Chiloglottis*, *Drakaea,* and *Paracaleana* (Linde *et al.* 2014), using eight loci analysed by a variety of methods (including phylogenies of individual loci, Bayesian coalescent based species delimitation, and population structure analysis) revealed five phylogenetic species: one associated with *Chiloglottis*, one with *Drakaea* and *Paracaleana,* and three with *Arthrochilus* (among which one was known from one isolate and another from two isolates). Analysis of the ITS alone recovered the same five

phylogenetic species as well-separated and well-supported clades, revealing congruence between the widely used ITS region and the more extensive multi-locus analysis (Linde *et al.* 2014). The phylogenetic species were not formally named in Linde *et al.* (2014).

Many of the orchid species associated with *Tulasnella* are rare or endangered (Hopper & Brown 2006), and the association between orchid and fungus has been and continues to be the subject of much research in Australia (Smith *et al.* 2010) and elsewhere (McCormick & Jacquemyn 2014). For *Tulasnella* associated with orchids identification by use of sequences is now the norm, rather than using cultural characters or features of the sporophore. It is therefore appropriate to supply formal names to three of the phylogenetic species (each known from more than two strains) already characterised on sequence data by Linde *et al.* (2014), along with a further phylogenetic species isolated from *Chiloglottis* associated with *Sphagnum*. After assessing information on *Tulasnella* from Australia, to determine if prior names exist for phylogenetic lineages, we describe four new species of *Tulasnella* here: *T. prima* and *T. sphagneti* spp. nov. from *Chiloglottis, T. secunda* sp. nov. from *Drakaea* and *Caleana* (inclusive of *Paracaleana*), and *T. warcupii* sp. nov. from *Arthrochilus oreophilus.*

MATERIALS AND METHODS

Fungal collections

Taxonomy of the orchid genera, which are all members of the subtribe *Drakaeinae*, follows Miller & Clements (2014), who accepted the genera *Arthrochilus*, *Caleana* (inclusive of *Paracaleana*), *Chiloglottis* (inclusive of *Simpliglottis*), and *Drakaea*. *Tulasnella* mycorrhizal associations as identified from previous studies on associations with Australian terrestrial orchids in *Arthrochilus, Caleana* (as *Paracaleana*), *Chiloglottis* and *Drakaea* (Roche *et al.* 2010, Phillips *et al.* 2011, Linde *et al.* 2014, Phillips *et al.* 2014), were investigated. Additionally, we treat a *Tulasnella* isolated from *Chiloglottis* aff. *valida* and *C. turfosa* growing in *Sphagnum* hummocks within the Kosciuszko National Park, New South Wales (Table 1). Some *Chiloglottis* orchids growing in *Sphagnum* were not in flower at the time of collection, and are thus referred to as "*Chiloglottis* sp." However, based on previous studies the *Chiloglottis* species involved are either *C.* aff. *valida, C. valida*, or *C. turfosa* (Peakall *et al.* 2010, Peakall & Whitehead 2014). Literature on *Tulasnella* from Australia was reviewed, and this literature along with GenBank and culture collection databases: CBS (CBS-KNAW Fungal Biodiversity Centre culture collection) and MAFF (culture collection, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan, searched via NIAS [National Institute of Agro-Environmental Sciences] Genebank - http://www.gene.affrc.go.jp/databases-micro_search_en.ph) were searched for isolates of *Tulasnella* from Australia (Tables 2 and 3).

Fungal isolation

Isolations were made within 7 d of the field collection of the plant tissue using a modified version of the protocol of Roche *et al.* (2010). We used two types of isolation media to grow mycorrhizal isolates: Fungal Isolation Media (FIM; Clements & Ellyard 1979) and 3MN+A-Z, which is a Melin-Norkrans medium (low CN MMN) (Wright *et al.* 2010) modified with 15g/L agar and human vitamin and mineral supplements (Centrum "Balanced Formula", Wyeth Consumer Healthcare, Baulkham Hills, NSW, Australia) instead of thiamine. One Centrum tablet was dissolved in 100 mL water, filter sterilised, and 10 mL added per litre of 3 MN medium (post autoclaving). Peloton-rich tissues (collars) of orchids were washed several times with sterile distilled water after which the tissue was macerated in sterile distilled water to release pelotons, which were plated onto agar plates containing antibiotics (FIM + tetracycline 25 mg/mL, and 3MN+A-Z + streptomycin 50 mg/mL). Germinating pelotons were transferred to either FIM or 3MN+A-Z media after 3–10 d. The medium chosen depended on which the pelotons germinated. After 3–4 wk all colonies were hyphal-tipped and subcultured to ensure colonies consisted of a single genotype. Cultures were stored at 5 °C on FIM or 3MN+A-Z agar slants covered with mineral oil. Voucher specimens of the fungi, as dried-down liquid cultures, are lodged at the National Herbarium of Victoria (MEL) and ex-type cultures are stored in the culture collection of the Department of Agriculture, Victoria (VPRI).

DNA extraction, sequencing and phylogenetic analysis

Small agar blocks cut from colony edges of isolates were briefly homogenised in 2 mL screw-cap tubes containing sterilise distilled water and glass beads. The blocks were homogenised in a FP120 (Thermo Scientific, Milford, MA) homogenizer for 5 s at 5.5 m/s. Petri dishes containing either half strength FIM or 3MN+A-Z broths were inoculated with the homogenised agar blocks and incubated at room temperature (approximately 23 °C) in the dark. Mycelium was harvested, stored at -4 °C, and lyophilized prior to DNA extraction. DNA extractions of the lyophilized-mycelium were performed using Qiagen DNeasy Plant Mini Kit or DNeasy 96 Plant Kit according to the manufacturer's instructions (Amersham Biosciences, Hilden, Germany).

As previously noted, in a comprehensive evaluation of eight nuclear and mitochondrial loci, Linde *et al.* (2014) sequenced *Tulasnella* isolates from orchids in the genera *Arthrochilus, Caleana* (as *Paracaleana*), *Chiloglottis,* and *Drakaea.* That study showed that within *Tulasnella* a single locus, ITS (nucR ITS), revealed congruent species delimitation and phylogenetic outcomes. Therefore, for the phylogenetic analysis of additional *Tulasnella* isolates from *Chiloglottis*, we only employed ITS. ITS sequences were amplified with the primers ITS1 and ITS4 (White *et al.* 1990) following methods described in Roche *et al.* (2010) for the PCR reaction, thermal cycling, purification of PCR and extension products. Products were sequenced bi-directionally with ABI PRISM BigDye Terminator v. 3.1 sequencing kit (Applied Biosystems, Foster City, CA) on an ABI-3130 automated sequencer. Sequences were edited using the program Sequencher v. 4.7 (GeneCodes, Ann Arbor, MI to correct for base read ambiguities. Our sequences were aligned with the most similar sequences available from GenBank (http://www.ncbi.nlm.nih.gov). Alignments were performed in Geneious v. 8 (http://www.geneious.com; Kearse *et al.* 2012) using

Table 1. *Tulasnella* isolates examined in this study.

Identity	Isolate no.	Type	Host	GPS, if known	Origin	Habitat	Collectors*	GenBank accession no.	Reference
Tulasnella sphagneti	**12033 (CLM541)**	**Holotype**	*Chiloglottis* aff. *valida*	35.5238S, 148.3656E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY095117	This study
	12030 (CLM583)		*Chiloglottis turfosa*	35.5346S, 148.3225E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY445922	This study
	13058		*Chiloglottis* sp.	35.5544S, 148.3528E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY445927	This study
	13065_1		*Chiloglottis* sp.	35.5544S, 148.3528E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY445926	This study
	13065_2		*Chiloglottis* sp.	35.5544S, 148.3528E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY445925	This study
	13102_1		*Chiloglottis turfosa*	35.5346S, 148.3225E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY445924	This study
	13102_2		*Chiloglottis turfosa*	35.5346S, 148.3225E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY445923	This study
	13139		*Chiloglottis* sp.	35.5336S, 148.2647E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY445928	This study
	13143		*Chiloglottis* sp.	35.5336S, 148.2647E	Kosciuszko NP, NSW	Alpine *Sphagnum* hammocks	YT	KY445929	This study
Tulasnella prima	**CLM159**	**Holotype**	*Chiloglottis trilabra*	34.1385S, 149.9722E	Blue Mountains, NSW	*Eucalyptus* woodland	RP	KF476556	(Roche *et al.* 2010)
	07033-45.II.2		*Chiloglottis seminuda*	34.6295S, 150.1539E	Exeter, NSW	*Eucalyptus* woodland	RP	HM196800	(Roche *et al.* 2010)
	CLM306		*Chiloglottis formicifera*	34.6537S, 150.6016E	Upper Kangaroo Valley, NSW	*Eucalyptus* woodland	RP	KF476550	(Roche *et al.* 2010)
	CLM308		*Chiloglottis formicifera*	34.6537S, 150.6016E	Upper Kangaroo Valley, NSW	*Eucalyptus* woodland	RP	KF476551	(Roche *et al.* 2010)
	CLM309		*Chiloglottis formicifera*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	KF476543	(Roche *et al.* 2010)
	CLM310.1		*Chiloglottis* aff. *jeanesii*	35.5056S, 149.5351E	Tallaganda State Forest, NSW	*Eucalyptus* woodland	RP	HM196792	(Roche *et al.* 2010)
	CLM310.2		*Chiloglottis* aff. *jeanesii*	35.5056S, 149.5351E	Tallaganda State Forest, NSW	*Eucalyptus* woodland	RP	HM196791	(Roche *et al.* 2010)
	CLM316.1		*Chiloglottis seminuda*	34.6295S, 150.1539E	Exeter, NSW	*Eucalyptus* woodland	RP	HM196797	(Roche *et al.* 2010)
	CLM316.2		*Chiloglottis seminuda*	34.6295S, 150.1539E	Exeter, NSW	*Eucalyptus* woodland	RP	HM196798	(Roche *et al.* 2010)
	CLM346		*Chiloglottis reflexa*	33.5211S, 150.3707E	Mt Wilson, NSW	*Eucalyptus* woodland	RP	HM196805	(Roche *et al.* 2010)
	CLM361		*Chiloglottis diphylla*	33.5154S, 150.4886E	Bilpin, NSW	*Eucalyptus* woodland	RP	HM196803	(Roche *et al.* 2010)

Table 1. (Continued).

Identity	Isolate no.	Type	Host	GPS, if known	Origin	Habitat	Collectors*	GenBank accession no.	Reference
	CLM366		*Chiloglottis trapeziformis*	35.2749S, 149.0976E	Black Mountain, ACT	*Eucalyptus* woodland	CCL	HM196794	(Roche *et al.* 2010)
	CLM371		*Chiloglottis trapeziformis*	35.2749S, 149.0976E	Black Mountain, ACT	*Eucalyptus* woodland	CCL	HM196799	(Roche *et al.* 2010)
	CLM372		*Chiloglottis trapeziformis*	35.2749S, 149.0976E	Black Mountain, ACT	*Eucalyptus* woodland	CCL	HM196789	(Roche *et al.* 2010)
	CLM377		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196782	(Roche *et al.* 2010)
	CLM380.1		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196779	(Roche *et al.* 2010)
	CLM380.2		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196788	(Roche *et al.* 2010)
	CLM381.1		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196783	(Roche *et al.* 2010)
	CLM381.2		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196784	(Roche *et al.* 2010)
	CLM388		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196787	(Roche *et al.* 2010)
	CLM389		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196795	(Roche *et al.* 2010)
	CLM390		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196785	(Roche *et al.* 2010)
	CLM391		*Chiloglottis* aff. *jeanesii*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196786	(Roche *et al.* 2010)
	CLM393		*Chiloglottis valida*	35.5056S, 149.5351E	Tallaganda State Forest, NSW	*Eucalyptus* woodland	RP	HM196804	(Roche *et al.* 2010)
	CLM395		*Chiloglottis valida*	33.9409S, 150.0552E	Kanangra Boyd NP, NSW	*Eucalyptus* woodland	RP	HM196801	(Roche *et al.* 2010)
	CLM405		*Chiloglottis trapeziformis*	35.2751S, 149.1097E	ANBG, ACT	*Eucalyptus* woodland	SR	HM196796	(Roche *et al.* 2010)
	SRBG01.II.3		*Chiloglottis trapeziformis*	35.2751S, 149.1097E	ANBG, ACT	*Eucalyptus* woodland	SR	HM196790	(Roche *et al.* 2010)
	CLM407		*Chiloglottis trapeziformis*	35.2751S, 149.1097E	ANBG, ACT	*Eucalyptus* woodland	SR	HM196793	(Roche *et al.* 2010)
	CLM416		*Chiloglottis trapeziformis*	35.2749S, 149.0976E	Black Mountain, ACT	*Eucalyptus* woodland	SR	HM196809	(Roche *et al.* 2010)
	SRBG03.I.8		*Chiloglottis trapeziformis*	35.2751S, 149.1097E	ANBG, ACT	*Eucalyptus* woodland	SR	HM196807	(Roche *et al.* 2010)

Table 1. (Continued).

Identity	Isolate no.	Type	Host	GPS, if known	Origin	Habitat	Collectors*	GenBank accession no.	Reference
	SRBG03.III.12		*Chiloglottis trapeziformis*	35.2751S, 149.1097E	ANBG, ACT	*Eucalyptus* woodland	SR	HM196810	(Roche *et al.* 2010)
	SRBG03.IV.5		*Chiloglottis trapeziformis*	35.2751S, 149.1097E	ANBG, ACT	*Eucalyptus* woodland	SR	HM196806	(Roche *et al.* 2010)
	CLM068		*Chiloglottis diphylla*	33.5154S, 150.4886E	Blue Mountains, NSW	*Eucalyptus* woodland	RP	KF476552	(Roche *et al.* 2010)
Tulasnella secunda	**CLM009**	**Holotype**	*Drakaea elastica*	NA	Karnup, WA	*Kunzea* woodland	RDP	KF476575	(Linde *et al*. 2014)
	CLM222		*Paracaleana minor*	35.2690S 149.0920E	Black Mountain, ACT	*Eucalyptus* woodland	CCL	KF476568	(Linde *et al*. 2014)
	CLM251		*Drakaea concolor*	NA	Mt Gregory, WA	Sandplain heath	RDP	KF476588	(Linde *et al*. 2014)
	CLM253		*Drakaea confluens*	NA	Lake Gnartaminny, WA	Jarrah forest	RDP	KF476592	(Linde *et al*. 2014)
	CLM255		*Drakaea livida*	NA	Walpole WA	Jarrah forest	RDP	KF476590	(Linde *et al*. 2014)
	CLM257		*Drakaea glyptodon*	NA	Moore River, WA	*Banksia* woodland	RDP	KF476583	(Linde *et al*. 2014)
	CLM258		*Drakaea glyptodon*	NA	Margaret River, WA	*Eucalyptus* woodland	RDP	KF476586	(Linde *et al*. 2014)
	CLM259		*Drakaea glyptodon*	NA	Ruabon, WA	*Kunzea* woodland	RDP	KF476577	(Linde *et al*. 2014)
	CLM260		*Drakaea elastica*	NA	Nambeelup, WA	*Kunzea* woodland	RDP	KF476593	(Linde *et al*. 2014)
	CLM261		*Drakaea gracilis*	NA	Westdale, WA	*Eucalyptus* woodland	RDP	KF476591	(Linde *et al*. 2014)
	CLM266		*Drakaea confluens*	NA	Lake Gnartaminny, WA	Jarrah forest	RDP	KF476579	(Linde *et al*. 2014)
	CLM267		*Paracaleana hortiorum*	NA	Talbot West, WA	*Eucalyptus* woodland	RDP	KF476584	(Linde *et al*. 2014)
	CLM268		*Paracaleana terminalis*	NA	Mt Gregory, WA	Sandplain heath	RDP	KF476574	(Linde *et al*. 2014)
	CLM272		*Paracaleana lyonsii*	NA	Eurardy, WA	Sandplain heath	RDP	KF476573	(Linde *et al*. 2014)
	CLM273		*Drakaea livida*	NA	Canning Mills, WA	Jarrah forest	RDP	KF476576	(Linde *et al*. 2014)
	CLM274		*Paracaleana triens*	NA	Talbot West, WA	*Kunzea* woodland	RDP	KF476580	(Linde *et al*. 2014)
	CLM276		*Drakaea isolata*	NA	Lake Chinocup, WA	Mallee woodland	RDP	KF476585	(Linde *et al*. 2014)
	CLM277		*Drakaea gracilis*	NA	Westdale, WA	*Eucalyptus* woodland	RDP	KF476578	(Linde *et al*. 2014)
	I3DLIsl6		*Drakaea livida*	NA	Walpole, WA	Jarrah forest	RDP	HQ386778	(Phillips *et al.* 2011)
	B9DGDen1		*Drakaea glyptodon*	NA	Denbarker WA	Jarrah forest	RDP	HQ386743	(Phillips *et al.* 2011)
	JS4		*Drakaea glyptodon*	NA	Southern WA			JX138567	(Sommer *et al.* 2012)

Table 1. (Continued).

Identity	Isolate no.	Type	Host	GPS, if known	Origin	Habitat	Collectors*	GenBank accession no.	Reference
Tulasnella warcupii	**CLM027**	**Holotype**	*Arthrochilus oreophilus*	17.2697S, 145.4535E	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476596	(Linde *et al*. 2014)
	CLM007		*Arthrochilus oreophilus*	17.3402S, 145.4210E	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476600	(Linde *et al*. 2014)
	CLM022		*Arthrochilus oreophilus*	17.3402S, 145.4210E	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476601	(Linde *et al*. 2014)
	CLM028		*Arthrochilus oreophilus*	17.3402S, 145.4210E	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476599	(Linde *et al*. 2014)
	CLM091		*Arthrochilus oreophilus*	17.2697S, 145.4535E	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476598	(Linde *et al*. 2014)
	CLM092		*Arthrochilus oreophilus*	17.3402S, 145.4210E	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476597	(Linde *et al*. 2014)
									(Linde *et al*. 2014)
Unassigned	CLM084		*Arthrochilus oreophilus*	17.3311E 145.4131S	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476594	(Linde *et al*. 2014)
Unassigned	CLM085		*Arthrochilus oreophilus*	17.3311E 145.4131S	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476595	(Linde *et al*. 2014)
Unassigned	CLM031		*Arthrochilus oreophilus*	17.3311E 145.4131S	Atherton Tablelands, Qld	*Eucalyptus* woodland	DG	KF476602	(Linde *et al*. 2014)
Unassigned	BB0002_2_A		Terrestrial orchid		Australia		Howard,C.G. and Clements,M.A.	JN015192	Unpublished

ANBG = Australian National Botanic Gardens; NP = National Park.

*CCL = Celeste Linde; YT = Yann Triponez; RP = Rod Peakall; RDP = Ryan Phillips; DG = Don Gomez.

Table 2. Isolates of *Tulasnella* (some as *Tulasnellaceae* or *Epulorhiza*) from Australian orchids, additional to those analysed from orchid hosts in *Drakaeinae* by Roche *et al.* (2010) and Linde *et al.* (2014). All isolates collected by Warcup currently present in culture collections are shown, along with all isolates from which sequences have been obtained. Orchid hosts in *Drakaeinae* are in bold. Note that AY643803 derived from isolate PN1 from *Pterosylis nutans* is given in GenBank as "asexual morph: *Epulorhiza repens*", but Bougoure *et al.* (2005) considered the isolate was most likely a *Thanatephorus* (and it is therefore omitted below).

***Tulasnella* species**	**Host**	**Original isolate**	**Reference for original isolate**	**Culture collection strain**	**Sequence ITS***	**Sequence LSU**	**Reference for sequence**
T. asymmetrica	*Thelymitra nuda*	JH Warcup 0267	(Warcup 1973)	MAFF 305807 AFTOL ID 1678	DQ520101		Garnica & Weiss (unpub.)
T. asymmetrica	*Thelymitra luteocilium*	JH Warcup 085	(Warcup & Talbot 1967)	MAFF 305806 (ex-type)	DQ388046		(Suarez *et al*. 2006)
					KC152339–44 [clones c001–c006]		(Cruz *et al*. 2014)
T. asymmetrica	*Thelymitra epipactoides*	JH Warcup 0302	(Warcup 1973)	MAFF305808	DQ388047		(Suarez *et al*. 2006)
					KC152347–49,51,52,56 [clones c001–c005, c009]		(Cruz *et al*. 2014)
T. asymmetrica	*Thelymitra epipactoides*	JH Warcup 0591	(Warcup 1973)	NIAES 5809			(Bidartondo *et al*. 2003)
				MAFF P305809 = NIAES 5809	DQ388048		(Suarez *et al*. 2006)
				NIAES 5809	KC152345–46, KC152350, KC152353–55, [clones c001, c003, c005, c008–c010]		(Cruz *et al*. 2014)
T. calospora	*Acianthus exsertus*	JH Warcup 07	(Warcup & Talbot 1967)	MAFF305801			no sequences
T. calospora	*Diuris maculata*	JH Warcup 0388	(Warcup 1973)	MAFF305802			no sequences
T. calospora	*Thelymitra aristata*	JH Warcup 0584	(Warcup 1973)	MAFF305803			no sequences
T. calospora	*Thelymitra* sp.	JH Warcup 0638	(Warcup 1973)	MAFF305804			no sequences
T. calospora	host not specified	JH Warcup 0689	(Warcup 1973)	MAFF305805			no sequences
T. calospora	*Caladenia reticulata*	JH Warcup 062		CBS 573.83		AY243521	(Taylor *et al*. 2003)
T. irregularis	*Dendrobium dicuphum*	JH Warcup 0632		CBS 574.83 [ex type] = JCM 9996		AF345560	(Kristiansen *et al*. 2001)
						AY243519	(Taylor *et al*. 2003)
T. calospora	*Microtis parviflora*	TM1	(Perkins *et al*. 1995)		AY643804		(Bougoure *et al*. 2005)
Tulasnella sp.	[presume from orchid]	JT Otero 306			DQ061110		Otero (unpub.)
Tulasnella sp.	[presume from orchid]	JT Otero 307			DQ061111		Otero (unpub.)
Epulorhiza 'possibly' [in GenBank as 'Fungi']	*Acianthus pusillus*	AP2			AY643806		(Bougoure *et al*. 2005)

Table 2. (Continued).

***Tulasnella* species**	**Host**	**Original isolate**	**Reference for original isolate**	**Culture collection strain**	**Sequence ITS***	**Sequence LSU**	**Reference for sequence**
Epulorhiza sp.	*Diuris corymbosa*	Kings_Park_ Dm01			EF160068		(Bonnardeaux *et al*. 2007)
Epulorhiza sp.	*Prasophyllum giganteum*	Kings_Park_ Pg01			EF160067		(Bonnardeaux *et al*. 2007)
Epulorhiza sp.	*Pyrorchis nigricans*	7 isolates			EF176464–66, 69–72		(Bonnardeaux *et al*. 2007)
Epulorhiza sp.	*Disa bracteata*	11 isolates			EF176473–77, 79–83, 85		(Bonnardeaux *et al*. 2007)
Tulasnella sp.	'terrestrial orchid'	BB0002_2_A			JN015192		Howard & Clements (unpub.)
T. calospora	*Diuris magnifica*	DR88			KT601561		Davis *et al.* (unpub)
T. calospora	*Disa bracteata*	DR28			KT601562		Davis *et al.* (unpub)
T. calospora	*Microtis media*	DR126			KT601563		Davis *et al.* (unpub)
Tulasnellaceae sp. RP-2011	***Drakaea elastica, D. glyptodon, D. livida, D. micrantha, D. thynniphila***	50 isolates			HQ386734–83		(Phillips *et al*. 2011)
Tulasnellaceae sp. 1–3	*Thelymitra macrophylla*	JP15, JP44, JP49			JX138557–59		(Sommer *et al*. 2012)
Tulasnellaceae sp. 4–5	*Disa bracteata*	JP24, JP26			JX138560–61		(Sommer *et al*. 2012)
Tulasnellaceae sp. 6–7	*Pyrorchis nigricans*	JP33, JP37			JX138562–63		(Sommer *et al*. 2012)
Tulasnellaceae sp. 8–9	*Diuris magnifica*	JP40, JP60			JX138564–65		(Sommer *et al*. 2012)
Tulasnellaceae sp. 10	*Microtis* sp.	JP63			JX138566		(Sommer *et al*. 2012)
Tulasnellaceae sp. 11	***Drakaea glyptodon***	JS4			JX138567		(Sommer *et al*. 2012)
Tulasnellaceae sp. 12	***Spiculaea ciliata***	JS43			JX138568		(Sommer *et al*. 2012)
Tulasnellaceae sp. 13	*Lyperanthus serratus*	JS64			JX138569		(Sommer *et al*. 2012)
Tulasnellaceae sp. 14	*Microtis capularis*	JS66, JS68			JX138570–71		(Sommer *et al*. 2012)
Tulasnellaceae sp. 16	*Microtis media*	JS163			JX138572		(Sommer *et al*. 2012)
Tulasnella sp. [as 'Uncultured mycorrhizal fungus']	*Diuris fragrantissima*	30 isolates			DQ790719–38,86-95	DQ790751–60,84-85	(Smith *et al*. 2010)
Tulasnella sp. [as 'Uncultured mycorrhizal fungus']	*Diuris punctata*	7 isolates			DQ790798	DQ790763,65, 72, 77,79,82,98	(Smith *et al*. 2010)
Tulasnella sp. [as 'Uncultured mycorrhizal fungus']	*Diuris punctata* var. *daltonii*	2 isolates			DQ790804,08	DQ790769,73	(Smith *et al*. 2010)

Table 2. (Continued).

***Tulasnella* species**	**Host**	**Original isolate**	**Reference for original isolate**	**Culture collection strain**	**Sequence ITS***	**Sequence LSU**	**Reference for sequence**
Tulasnella sp. [as 'Uncultured mycorrhizal fungus']	*Diuris dendrobioides*	1 isolate			DQ790802	DQ790767	(Smith *et al*. 2010))
Tulasnella sp. [as 'Uncultured mycorrhizal fungus']	*Diuris chryeopsis*	3 isolates			DQ790796,99 DQ790815	DQ790761,64, 80	(Smith *et al*. 2010)
*some sequences are ITS plus partial LSU.							

Table 3. *Tulasnella* species isolated from Australian orchids by JH Warcup and PHB Talbot and other studies. Sporophore morphology was from sporophores (i.e. the "perfect state") initiated from cultures. *Tulasnella* species in bold were newly described by Warcup and Talbot. Orchid genera in *Drakaeinae* are given in bold. References do not include publications where original isolates of Warcup were later sequenced (as cited in Table 2). Unpublished observations derive from sequences in GenBank as detailed in Table 2.

***Tulasnella* species**	**Orchid genera**	**Identification method**	**Comments**	**References**
T. allantospora	***Chiloglottis***, *Corybas*	sporophore morphology		(Warcup & Talbot 1971, Warcup 1981)
T. asymmetrica	***Chiloglottis***, *Cryptostylis*, *Dendrobium*, *Thelymitra*	sporophore morphology	also as *Tulasnella* sp., isolate 086 (Warcup & Talbot 1967); see Warcup & Talbot (1971)	(Warcup & Talbot 1967, 1971, Warcup 1973, 1981)
T. calospora	*Acianthus*, *Caladenia*, *Corybas*, *Cymbidium*, *Dendrobium*, *Diuris*, *Eriochilus*, *Lyperanthus*, *Microtis*, *Orthoceras*, *Thelymitra*	sporophore morphology		(Warcup & Talbot 1967, Warcup 1971, 1973, 1981, 1990)
	Disa, Diuris, Microtis	sequence		(Bougoure *et al.* 2005)
T. cruciata	*Acianthus*, ***Chiloglottis***, *Thelymitra*	sporophore morphology		(Warcup & Talbot 1971, Warcup 1973, 1981, 1990)
T. deliquescens [as *Epulorhiza repens*]	*Acianthus*, *Microtis*	culture morphology		(Perkins et al. 1995)
T. irregularis	*Dendrobium*	sporophore morphology	also as *Tulasnella* sp. 1, isolate 0632 (Warcup 1973); see Warcup & Talbot (1980)	(Warcup & Talbot 1980, Warcup 1981)
T. violea	***Drakaea***	culture morphology		(Warcup 1981)
	Thelymitra	sporophore morphology		(Warcup & Talbot 1971, Warcup 1973, 1990)
Tulasnella sp. (some as *Epulorhiza* sp. or Tulasnellaceae sp.)	***Arthrochilus***, *Caladenia*, ***Caleana***, *Calochilus*, ***Chiloglottis***, *Corybas*, *Cryptostylis*, *Cymbidium*, *Dendrobium*, *Dipodium*, ***Drakaea***, *Microtis, Thelymitra*	culture morphology	"culturally seem *Tulasnella*, perfect states not seen"	Warcup 1973, 1981, 1990, Perkins & McGee 1995, Perkins et al. 1995)
	Acianthus, ***Caleana*** (as *Paracaleana*), *Disa*, *Diuris*, ***Drakaea***, *Lyperanthus*, *Microtis*, *Prasophyllum*, *Pyrorchis*, ***Spiculaea***, *Thelymitra*	sequence		(Bougoure *et al.* 2005, Bonnardeaux *et al.* 2007, Smith *et al.* 2010, Phillips *et al.* 2011, Sommer *et al.* 2012)

ClustalW followed by manual adjustments to optimise indel locations.

Sequences for phylogenetic analysis included representatives of species-level clades in one of the two main subclades of phylogenetic group IV of the phylogeny of *Tulasnella* constructed by Cruz *et al.* (2011). This subclade contains *Tulasnella* sp. ECU 6 and *T. eichleriana*. To these sequences were added a selection of previously sequenced isolates from Australian orchids (Table 1) representing the phylogenetic breadth of the OTUs identified by Linde *et al.* (2014) along with new sequences from *Chiloglottis* associated with *Sphagnum* (Table 1). BLAST matches were carried out for representative sequences of putative OTUs from Australian orchids from our analysis to recover related sequences in GenBank. Phylogenies were estimated using Bayesian inference with MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) and maximum likelihood (ML) analysis through the RAxML Blackbox (Stamatakis *et al.* 2008). Support for the nodes was assessed with Bayesian Posterior Probabilities (BPP) in MrBayes and for ML trees using 1000 pseudoreplicates of nonparametric bootstrapping. A GTR+G substitution model was used for all analyses as other models are nested within these. Trees were visualised using FigTree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and rooted to *Tulasnella eichleriana* sequences. Trees include identical sequences from different isolates; however the identical sequences were removed when nodes support was assessed. Pairwise sequence divergence of the ITS sequences within and among lineages were estimated with the Kimura-2-parameter distances with gap deletion in MEGA5 (Tamura *et al.* 2011).

RESULTS

Tulasnella species from Australian orchids

In placing formal names on phylogenetic species of *Tulasnella*, it is necessary to consider any names from previous work on the genus. Essentially, type specimens (that anchor names) need to be placed into the phylogenetic framework. However, given the lack of diagnostic morphological characters for recently isolated strains, a significant issue is whether types or suitable reference material exists and if sequence data are available for that material. Three species of *Tulasnella* have been described from Australian orchids: *T. cruciata*, originally from *Acianthus* and *Dendrobium*; *T. irregularis* from *Dendrobium*; and *T. asymmetrica* originally from *Thelymitra*. May *et al.* (2003) collated records of *Tulasnella* from all sources from Australia, including reports of a further four species: *Tulasnella allantospora*, *T. calospora*, *T. deliquescens*, and *T. violea*. According to Roberts (1994), *T. asymmetrica* was morphologically indistinguishable from *T. pinicola*, and was listed by Roberts (1999) as a synonym of the latter species. Furthermore, Roberts (1999) noted that the Australian report of *T. allantospora* by Warcup & Talbot (1971) was possibly misidentified, and might represent *T. rubropallens*; and *T. calospora* in the sense of Warcup & Talbot (1967) was deemed to be *T. deliquescens*. In making redispositions of Australian *Tulasnella* names, Roberts (1999) noted that he had not examined type material or voucher collections for reports by Warcup & Talbot (1967, 1971) of *T. allantospora* and *T. calospora*. Indeed, type material of *T. cruciata* or *T. irregularis* could not be located in ADW (Roberts 1999), and although the type of *T. asymmetrica* is listed by Roberts (1999) as housed at ADW, it was not examined. Warcup's collections were originally in ADW and subsequently transferred to AD (macrofungi) and DAR (microfungi). There are ex-type cultures of *T. asymmetrica* (Warcup 085, MAFF 305806) and *T. irregularis* (Warcup 0632, CBS 574.83 = JCM 9996), but apparently none of *T. cruciata*.

Tulasnella isolates have been obtained from 21 terrestrial orchid genera and one lithophytic/epiphytic orchid genus (*Dendrobium*) in Australia (Table 3) (Warcup & Talbot 1967, 1971, 1980, Warcup 1971, 1973, 1981, 1990). For the genera *Arthrochilus*, *Caleana* (or from *Paracaleana*, under which name *Caleana* was formerly placed), *Chiloglottis*, and *Drakaea* that were the source of the apparently novel phylogenetic species delimited by Linde *et al.* (2014), the only previous reports are of unidentified *Tulasnella* isolates. An exception is a report of *Tulasnella violea* from *Drakaea*, identified only from cultural characteristics (Warcup 1981). However, for *Chiloglottis* there are reports of *T. allantospora*, *T. asymmetrica, T. cruciata* and also an unidentified species (Warcup 1973, 1981). For the two *Tulasnella* species described from Australia (*T. asymmetrica* and *T. cruciata*), the types are from other orchid genera, and the isolates of these two species from *Chiloglottis* were collected after the species were described.

Phylogenetic analysis of isolates from *Arthrochilus*, *Caleana, Chiloglottis,* and *Drakaea*

GenBank BLAST searches using query ITS sequences of *Tulasnella* isolates in this study revealed these sequences were related to *T. eichleriana, T. tomaculum,* and two *Tulasnella* lineages (ECU 5 and ECU 6) isolated from decaying branches in Ecuador. Representative sequences of these four lineages were added to the 72 fungal sequences from the Australian orchid genera *Arthrochilus*, *Chiloglottis*, *Drakaea,* and *Caleana.* The resulting phylogram show high bootstrap (100 %) and posterior probability (1) support for the three phylogenetic species of *Tulasnella* from (a) *Arthrochilus oreophilus*, (b) *Chiloglottis,* and (c) *Drakaea* and *Caleana*, that had previously been delimited on multi-gene data by Linde *et al.* (2014). Further sequences from *Chiloglottis* (exclusively associated with *Chiloglottis* growing in *Sphagnum*) formed a well-separated clade, sister to the other sequences from *Chiloglottis* (Fig. 1). ITS sequences from the ex-type culture of *T. asymmetrica* fall outside of the clades depicted in Fig. 1, as do all other sequences from Australian orchids (data not shown) with the exception of HQ386778, HQ386743 and JX138567 from *Drakaea*, that fell within the clade of isolates from *Drakaea* and *Caleana* and JN015192 from an Australian terrestrial orchid that is sister to the two clades consisting of isolates from *Chiloglottis*. A number of additional sequences from *Drakaea* (Phillips *et al.* 2011) all cluster within the clade from *Drakaea* with 100% bootstrap support. Those matching sequences were subsequently excluded from the final analysis. The LSU sequence from the ex-type culture of *T. irregularis* is only distantly related to LSU sequences

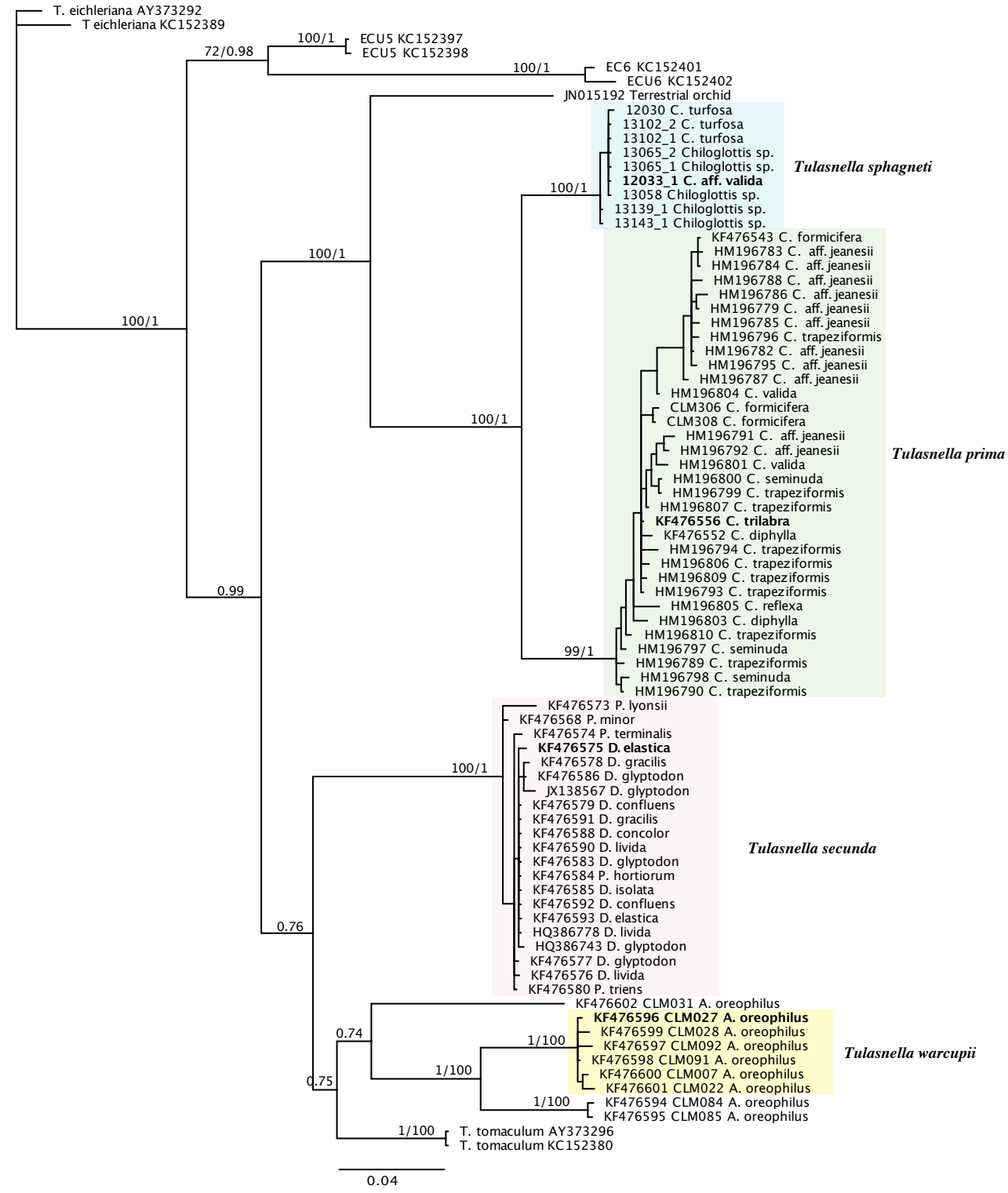

Fig. 1. Rooted MrBayes tree for *Tulasnella* obtained for ITS. The tree with the highest log likelihood is shown. The numbers above the branches are maximum likelihood bootstrap values/Bayesian posterior probabilities. Bootstrap values of ≥ 70 % and Bayesian posterior probabilities of ≥ 0.70 are shown. The branch length is proportional to the inferred divergence level. Host from which the *Tulasnella* isolate was collected from is indicated after the isolate number or GenBank number. Sequences from the holotype of each species is indicated in bold.

from Australian isolates of *Tulasnella* from *Arthrochilus*, *Chiloglottis*, *Drakaea,* and *Caleana* (data not shown).

The percentage sequence divergence between the two lineages from *Chiloglottis* was 6.3 %. Sequence divergence between all other Australian *Tulasnella* lineages and close relatives ranged from 9.8–20.6 % (Table 4). The natural ITS barcode gap between all *Tulasnella* lineages studied here is between 4–6 % sequence divergence (Fig. 2).

Recognition of novel taxa

Support for three of the novel taxa was high across the eight loci analysed by Linde *et al.* (2014) (Table 5) and all

Table 4. Within host group and between host group Kimura -2P distances for *Tulasnella* as calculated from ITS. All positions containing gaps and missing data were eliminated. There were a total of 601 positions in the final dataset.

	Within taxa	*T. prima*	*T. sphagneti*	*T. warcupii*	*T. secunda*	*T. tomaculum*	*T. eichleriana*	T. ECU5
Tulasnella prima	1.2 ± 0.3							
Tulasnella sphagneti	0.1 ± 0.1	6.3 ± 1.0						
Tulasnella warcupii	3.8 ± 0.4	18.1 ± 1.7	16.7 ± 1.6					
Tulasnella secunda	0.2 ± 0.1	14.8 ± 1.6	15.1 ± 1.5	13.9 ± 1.5				
Tulasnella tomaculum	0	15.6 ± 1.6	13.3 ± 1.5	11.6 ± 1.3	9.8 ± 1.3			
Tulasnella eichleriana	2.2 ± 0.6	15.4 ± 1.6	15.1 ± 1.6	15.0 ± 1.5	12.7 ± 1.5	11.4 ± 1.4		
Tulasnella ECU5	0.2 ± 0.2	15.2 ± 1.6	13.8 ± 1.6	14.9 ± 1.6	14.3 ± 1.7	10.5 ± 1.4	11.2 ± 1.4	
Tulasnella ECU6	1.5 ±0.5	20.6 ± 2.0	18.3 ± 1.9	17.6 ± 1.7	18.6 ± 1.9	14.6 ± 1.6	17.0 ± 1.7	14.5 ± 1.6

clades had long subtending basal stems in the phylogenies generated. Base-pair differences and their positions for each lineage are given in Table 6. Therefore we conclude that each can be regarded as a well-supported phylogenetic species. The additional clade consisting of isolates from *Chiloglottis* associated with *Sphagnum* was also well-supported in the ITS tree (Fig. 1) and well-separated from the sister clade, above the divergence established between the three phylogenetic species delimited on multi-locus concordance, and is therefore recognised as a fourth phylogenetic species.

None of the clades for these four phylogenetic species contain sequences from material of *Tulasnella* previously described from Australia, or indeed any other sequences of described species in GenBank. In addition, the ITS sequences from ex-type cultures of *T. asymmetrica* and *T. irregularis* do not cluster with or are close to any of the four phylogenetic species described here. Therefore, we conclude that these four phylogenetic species are previously unrecognized, and consequently they are formally described below. Two further putative new phylogenetic species (*Tulasnella* sp. Arthrochilus II and *Tulasnella* sp. Arthrochilus III) (Linde *et al.* 2014) that were represented by only two and one isolates respectively, are not formally described here pending discovery of further isolates. Previous morphology-based identifications of various *Tulasnella* species from hosts in *Drakaeinae* (Table 3) will all need to be re-visited and confirmed with sequence data, if voucher specimens or cultures still exist.

Here we diagnose the new species on the basis of both sequence-based synapomorphies and clade-based definitions from molecular phylogenies (Hibbett *et al.* 2011, Renner 2016). This is because it is not possible to be certain as to which morphological characters are actually diagnostic. In time, certain morphological features may turn out to be unique for particular taxa, but this can only be known if morphological data are comprehensive across known species of the genus. In addition, it is sequence data that are routinely used to identify isolates of *Tulasnella*, and hence we are providing both rigorous species delimitation and the means to identify further isolates with certainty. Therefore, our descriptions of the morphology of cultures and of hyphal characters are provided for completeness, rather than as species characteristics.

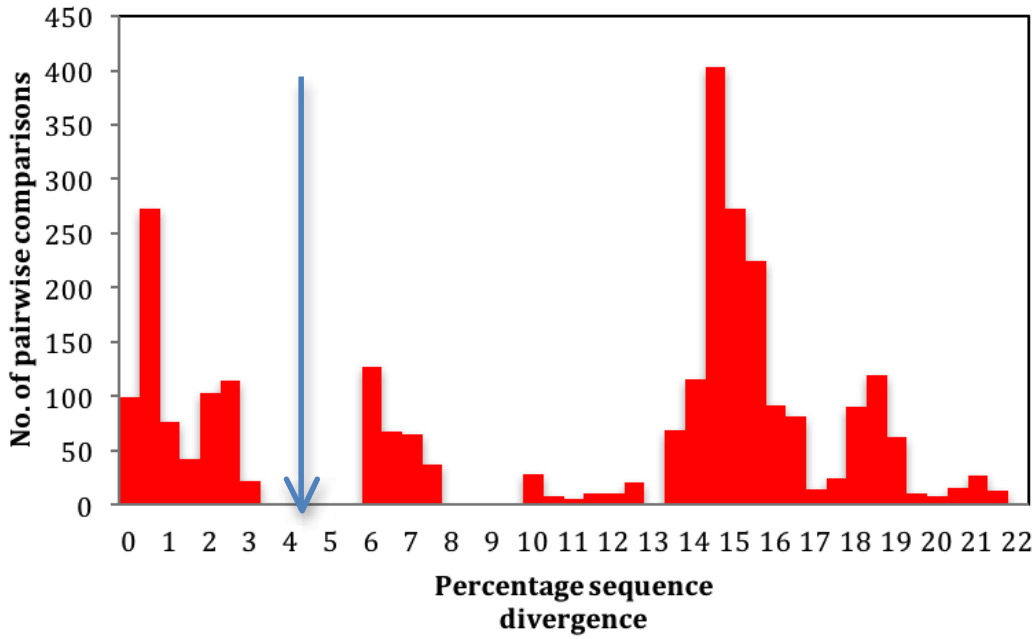

Fig. 2. Barcode gap; percentage sequence divergence among *Tulasnella* isolates. The vertical arrow indicates the ~3.3 to 5.7 % ITS sequence divergence threshold for this dataset.

Table 5. Presence of and support for clades of six phylogenetic species of *Tulasnella* from Australian orchids in the genera *Arthrochilus*, *Chiloglottis*, *Drakaea* and *Caleana* in phylogenetic trees constructed separately for each of eight loci, as indicated on trees presented as Fig. 2 and Supporting information figures S2-S8 of Linde *et al*. (2014). Values are bootstrap/Bayesian posterior probability. +: clade present (support less than BS 70% and BPP 0.80); *: one isolate (CLM417) fell outside of the clade, basal to all other sequences; **: support values are from Fig. 1 of the present work (all other clades in this tree representing the phylogenetic species are also 100/1.00); n=1: one isolate only, falls outside of other clades, and separate to any other singletons; na: not present in analysis.

	no. isolates	ITS	mtLSU	C4102	C12424	C14436	C3304	C4722	C10499
T. prima	33	100/1.00	97/1.00	100/1.00	100/1.00	85/0.99*	100/1.00	100/1.00	99/1.00
T. sphagneti	9	100/1.00**	na	na	na	na	na	na	na
T. secunda	21	100/1.00	+	95/1.00	86/0/96	96/1.00	100/1.00	99/1.00	94/1.00
T. warcupii	6	100/1.00	94/0.88	100/1.00	100/1.00	-/0.94	99/1.00	n=1	100/1.00
T. sp. Arthrochilus II	2	100/1.00	98/0.99	100/1.00	100/1.00	98/1.00	+	n=1	na
T. sp. Arthrochilus III	1	n=1	n=1	n=1	n=1	n=1	n=1	na	n=1

TAXONOMY

Tulasnella prima Linde & T.W. May, **sp. nov.**
MycoBank MB817404
(Fig. 3A)

Etymology: Referring to the first *Tulasnella* found in the host, *Chiloglottis*.

Type: **Australia**: *New South Wales*: Blue Mountains, Mt Werong, Ranger Fire Trail, isolated from *Chiloglottis trilabra*, 22 Mar. 2007, *C.C. Linde & R. Peakall CLM159* (MEL 2402822 – holotype; ex-type culture VPRI 42810).

Diagnosis: *Tulasnella prima* can be diagnosed by the following nucleotide characters, which are fixed between *T. tomaculum* and *T. prima* respectively: Locus ITS: **ITS1** upstream from the 18S at position 18 (G:T), 23-25 (TGCT:CTGA), 32-33 (CG:--), 38 (G:T/A), 41 (A:T/C), 44 (G:T), 58 (T:C), 61 (-:T), 68 (-:T), 80 (G:T), 101 (T:A), 117 (T:C), 123 (G:A), 127 (C:T), 130 (G:T), 132-133 (CT:TC), 140 (A:T), 144-145 (AG:TT), 152 (C:T), 156 (T:G), 158 (-:A/G), 163 (A:G), 165 (C:-), 168 (T:C), 180 (G:A/T), 190 (A:T/C), 192 (C:T), 203-204 (CT:TC), 214-215 (AC:GT), 217-218 (TG:CT), 224-225 (TA:--), 237)C:A/G). **5.8S** starting from ITS1 end: Position 4 (-:T/-), 22 (T:C), 139 (T:C), 141 (T:C), 154 (C:T). **ITS2** starting from 5.8S end: Position 14 (T:-), 16 (C:A), 25 (T:C), 27 (A:T/C), 32-33 (CT:TC), 37 (C:T), 49 (T:C), 55-56 (CT:TC), 59 (C:T), 69 (A:G), 71-72 (CA:TG), 75 (T:C), 77-81 (TCTGA:CTAT/CG), 84 (T:C), 88 (A:G), 91 (G:C/T), 93 (C:T), 96-98 (GTT:AAA), 105 (A:-), 107 (A:T), 109-110 (--:CT), 116-117 (TA:--), 120 (T:C), 126 (T:C), 136 (G:T), 139 (C:T), 143-144 (AT:GA), 147-148 (CC:TA/G), 150 (-:C/T), 154 (G:A), 157 (T:G), 163 (C:T), 180 (T:G), 187 (-:T/C), 191 (G:T/C), 215 (T:G), 222 (T:-), 237 (T:-), 241 (-:G/A), 251 (C:G), 253 (G:A), 256 (G:T, 259 (T:A), 262 (C:T), 285 (G-:AC), 290-293 (TCCG:CTGC), 295-296 (CG:TT), 298-299 (TG:AT), 302-303 (CG:GT/C), 305 (A:G), 307-308 (AC:TT), 328 (T:C), 331 (G:A), 338 (G:T).

Clade-based diagnosis: The least inclusive clade in the ITS phylogeny in Fig. 1 containing HM196790 and HM196786.

Substrate or host: Roots and underground stem-collars of *Chiloglottis* orchid species.

Distribution: High rainfall parts of south-eastern Australia and Tasmania in *Eucalyptus* woodlands and forests. Current known distribution coincides with that of the *Chiloglottis* hosts.

Notes: Cultures on quarter strength PDA show fine concentric rings. Culture edges lack concentric rings, are broad and diffused. Culture appearance is quite variable, with some cultures showing aerial mycelium. Not all cultures grow on 3MN +A-Z. Hyphae from cultures are cylindrical, 2–5 µm diam, branched, often at right angles, septate, lacking clamp connections; wall slightly thickened (to 0.25 µm); rarely with refractive internal bodies, and then small; sometimes uneven (with undulate outline); sometimes with swollen elements to 9.5 µm diam that are thick-walled (to <0.5 µm thick), clavate, terminal or intercalary, sometimes in short chains.

Additional material examined: See Table 1.

Tulasnella secunda Linde & T.W. May, **sp. nov.**
MycoBank MB817406
(Fig. 3C)

Etymology: Referring to the second *Tulasnella* described that associates with *Drakaeinae* orchids.

Type: **Australia**: *Western Australia*: Paganoni Swamp Reserve, Karnup, isolated from *Drakaea elastica*, 2008, *R.D. Phillips* [*C.C. Linde CLM009*], (MEL 2402819 – holotype; ex-type culture VPRI 42808).

Diagnosis: *Tulasnella secunda* can be diagnosed by the following nucleotide characters, which are fixed between *T. tomaculum* and *T. secunda* respectively. Locus ITS: **ITS1** upstream from the 18S at position 34-35 (TT:-A), 41 (A:C/T), 54 (G:A), 77 (C:T), 128 (G:A), 130 (C:A), 132 (G:T), 152-153 (-C:TT), 155-156 (TC:CT), 158 (T:C), 163 (C:-), 166 (T:-), 170 (-:C), 179 (G:A), 181 (C:T), 189 (A:C), 202 (C:A), 211-212 (AC:--), 219-220 (CA:AC), 223 (T:C), 238 (T:C). **5.8S** no differences. **ITS2** starting from 5.8S end: Position 23 (C:T), 27 (A:T), 32 (C:T), 35-37 (GGC:AAT), 48 (G:A), 55-56 (CT:T/

Table 6. Basepair differences and their positions, among *Tulasnella tomaculum, T. sphagneti, T. prima, T. secunda* and *T. warcupii*. Polymorphic basepair differences in two or more isolates of a species are given by the most common basepair/alternative basepair.

	1	5	6	7	8	9	15	16	17	18	19	24	27	28	30	37	41	44	51	62	63	64	74	90	92
T. tomaculum	**G**	**G**	**T**	**G**	**C**	**T**	**C**	**G**	**T**	**T**	**T**	**A**	**G**	**C**	**C**	**G**	**T**	**-**	**-**	**T**	**C**	**G**	**-**	**T**	**T**
T. sphagneti	T	.	C	T	G	A	T	C	.	C	C	C	T	.	.	.	C	T	C	C	.	T	-	A	.
C. prima	T	.	C	T	G	A	T	T	.	C	.	T	T	.	.	.	C	T	T	.	.	T	-	A	.
T. secunda	.	.	C	A/.	.	.	.	.	A	.	C	C	.	.	.	A	.	-	-	.	T	.	-	.	.
T. warcupii	.	A	C	.	.	C	.	C	.	.	.	.	.	./T	T	.	.	-	-	.	.	.	C	.	C

	97	99	105	107	111	113	117	120	121	122	123	124	125	126	129	131	135	136	144	145	146	147	149	150	152
T. tomaculum	**C**	**A**	**C**	**T**	**C**	**G**	**C**	**G**	**G**	**C**	**-**	**T**	**G**	**G**	**C**	**A**	**A**	**G**	**C**	**C**	**G**	**-**	**T**	**C**	**T**
T. sphagneti	T	C	.	.	.	A	T	.	-	T	C	C	.	A	.	T	T	.	T	.	.	G	.	.	C
T. prima	T	.	C	C	.	A	T	T	.	T	-	C	.	./A	.	T	T	T	T	.	.	G	C	A	.
T. secunda	.	.	.	.	.	.	.	A	.	A	-	.	T	.	T/.	.	.	.	.	T	T	G	C	T	C
T. warcupii	.	.	.	.	T	.	.	.	T	T	-	.	.	.	.	.	.	.	.	.	T	-	.	.	C

	153	155	160	162	163	172	174	180	182	183	184	195	196	197	209	212	214	215	218	231	232	233	234	259	376
T. tomaculum	**G**	**A**	**T**	**C**	**T**	**G**	**C**	**T**	**A**	**A**	**C**	**C**	**T**	**T**	**T**	**-**	**A**	**-**	**T**	**C**	**A**	**T**	**A**	**T**	**T**
T. sphagneti	A	G	C	.	C	A	.	.	T	.	T	T	C	C	.	A	T	T	C	.	.	.	.	.	C
T. prima	.	G	C	.	./C	A/T	.	.	C	.	T	T	C	.	./C	A	T	T	C	A	.	.	.	C	C
T. secunda	.	.	C	T	C	A	T	.	C	.	.	A	.	.	.	A	-	-	C	.	.	C	.	.	.
T. warcupii	.	.	C	.	C	T	.	C	.	G	.	T	.	A	C	A	.	T	.	.	G	.	T	.	.

	378	391	410	411	414	421	422	423	424	425	426	431	432	434	435	436	438	445	447	448	449	452	454	455	456
T. tomaculum	**T**	**C**	**T**	**C**	**C**	**C**	**C**	**T**	**G**	**A**	**T**	**C**	**T**	**G**	**G**	**C**	**G**	**C**	**G**	**T**	**G**	**C**	**C**	**T**	**C**
T. sphagneti	C	T	.	.	A	.	.	C	T	C	.	T	C	A/.	.	.	.	.	.	.	.	.	T	C	T
T. prima	C	T	.	.	A	.	.	C	A/T	T/C	.	T	C	A/.	.	T	.	.	.	C	A/.	T	T	C	T
T. secunda	.	.	.	.	.	T	.	.	.	T	.	T	.	A	A	T	.	.	A	.	.	.	T	C	.
T. warcupii	.	.	C	G	.	.	T	.	A	.	A	.	.	.	.	.	A	T	.	C	.	.	T	C	.

	457	458	459	460	463	469	470	471	472	475	481	482	484	486	490	492	493	494	497	501	503	504	505	506	508
T. tomaculum	**G**	**C**	**G**	**T**	**T**	**A**	**C**	**C**	**A**	**T**	**-**	**-**	**-**	**G**	**T**	**G**	**C**	**A**	**G**	**C**	**C**	**G**	**T**	**T**	**G**
T. sphagneti	.	.	A	.	C	G	.	.	G	.	C	A	-	C	C	.	.	G	.	T	T	.	G	.	.
T. prima	.	T	.	.	.	G	.	T	G	C	A	T	-	.	C	.	T	G	T	T	.	A	A	A	./A
T. secunda	A	.	A	C	.	G	.	.	G/.	C	C	T	C/T	.	.	.	./T	G	.	T	T	.	.	.	A
T. warcupii	.	T	.	C	C	G	T	.	G	-	C	T	-	.	C	A	.	G	.	.	.	.	.	.	A

	509	510	511	513	514	515	516	517	518	519	521	522	523	525	526	527	532	540	542	545	548	549	550	551	553
T. tomaculum	**C**	**G**	**G**	**A**	**G**	**A**	**T**	**G**	**T**	**G**	**T**	**A**	**-**	**G**	**T**	**T**	**T**	**C**	**G**	**C**	**C**	**A**	**T**	**C**	**C**
T. sphagneti	.	.	T	G	C	T	C	C	A	T	A	G	T	.	C	.	C	.	T	T	.	G	G	T	.
T. prima	.	A	./T	G	T	T	C	T	G/A	C	.	G	T	.	C	.	C	.	T	T	.	G	A	T/.	.
T. secunda	.	.	.	.	A	.	C	C	.	T	.	G	-	A	C	.	.	T	.	.	A	G	A	-	.
T. warcupii	T	.	.	G	.	G	C	T	.	T	.	G	-	.	C	G	C	.	.	T	.	G	A	.	A

	554	555	557	558	562	565	566	571	577	587	588	590	592	594	595	598	599	600	606	607	623	644	645	646	647
T. tomaculum	**C**	**C**	**-**	**-**	**G**	**T**	**G**	**C**	**C**	**C**	**-**	**T**	**C**	**T**	**G**	**A**	**G**	**A**	**T**	**T**	**T**	**C**	**T**	**C**	**T**
T. sphagneti	T	A	C	C	A	A	.	.	.	.	G	C	T	G	T	G	C	G	.	.	G	.	C	.	.
T. prima	T	A	-	C	A	G	.	T	./T	.	G	C	T	G	T	.	T	.	.	.	G	.	C	T	G
T. secunda	T	T	C	C	-	C	A	.	.	T	G	.	.	.	.	.	.	.	.	./C	G	.	C	T	G
T. warcupii	T	.	-	C	A	C	.	.	.	.	G	.	-	C	.	.	.	.	C	C	.	T	C	.	.

	657	658	659	660	662	663	664	665	667	668	669	670	671	675	680	681	686	687	688	689	690	695	696	698	700
T. tomaculum	**T**	**C**	**G**	**G**	**T**	**C**	**-**	**G**	**G**	**T**	**G**	**C**	**C**	**G**	**C**	**T**	**C**	**T**	**C**	**G**	**-**	**G**	**A**	**C**	**T**
T. sphagneti	.	G	.	A	.	.	-	T	.	A	C	.	T	A	.	.	.	.	.	.	-	A	.	A	-
T. prima	.	G	.	A	.	.	-	T	.	A	.	.	T	.	.	.	.	.	.	.	-	A	C	A	-
T. secunda	C	T	.	A	.	.	T	T	.	C	.	.	.	.	A	C	T	.	.	.	-	A	.	T	G
T. warcupii	G	A	T	.	C	G	-	A	A	C	.	T	.	.	.	C	T	C	T	A	C	A	.	.	-

	703	707	708	709	710	711	712	713	714	717	719	720	721	723	724	725	726	727	728	746	749	750	756
T. tomaculum	**-**	**G**	**C**	**T**	**G**	**T**	**G**	**C**	**-**	**-**	**A**	**-**	**A**	**-**	**C**	**-**	**T**	**C**	**A**	**T**	**G**	**T**	**G**
T. sphagneti	T	T	T	C	C	.	C	G	-	T	G	G	G	-	-	T	.	.	.	C	A	.	T
T. prima	T	T	T	C	A	.	T	G	-	C	G	-	-	-	T	T	.	.	.	C	A	C	T
T. secunda	A	T	T	-	T	G	T	-	-	T	G	-	.	G	.	T	C	.	.	.	.	.	.
T. warcupii	-	T	T	C	-	G	T	G	C	G/-	G	-	.	-	.	-	C	.	T	.	.	.	.

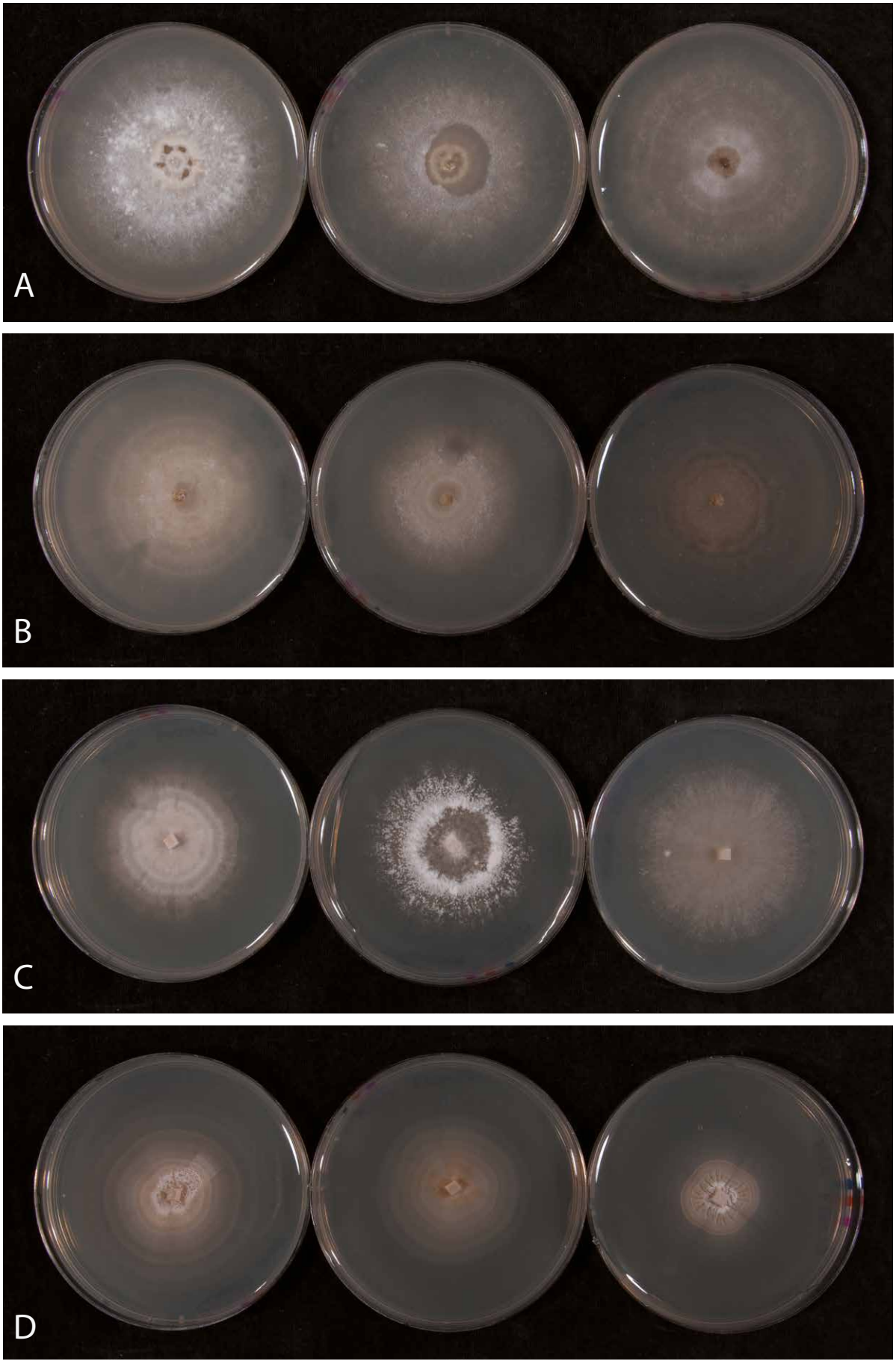

Fig. 3. *Tulasnella* cultures on quarter strength PDA (left), half strength FIM (middle) and 3MN +A-Z (right) media. **A.** *Tulasnella prima* (CLM159); **B.** *T. sphagneti* (CLM541); **C.** *T. secunda* (CLM009) and **D.** *T. warcupii* (CLM027).

AC), 58 (G:A), 60-61 (GT:AC), 69 (A:G), 73 (T:C), 76 (T:C), 80 (-:C/T), 89 (A:G), 94 (C:T), 96 (C:T), 101 (G:A), 107 (G:A), 109-110 (TG:CC), 112 (G:-), 115 (A:T/C), 118-119 (-T:AC), 133 (C:T), 141 (C:-), 143-147 (TCCC:--GA), 149 (-:T), 151 (T:C), 156-157 (TG:CA), 178-179 (-C:TG), 214 (T:G), 235-237 (TCT:CTG), 248-249 (TC:CT), 251 (G:A), 254-255 (G-:TT), 258 (T:C), 270-271 (CT:AC), 276 (C:T), 284 (G:A), 287 (CT:TG), 291 (G:A), 294-295 (GC:TT), 300 (C:T), 305 (A:G), 308 (-:C).

Clade-based diagnosis: The least inclusive clade in the ITS phylogeny in Fig. 1 containing KF476573 and JX138567.

Substrate or host: Underground stem-collars of *Caleana* and *Drakaea* orchid species.

Distribution: South-western and south-eastern Australia, extending from high rainfall areas to the margin of the arid zone, occurring in open areas within eucalypt forests and woodlands, *Banksia* woodlands and sandplain heath. Most records are from well-drained grey sandy soils, but also known from yellow sands, laterite, sandy clay soil, etc. Current known distribution coincides with that of *Caleana* (inclusive of *Paracaleana*) and *Drakaea*.

Notes: This taxon was referred to as "Tulasnellaceae sp. RP-2011" by Phillips *et al.* (2011). Cultures often have a rose-pink colour due to bacterial associates that are not affected by streptomycin in the isolation medium. Application of tetracyclin eliminates bacteria and cultures then assume an off-white colour. On quarter strength PDA, cultures show some aerial mycelium giving it a velvety look. Cultures also have concentric rings with culture edges diffused. Cultures often show scalloped edges. Hyphae from cultures are cylindrical, 2-5 µm diam, frequently branched, often at right angles, septate, lacking clamp connections; wall slightly thickened to thickened (to 0.25 µm); often with refractive internal bodies; sometimes uneven (with undulate outline); often with swollen elements to 10.5 µm diam that are thick-walled (to 0.5 µm thick) and globose to clavate when terminal, and globose to ellipsoid when intercalary; when terminal, subtended by one or two swollen, clavate elements, but not in chains. Refractive bodies within the hyphae are more common and obvious in this and *T. warcupii* than in the other two species.

Additional material examined: See Table 1.

Tulasnella sphagneti Linde & T.W. May, **sp. nov.**
MycoBank MB817405
(Fig. 3B)

Etymology: Referring to the *Sphagnum* habitat of the orchid host.

Type: **Australia**: *New South Wales*: Kosciuszko NP, alongside Tantangara Road, isolated from *Chiloglottis* aff. *valida* growing in a *Sphagnum* hummock, 19 Jan. 2012, *C.C. Linde CLM541 & E. Triponez* (MEL 2402823 – holotype; ex-type culture VPRI 42811).

Diagnosis: *Tulasnella sphagneti* can be diagnosed by the following nucleotide characters, which are fixed between between *T. tomaculum* and *T. sphagneti* respectively: Locus ITS: **ITS1** upstream from the 18S at position 18 (G:T), 23 (-:C), 26 (C:-), 27 (T:A), 33 (C:-), 34 (G:), 39 (G:C), 42 (A:C), 45 (G:T), 59 (T:C), 62 (-:T), 69 (:C), 79 (T:C), 81 (G:T), 102 (T:A), 108 (C:T), 110 (A:C), 124 (G:A), 128 (C:T), 132 (G:T), 134 (T:C), 136 (G:A), 141 (A:T), 145 (A:T), 154 (C:T), 156 (-:G), 161 (:-C), 162 (-:A), 166 (A:-), 167 (T:-), 168 (C:-), 171 (T:C), 174 (T:C), 183 (G:A), 193 (A:T), 195 (C:T), 206 (C:T), 207 (T:C), 216 (C:T), 217 (A:G), 218 (C:T), 220 (T:C), 221 (G:T), 227 (T:), 228 (A:). **5.8S** starting from ITS1 end: Position 138 (T:C), 140 (T:C), 153 (C:T). **ITS2** starting from 5.8S end: Position 14 (T:), 16 (C:A), 25 (T:C), 26 (G:T), 27 (A:C), 32 (C:T), 33 (T:C), 55 (C:T), 56 (T:C), 57 (G:T), 60 (G:A), 64 (T:C), 69 (A:G), 72 (A:G), 77 (-:C), 80 (T:A), 81 (G:C), 85 (T:C), 89 (A:G), 94 (C:T), 96 (C:T), 98 (T:G), 104 (G:T), 106-115 (AGATGTGTTA:GCTCCATAGT), 118 (T:C), 124 (T:C), 134 (G:T), 137 (C:T), 141-142 (-A:GG), 145-147 (-CC:TAT), 149 (T:C), 153 (G:A), 156 (T:A), 179 (T:G), 187-188 (CA:TC), 190-191 (A-:CG), 214 (T:G), 221 (T:), 236 (T:C), 249 (C:G), 251 (G:A), 254 (G:T), 257-258 (TG:AC), 260 (C:T), 264 (G:A), 283 (G:A), 286-287 (CT:AC), 293-295 (G--:TTC), 298-299 (GT:CG), 301 (C:T), 304-305 (AA:GG), 307 (C:T), 327 (T:C), 330 (G:A), 337 (G:T).

Clade-based diagnosis: The least inclusive clade in the ITS phylogeny in Fig. 1 containing 13143 and 12030.

Substrate or host: Roots and collars of *Chiloglottis valida*, *C.* aff. *valida,* and *C. turfosa* growing in *Sphagnum* hammocks in alpine areas in eastern Australia.

Distribution: South-eastern Australia, occurring in alpine habitats associated with *Sphagnum* hummocks. Current known distribution coincides with that of *Chiloglottis* hosts within this particular habitat.

Notes: Cultures on quarter strength PDA show fine concentric rings. Culture edges lack concentric rings, are broad and diffused. Hyphae from cultures cylindrical, 2–5.5 µm diam, branched, often at right angles, septate, lacking clamp connections; wall slightly thickened (to 0.25 µm); rarely with refractive internal bodies, and then in narrower hyphae; sometimes uneven (with undulate outline); rarely with swollen elements to 7 µm diam that are slightly thick-walled, subglobose and terminal. Swollen elements are less common in this species than in the other three.

Additional material examined: See Table 1.

Tulasnella warcupii Linde & T.W. May, **sp. nov.**
MycoBank MB817407
(Fig. 3D)

Etymology: After J. H. Warcup who was instrumental in studying mycorrhizal fungi associated with orchids in Australia.

Type: **Australia**: *Queensland*: Atherton Tablelands, Herberton Range State Forest, Atherton, isolated from *Arthrochilus*

oreophilus, 1 Apr. 2010, *C.C. Linde & D. Gomez CLM027* (MEL 2402821 – holotype; MEL 2402820 – isotype; ex-holotype culture VPRI 42809).

Diagnosis: *Tulasnella warcupii* can be diagnosed by the following nucleotide characters, which are fixed between *T. tomaculum* and *T. warcupii* respectively: Locus ITS: **ITS1** upstream from the 18S at position 22-23 (GT:AC), 26 (T:C), 33 (G:C), 37 (C:-), 47 (C:T), 101 (T:C), 120 (C:T), 130 (G:T/A), 131 (C:T), 154 (G:T), 158 (T:C), 163 (C:-), 166 (T:C), 169 (T:C), 178 (G:T), 186 (T:C), 189 (A:G), 201 (C:T), 203 (T:A), 212-213 (--:G/AT), 218-221 (GTCA:----), 238 (A:G), 240 (A:T). **5.8S** starting from ITS1 end: Position 1 (T:-). **ITS2** starting from 5.8S end: Position 13 (C:G), 24 (C:T), 26 (G:A), 28 (T:A), 39 (G:A), 46 (C:T), 49 (T:C), 55-56 (CT:TC), 59 (C:T), 61 (T:C), 64 (T:C), 69-70 (AC:GT), 72 (A:G), 76 (T:C), 84 (T:C), 86 (G:A), 88 (A:G), 92 (-:-/G), 93 (-:-/C), 102-103 (GC:AT), 107 (A:G), 109-111 (ATG:GCT), 113 (G:T), 116 (A:G), 119-120 (TT:CG), 125 (T:C), 138 (C:T), 142-143 (AT:GA), 145-146 (CC:AT), 149 (-:C), 153 (G:A), 156 (T:C), 179 (T:G), 181 (C:T), 184 (T:C), 196-197 (TT:CC), 234-235 (CT:TC), 247-249 (TCG:GAT), 251-253 (TCG:CGA), 255-256 (GT:AC), 258 (C:T), 269 (T:C), 274 (-:T), 277 (C:A), 278 (G:C/T), 283 (G:A/-), 287 (T:-), 293-295 (GCT:TTC), 302 (-:-/G), 303 (A:-/G), 307 (T:C), 309 (A:T).

Clade-based diagnosis: the least inclusive clade in the ITS phylogeny in Fig. 1 containing KF476596 and KF476601.

Substrate or host: Roots and collars of *Arthrochilus oreophilus*.

Distribution: Atherton Tablelands in Queensland, Australia, in association with *Arthrochilus oreophilus* in *Eucalyptus* woodland.

Notes: On PDA cultures show fine concentric rings with a velvety edge. Usually no aerial mycelium is visible. Cultures are off-white to yellowish. Of the four *Tulasnella* species described here, it is the slowest growing. Hyphae from cultures are cylindrical, 1–2.5(–4) µm diam, branched, often at right angles, septate, lacking clamp connections; wall slightly thickened to thickened (to 0.5 µm); often with refractive internal bodies; sometimes uneven (with undulate outline) to distinctly monilioid, with short, repeated, globose to subglobose elements to 7 µm diam. The minimum diameter of hyphae is noticeably thinner than in the other three species, and this is the only one of the four species to show chains of globose elements.

Additional material examined: See Table 1.

DISCUSSION

Here we describe four new species of *Tulasnella* that are found in association with Australian terrestrial orchids belonging to the *Diurideae*, using diagnostic DNA characters as advocated by Renner (Renner 2016). Three of these species (*T. prima*, *T. secunda*, and *T. warcupii*) were initially revealed by an in depth study using eight sequence loci and three different methods of species delimitation (Linde *et al.* 2014). These three species were shown to successfully germinate seed of members of the orchid genus they associate with (Linde *et al.* 2014). Our addition of *Tulasnella* isolates from *Chiloglottis* growing in *Sphagnum* hummocks in alpine areas in eastern Australia revealed the fourth species, *T. sphagneti*. This represents the second *Tulasnella* species to be found associated with *Chiloglottis* orchids. Based on the widely accepted 3 % sequence divergence cut-off value for species delimitation (Nilsson *et al.* 2008), or the 3–5 % divergence proposed for delimiting *Tulasnella* species (Girlanda *et al.* 2011, Jacquemyn *et al.* 2011), the sequence divergence between the four new species described in this study, exceeds these cut-off thresholds (6.3 %).

Tulasnella is representative of the complexity of contemporary taxonomic mycology. Some species are rigorously defined on multi-gene data, or on the single region (ITS) that has been confirmed as having utility as a barcode in this genus, while other species have been and are being described with excellent details of morphological characters. Unfortunately, few species are well known from both morphology and molecular sequence data. Ideally, all type material should be sequenced, which would allow integration of the two approaches. However, Cruz *et al.* (2016) point out that "sequencing of old fungarium specimens of *Tulasnella* spp. has been unsuccessful probably due to inappropriate conservation of DNA" and they consider this could well remain the case even with improvements in techniques. Therefore, sequenced epitypes will need to be designated where the strict application of names without sequences is ambiguous, but the challenge will be to match modern cultures or collections to old names.

Apart from their association with orchids, the ecology and distribution of the new *Tulasnella* species described here remains poorly known. Interestingly, all orchid species investigated, within the orchid genera *Chiloglottis*, *Drakaea,* and *Caleana,* associate with a single *Tulasnella* species with one exception. The one exception is in *Chiloglottis* where both *T. prima* and *T. sphagneti* associate with *Chiloglottis* orchids, but *T. sphagneti* is so far only found in *Chiloglottis* species growing in *Sphagnum*. Where orchids in the *Drakaeinae* are host to multiple *Tulasnella* species, the fungi are closely related. *Tulasnella prima* and *T. sphagneti* from *Chiloglottis* are sister taxa and the three *Tulasnella* species from *Arthrochilus* form a clade. However, the overall phylogeny of the *Tulasnella* species from *Drakaeinae* does not appear to match that of the hosts (Miller & Clements 2014), where *Chiloglottis* is sister to *Drakaea*, and these form a clade sister to the remaining genera, including *Arthrochilus* and *Caleana*. Remarkably, in *Caleana*, the only orchid genus in this group to be found in both eastern and western Australia, this association extends across the continent. In contrast to the orchid genus-wide association of most *Tulasnellas* in this study, mycorrhizal associations of the tropical *Arthrochilus oreophilus* appear far more diverse. Previously, three *Tulasnella* OTUs were shown to occur in a narrow sample of this subtropical species (Linde *et al.* 2014). Because two of the OTUs are represented by only one or two sequences, and lack living cultures, only one (*T. warcupii*) is described here. Our findings raise the question of why only a small diversity

of *Tulasnella* fungi associates with a large number of orchid species across a vast geographic range.

The pattern of one fungal species to many orchid species appears to be in stark contrast to studies of orchid-mycorrhizal interactions outside Australia, which have consistently found a number of mycorrhizal OTUs associating with sympatric as well as allopatric orchid congeners (Jacquemyn *et al* 2015). For example, 15 OTUs from *Tulasnellaceae* were associated with four species of *Anacamptis* orchids. Of those 15 OTUs, 13 associated with seven species of *Ophrys* and two *Orchis* species, whereas nine OTUs associated with three *Serapias* species (Pellegrino *et al.* 2014). The high diversity of *Tulasnella* was such that within sites up to 15 OTUs were co-occurring and 85 % of plants associated with more than three different OTUs (Pellegrino *et al.* 2014). A corresponding result was found along a single 1000 m transect with the same orchid genera where 16 *Tulasnellaceae* OTUs were recovered for 20 species of orchids (Jacquemyn *et al.* 2015). The same pattern is found in Andean tropical rainforests where up to six *Tulasnella* OTUs may associate with *Stelis* orchid species and *Pleurothallis lilijae* (Suarez *et al.* 2006, Kotte *et al.* 2008). Consistent among these studies and ours, is the finding that multiple species of an orchid genus can share the same fungal OTU. However, the ability to germinate orchid seed was not shown in other studies, making it difficult to ascertain the real mycorrhizal diversity associating with the orchids.

Our description of four new species of *Tulasnella*, all associated with Australian orchids, extends the number of formally described species known as mycorrhizal agents of orchids. However, it is evident that more *Tulasnella* species await DNA analysis and formal description. For example, previous studies on *Tulasnella* ITS diversity associated with *Diuris* orchids have uncovered a large number of OTUs (Smith *et al.* 2010), likely to represent many undescribed *Tulasnella* species. It is further evident that earlier morphologically based studies by Warcup and co-workers prior to the advent of DNA sequencing grossly underestimated the *Tulasnella* species diversity associated with orchids in Australia.

Although *Tulasnella* is most commonly detected in association with orchids, orchids are not essential for *Tulasnella* existence. To understand issues such as the ecology, habitat, and geographic range of these fungi, it is essential to develop detection methods that are independent of the orchid, such as a metagenomic approach. This may not only uncover further *Tulasnella* diversity, but will also shed light on the lives of these fungi independent of orchids.

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